JOURNAL OF BACTERIOLOGY, Dec. 1988, p. 5870–5876
0021-9193/88/125870-07$02.00/0

Vol. 170, No. 12

Genetic Manipulation of Major P-Fimbrial Subunits and Consequences for Formation of Fimbriae

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Received 11 April 1988/Accepted 18 September 1988

The influence of genetic manipulation of the structural genes coding for major P-fimbrial subunits on the formation of fimbriae in *Escherichia coli* was studied. Deletion of two regions that code for hypervariable parts of the P fimbrillin resulted in strong reduction or total absence of fimbria production. Replacement of deleted amino acids by other amino acid residues restored the formation of fimbriae. The hypervariable regions may be important for biogenesis of fimbriae by imposing correct spacing between conserved regions of the protein. The potential for substituting amino acids in the P-fimbrial subunit opens interesting possibilities for use of fimbriae as carriers of foreign antigenic determinants. An antigenic determinant of foot-and-mouth disease virus (FMDV) was incorporated in the F11 fimbrial subunit. Hybrid fimbriae, recognized by an FMDV-specific neutralizing monoclonal antibody directed against FMDV, were formed.

Fimbriae are long filamentous appendages that occur on many bacteria and consist of about 1,000 protein subunits (for a review, see reference 14). Many uropathogenic *Escherichia coli* strains expose on their cell surfaces P fimbriae that mediate adherence of the pathogen to the uroepithelium (11, 31, 32). P fimbriae recognize the α-D-galactose-(1,4)-β-D-galactose moiety of antigens of the P blood group (13, 18). Among P fimbriae, the serotypes F7 through F13 have been distinguished (27). From uropathogenic *E. coli*, the genes responsible for synthesis of various serologically different P fimbriae, i.e., $F7_1$, $F7_2$, F8, F9, F11, and F13, have been cloned (5, 6, 10, 12, 28, 36, 37). It has been shown that these fimbrial gene clusters are very similar in general organization (25, 36, 38), which suggests that the biogenesis of P fimbriae may follow a general concept.

The composition of P fimbriae is very complex. The major subunit, or P fimbrillin, is predominant and determines the antigenic properties (35). Several minor components, among them the adhesin protein, are also present in the fimbrial structure (19, 20, 29). Localization and biogenesis of the minor fimbrial components have been studied by Lindberg et al. (19) and Riegman et al. (29). They showed that these minor fimbrial components are located at the tip of the fimbrial structure. Riegman et al. (29) suggested that at least some of these minor proteins are important for the initiation of polymerization of the minor and major subunits into a fimbrial structure.

On the basis of comparisons of the amino acid sequences of various P fimbrillins, a model has been presented for the functional domains of the major fimbrial subunit (34). Conserved regions occur in the P fimbrillins that were suggested to be important for biogenesis. In addition, there are indications of hypervariable domains that are involved in determination of antigenic specificity (34, 35).

In this study, we describe experiments indicating that hypervariable regions, simply by acting as spacers between conserved regions, may be important for correct processing of the major fimbrial subunit. The amino acid sequence present in this region might be relatively unimportant for biogenesis. This observation offers interesting possibilities for the exploitation of the major subunit as a carrier of foreign antigenic determinants.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* K-12 HB101 (2), a strain deficient in the production of type 1 fimbriae, was used as the host strain in all experiments unless otherwise indicated. JM101 was used as the host strain for M13mp8 derivatives (24). HB2154 (3) was used as the host strain for M13mp18 (26) derivatives in the localized mutagenesis experiments. The mutant plasmids described were derived from pPIL110-75, carrying the *fso* gene cluster, and pPIL291-15, carrying the *fel* (F-eleven) gene cluster (6, 29, 36). Bacteria were cultivated in L broth or on yeast broth agar as described previously (37). Selective pressure against loss of plasmids was imposed by addition of ampicillin (50 μg/ml).

Recombinant DNA techniques and enzymes. Recombinant DNA techniques were performed essentially as described by Maniatis et al. (22). Restriction enzymes and other DNA-modifying enzymes (Pharmacia, Uppsala, Sweden) were used under conditions recommended by the manufacturer.

Localized mutagenesis was performed by the gapped-duplex method (16), with M13mp8 carrying the *felA* gene (coding for the F11 major fimbrial subunit) of pPIL291-15 (6) as a template. A 30-mer primer consisting of the flanking regions of hypervariable region 4 of the *felA* gene separated by the sequence containing the recognition site for *Hpa*I, as well as linearized M13mp18, were hybridized with the template DNA. After extension, ligation, and transformation into the *mutL* strain HB2154, the desired plaques were selected. The *felA* gene of pPIL291-15 was then replaced with the mutant *felA* gene, resulting in plasmid pPIL291-1529. The oligodeoxynucleotides used for the localized and insertion mutagenesis studies were synthesized on a Biosearch 8600 DNA synthesizer. Ligated DNA as well as isolated plasmids were introduced into recipient strains by transformation as described by Kushner (17). The chain termination method of Sanger et al. (30) was used to se-

quence the mutations after cloning of the relevant DNA fragments into M13mp8.

Electron microscopy and immunocytochemical labeling. Electron microscopy was performed by using a Philips 200 microscope with nickel grids coated with Parlodion films (Mallinckrodt, Inc., St. Louis, Mo.). Negative staining of whole cells was performed with 2% uranyl acetate for 60 to 90 s. Immunocytochemical labeling of whole cells with goat anti-mouse immunoglobulin G-gold complexes was carried out essentially as described previously (29). Cells were grown overnight on solid medium and suspended carefully in phosphate-buffered saline to a density of approximately 10^{10} cells per ml. The F11-specific monoclonal antibody (MAb) M7-6 has been described (7, 35).

Purification of fimbriae, SDS-PAGE, and Western blotting (immunoblotting). Fimbriae were purified essentially as described previously (37). Purified fimbriae were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 14% acrylamide gels as described by Lugtenberg et al. (21). Western blot analysis was performed according to Gershoni and Palade (9) as described by Agterberg et al. (1). The foot-and-mouth disease virus (FMDV)-specific MAb MA18 was isolated and described by Meloen et al. (23). Protein staining was performed with fast green.

ELISA. The enzyme-linked immunosorbent assay (ELISA) (8) was performed essentially as described by Van der Ley et al. (33). *ortho*-Phenylenediamine (0.4 mg/ml in 24 mM citric acid–51 mM disodium hydrogen phosphate [pH 5]) was used as a substrate for the peroxidase reaction. The A_{492} of the ELISA was read with the Easy Reader EAR 400.

RESULTS

Construction of deletions in the *fsoA* and *felA* genes. Deletions in hypervariable region 2 of the *fsoA* gene, coding for the $F7_1$ major fimbrial subunit, were obtained by linearization of plasmid pPIL110-703 (35) with the restriction enzyme *Xho*I, followed by treatment with exonuclease *Bal*31. Subsequently, the linear DNA molecules were circularized by ligation in the presence of a synthetic DNA linker containing the recognition site for the restriction enzyme *Sal*I. After transformation of *E. coli* K-12 HB101, transformants were selected that contained a *Sal*I restriction site in the *fsoA* gene. The sizes of the deletions in the plasmids were determined by nucleotide sequencing. Three plasmids in which the original reading frame of the *fsoA* gene had been conserved and that contained small deletions were selected for further analysis (Fig. 1A and B). In plasmid pDH3, the nucleotides coding for seven of the nine amino acids of hypervariable region 2 were deleted. In the mutated subunit encoded by plasmid pDH2, five amino acids of the hypervariable region and five amino acids of the N-terminal-flanking region were deleted. In the subunit encoded by plasmid pDH4, the complete hypervariable region and four amino acids of its C-terminal-flanking region were removed. In all three plasmids, insertion of a *Sal*I linker had occurred.

Two deletions in the *felA* gene, coding for the F11 major fimbrial subunit, were constructed (Fig. 1C and D). Plasmid pPIL291-1529 lacks the sequence coding for hypervariable region 4 of the *felA* gene, and six nucleotides providing the recognition site of the restriction enzyme *Hpa*I were inserted in that position. This mutation was obtained by localized mutagenesis of the *felA* gene of pPIL291-15 with the aid of a 30-base-pair primer. Plasmid pPIL291-1526 was constructed by fusion of plasmids pPIL291-1523 and pPIL291-1522 (35) on the *Xho*I restriction site that was present in these plasmids in hypervariable regions 3 and 4, respectively. In this way, a protein with a deletion of eight amino acids was obtained (Fig. 1D).

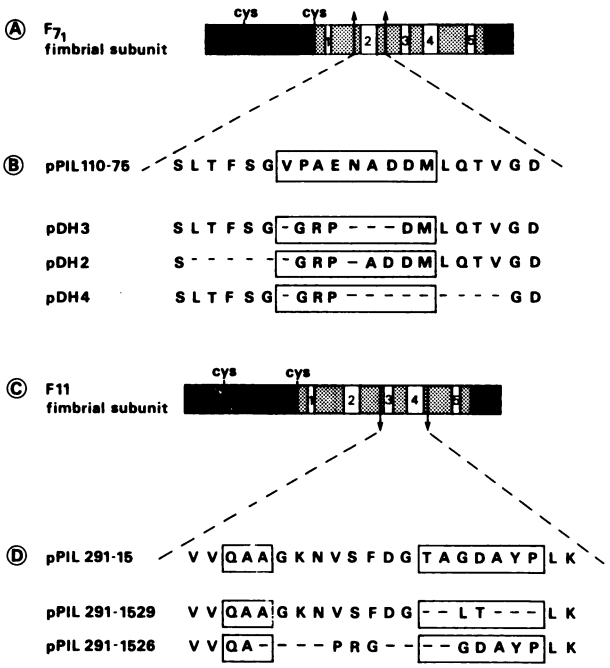

FIG. 1. Schematic representation of deletions in the FsoA and FelA fimbrillins. Deletions were obtained in hypervariable region 2 of the FsoA fimbrillin (A and B) and in hypervariable regions 3 and 4 of the FelA fimbrillin (C and D). The hypervariable regions are boxed. (A and C) Model of a P fimbrillin (34, 35). The hypervariable regions (white boxes) are numbered 1 through 5. (B) Schematic showing the amino acid sequences (one-letter codes) of hypervariable region 2 and its environment in the wild-type FsoA protein, encoded by pPIL110-75. pDH2, pDH3, and pDH4 are plasmids that differ from pPIL110-75 by having a deletion in hypervariable region 2, which was generated by *Bal*31. After *Bal*31 treatment, a DNA linker with the recognition sequence for the restriction enzyme *Sal*I was inserted, resulting in the amino acid sequence GRP. The amino acid sequences in the region of deletion and the flanking regions are shown. (D) Schematic showing the amino acid sequence (one-letter codes) of hypervariable regions 3 and 4 and environmental regions in the wild-type FelA protein. pPIL291-1526 and pPIL291-1529 are plasmids that differ from pPIL291-15 by having a deletion in the region shown. In pPIL291-1529, deletion of hypervariable region 4 was obtained by localized mutagenesis. In pPIL291-1526, part of the hypervariable regions 3 and 4 and the region between were deleted. Insertion of a DNA linker encompassing the recognition sequence for an *Xho*I restriction enzyme resulted in the addition of the amino acid sequence PRG.

Effect of the deletions in the *fsoA* and *felA* genes on the formation of fimbriae. The effect of the deletions in the fimbrial subunits on the formation of fimbriae of cells carrying the mutant plasmids was studied by electron microscopic examination of negatively stained cells and by ELISA. Formation of fimbriae appeared to be severely disturbed in cells with the *fsoA* deletion plasmids. No positive reaction was found by ELISA (results not shown). By electron microscopic examination, however, it was observed that a few cells (1 to 5%) of all three mutant strains had formed a few fimbriae (Fig. 2B).

HB101 cells harboring pPIL291-1526 or pPIL291-1529 showed no formation of fimbriae as determined by electron microscopy, in contrast to cells carrying the parental plasmid pPIL291-15, which showed normal fimbria formation

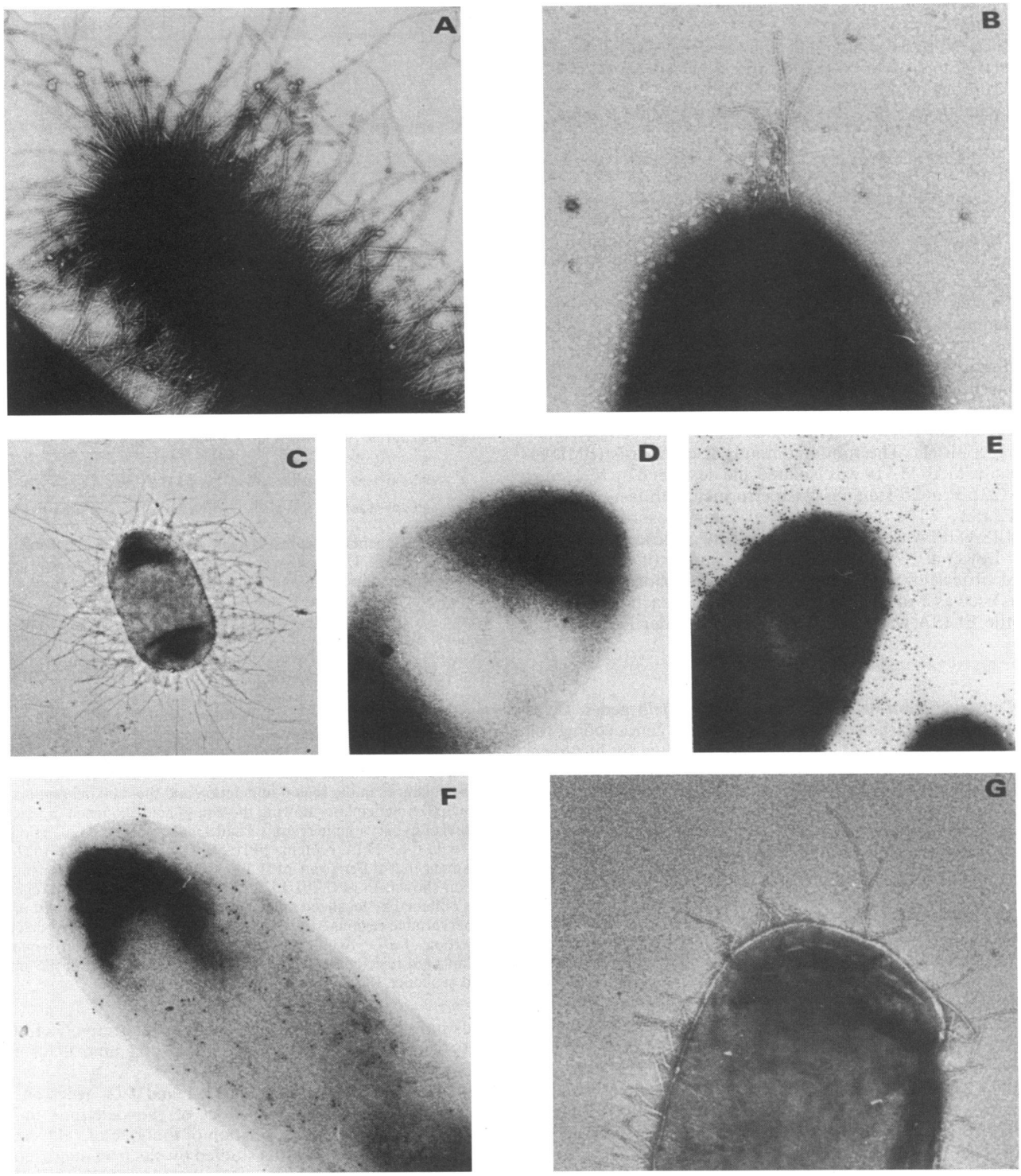

FIG. 2. Electron micrographs of HB101 cells harboring different plasmids. All electron micrographs show negatively stained HB101 cells harboring the following plasmids: (A) pPIL110-75 (36), (B) pDH4, (C) pPIL291-15 (6), (D) pPIL291-1526, (E) pPIL291-15, (F) pPIL291-1529, and (G) pPIL291-1569. Preparations shown in panels D through F were also immunocytochemically labeled with the F11-specific MAb M7-6 (7, 35).

(Fig. 2C and E). In an ELISA, no binding of the F11-specific MAb M7-6 (7, 35) to HB101(pPIL291-1526) was detected (Table 1). HB101 cells carrying pPIL291-1529 showed a very weak reaction in the same experiment. Cells harboring the *felA* mutant plasmids were also studied by immunocytochemical labeling of whole cells with MAb M7-6. A low amount of gold label was found on the surfaces of cells harboring pPIL291-1529, which indicated that the mutated FelA fimbrillin was present on the cell surface (Fig. 2F). This finding could suggest that transport of subunit protein to the

TABLE 1. ELISA of serial dilutions of cells with the F11-specific MAb M7-6 and the FMDV-specific MAb MA18

HB101 cells carrying plasmid:	A_{492} at given dilution[a]											
	M7-6						MA18					
	1	1/2	1/4	1/8	1/16	1/32	1	1/2	1/4	1/8	1/16	1/32
pPIL291-1526	0	0	0	0	0	0	—	—	—	—	—	—
pPIL291-1529	0.01	0.01	0.05	0.07	0.03	0.03	—	—	—	—	—	—
pPIL291-1549	0.49	0.31	0.14	0.06	0.01	0	—	—	—	—	—	—
pPIL291-1569	0.60	0.30	0.21	0.09	0.07	0.04	0.49	0.40	0.30	0.19	0.17	0.13
pPIL291-15	0.85	0.83	0.64	0.47	0.18	0.06	0	0	0	0	0	0

[a] Monoclonal antibodies were used at a dilution of 1/2,000. Cell suspensions with an optical density at 660 nm of 0.1 were serially diluted as indicated. *ortho*-Phenylenediamine (0.4 mg/ml) with 0.001% (vol/vol) peroxide (30%) was used as a substrate. —, Not determined.

cell surface still occurred but with a low efficiency. However, assembly of the subunits into fimbriae was completely disturbed. In the case of HB101(pPIL291-1526), no label was found on the cell surface (Fig. 2D). This finding implies either that the mutated FelA fimbrillin was not transported to the cell surface or that the epitope recognized by M7-6 antibodies had been disturbed by the deletion.

On the basis of these results, we conclude that the deletions of (part of) hypervariable regions of the major fimbrial subunit interfere with formation of fimbriae. In the case of HB101 harboring pDH2, pDH4, or pPIL291-1526, this could be due to the absence of amino acids flanking the hypervariable regions which are conserved in the serological variants of P fimbriae (34). In plasmids pDH3 and pPIL291-1529, however, only regions coding for hypervariable parts of the protein were removed. These parts were not expected to be essential for the biogenesis of fimbriae and have been shown to encompass F-specific antigenic determinants (35). Therefore, we suppose that deletion of a hypervariable part of the fimbrillin can influence the biogenesis of fimbriae in a more indirect way, by destroying correct spacing between regions of the protein that are involved in biogenesis.

Replacement of hypervariable region 4 of the FelA fimbrillin by other amino acid sequences and its effects on formation of fimbriae. To test whether the spacing between conserved regions of the fimbrial subunit could indeed be important for biogenesis of fimbriae, amino acids of hypervariable region 4 of the FelA fimbrillin were substituted by other amino acids. In plasmid pPIL291-1529, a 12-mer oligonucleotide containing the recognition site for the restriction enzyme *Xho*I was inserted in the *Hpa*I restriction site present in the *felA* mutant gene, resulting in plasmid pPIL291-1549 (Fig. 3). In the mutated FelA protein encoded by this plasmid, hypervariable region 4 contained six amino acids that were different, compared with the seven residues in the wild-type F11 protein encoded by pPIL291-15. Similarly, a 27-mer oligonucleotide was inserted in the *felA* gene of pPIL291-1529, coding for an amino acid sequence that represents an epitope of FMDV. Meloen et al. (23) have shown that this peptide (Tyr-Lys-Gln-Lys-Ile-Ile-Ala-Pro) is indistinguishable from FMDV in its ability to bind MAb MA18, a neutralizing monoclonal antibody raised with FMDV type A10. In the resulting plasmid pPIL291-1569 (Fig. 3), both the composition and the number of nucleotides of hypervariable region 4 of the *felA* gene were different than in the wild-type gene.

HB101 cells harboring pPIL291-1549 or pPIL291-1569 formed fimbriae, as shown by ELISA with the F11-specific MAb M7-6 and electron microscopy. In both cases, the amount of F11 antigen on the cell surface was less than that in the wild-type protein (Table 1). It was observed by electron microscopy that the fimbriae encoded by HB101(pPIL291-1569) appeared to be shorter than normal F11 fimbriae or pPIL291-1549-encoded fimbriae (Fig. 2C and G).

These results show that hypervariable region 4 can be replaced by other amino acid sequences without loss of the ability to form fimbriae. There appear to be no stringent demands as to the inserted amino acid sequence, since different sequences could be inserted without loss of the capacity for fimbria formation. This finding is consistent with the hypervariability found in this region in different serological variants of P fimbriae (34). Therefore, correct spacing of the conserved regions may be more essential for biogenesis of fimbriae than is the nature of the amino acid sequence present between the regions.

Characterization of the F11-FMDV hybrid fimbriae. The mutated F11 fimbriae carrying the FMDV peptide were further characterized by means of the FMDV-specific MAb MA18 (23). The hybrid fimbriae produced by HB101 (pPIL291-1569) as well as F11 fimbriae were isolated and analyzed by SDS-PAGE. The electrophoretic mobility of the hybrid fimbrillin was shown to be less than that of the wild-type F11 fimbrillin (Fig. 4B). Western blotting experiments showed that the hybrid F11-FMDV fimbrillins, in contrast to the F11 fimbrillins, reacted with the MA18 antibodies (Fig. 4A). In an ELISA, MA18 antibodies bound also to the hybrid fimbriae (Table 1) and not to F11 fimbriae, which shows that the exposure of the FMDV epitope in the fimbrial filament is such that the epitope is recognized by this antibody.

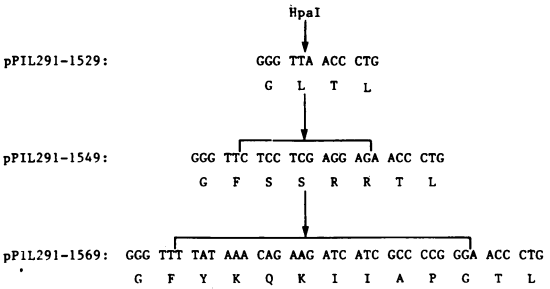

FIG. 3. Insertion of oligonucleotides into the deletion plasmid pPIL291-1529. Nucleotide and deduced amino acid sequences (one-letter codes) at the manipulated site of plasmid pPIL291-1529 and its derivatives, pPIL291-1549 and pPIL291-1569. pPIL291-1549 was constructed by insertion of a 12-base-pair DNA linker encompassing an *Xho*I restriction site in the *Hpa*I site present in pPIL291-1529 (see also Fig. 1D). pPIL291-1569 was constructed by insertion of a 27-base-pair oligonucleotide, encoding an FMDV antigenic determinant, in the same *Hpa*I site.

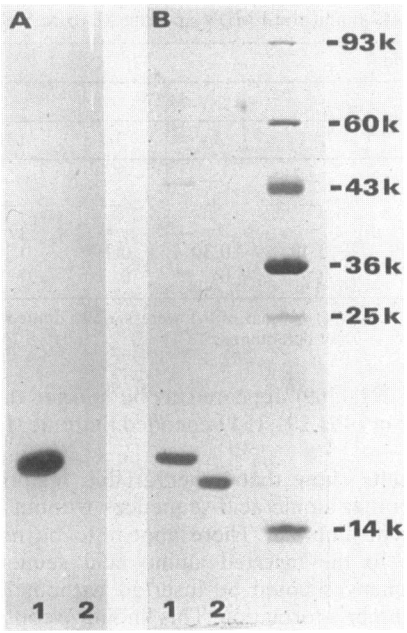

FIG. 4. SDS-PAGE patterns of purified fimbriae isolated from plasmid-containing HB101 cells. Lanes: 1, patterns of purified fimbriae isolated from HB101(pPIL291-1569) cells; 2, SDS-PAGE patterns of F11 fimbriae isolated from HB101(pPIL291-15) cells. (A) Western blot analysis of fimbrial proteins. After transfer of the proteins from a gel to the nitrocellulose filter, the filter was incubated with the FMDV-specific MAb MA18 (23), with goat anti-mouse immunoglobulin G and M peroxidase as the second antibody and 5 mM 5-amino-2-hydroxybenzoic acid–0.02% H_2O_2 as substrate. (B) SDS-PAGE patterns of the fimbrial proteins after protein staining with fast green of the blot shown in panel A. The positions of proteins used for standard molecular sizes (in kilodaltons [k]) are shown at the right.

DISCUSSION

Biogenesis of P fimbriae is a complex process that requires interaction among the major fimbrial subunit, minor fimbrial components, and accessory proteins (25). Studies of Lindberg et al. (19) and Riegman et al. (29) were focused on the role of the minor proteins in the adhesion process and biogenesis of fimbriae. Riegman et al. (29) proposed that initiation of the formation of a fimbrial filament depends on the presence of the major and minor subunits in an initiation complex in the periplasm. Further growth of the fimbrial structure appeared to be independent of minor subunits. We have already reported some characteristics of the major fimbrial subunit, and we proposed a model that predicted the presence of conserved regions that are important for biogenesis and hypervariable regions that determine the antigenic specificity of the P fimbriae (34). The hypervariable regions 2, 3, and 4 were shown to encompass P-fimbrial epitopes (35).

The present study was undertaken to survey the possibilities for exploiting the hypervariable regions for insertion of foreign antigenic determinants into the major fimbrial subunit. The major fimbrial subunit may be a suitable carrier of foreign antigenic determinants if insertion of such determinants into the protein does not interfere with biogenesis of the fimbrial structure and if the determinant is exposed in the chimeric fimbriae.

We have shown in this study that deletions in hypervariable regions 2 and 4 of the FsoA and FelA fimbrillins, respectively, drastically affect formation of fimbriae. Deletions in hypervariable region 2 generally result in unfimbriated cells. In about 1 to 5% of the cells, however, one or a few fimbriae of normal length are found. This observation may indicate that the mutated subunits have decreased stability. This supposition, however, cannot explain the occurrence of a few fimbriae of normal length. A similar phenotype was observed by Riegman et al. (29) with HB101 cells carrying pPIL110-752, a plasmid lacking the genes encoding FsoF and FsoG. In that case, subunit protein was found to accumulate in the periplasm (29). We therefore favor the hypothesis that the biogenesis of the mutants described here is disturbed at the level of initiation as well. Deletions in hypervariable region 2 or its flanking regions might result in a subunit protein that has reduced affinity for the initiation complex. This complex is not experimentally defined but may also contain the periplasmic protein D or the 75-kilodalton protein C or both. Formation of such a complex in the periplasm may be strongly retarded. However, when an initiation complex has been formed by binding of the first subunit protein, extension of the filament formation may occur normally, e.g., by coupling the subunits head to tail, resulting in the formation of a few fimbriae of normal length. The deletion in hypervariable region 4, or between regions 3 and 4, in the FelA protein completely destroys fimbria formation. In the case of HB101(pPIL291-1529), in which region 4 was deleted, fimbrial subunits are transported to the cell surface, as was deduced from immunocytochemical labeling of the cells. The low amount of gold label found on the cell surface may suggest that transport does not occur very efficiently. In this mutant, therefore, transport of the subunit protein seems to be affected to some extent and the assembly process apparently is completely disturbed. These results suggest that transport of the subunits to the cell surface and assembly of subunits into fimbriae might be two subsequent steps in the biogenesis of fimbriae that pose their own specific requirements on the subunit proteins. The minor P-fimbrial subunits, which show low but significant homology to the P fimbrillins (20, 25), may thus be regarded as fimbrial subunits that do not have the capacity to be assembled into fimbriae.

If the hypervariable regions are indeed essential for biogenesis of fimbriae by virtue of their spacer function, the nature of the amino acids present in these regions may be less important. Hypervariable region 4 could be replaced by other amino acids without interference with the formation of fimbriae. In the mutated FelA fimbrillin encoded by pPIL291-1569, the seven amino acids of hypervariable region 4 were replaced by other amino acid residues, and four additional amino acids were inserted compared with the wild-type FelA protein. HB101 cells harboring this plasmid form a reduced amount of short fimbriae, which indicates that biogenesis is affected in some way. In this case, spacing between other regions may be affected by the insertion of amino acids. One could expect, however, that insertions do not necessarily have to interfere as much as deletions with correct spacing. The effect of insertions may depend on the folding of the inserted oligopeptide.

The potential for replacing a stretch of amino acids in the fimbrial subunit without loss of the capacity for fimbria formation opens an interesting possibility for application of these fimbriae, i.e., to use them as carriers of foreign antigenic determinants. Such hybrid fimbriae might be valuable tools in the development of serodiagnostics or as components of a subunit vaccine.

Similarly, two outer membrane proteins of *E. coli*, the

LamB (4) and PhoE (1) proteins, have recently been used for expression of antigenic determinants. Fimbriae have the advantage of being extracellular proteins that are easily purified. In addition, it has been shown that fimbriae are very immunogenic (15). We show here that an antigenic determinant of FMDV, an example of a foreign antigenic determinant, can be incorporated into fimbriae. The binding of the FMDV-specific antibody with the hybrid fimbriae indicates that the FMDV antigenic determinant is exposed in this protein. Whether these hybrid fimbriae induce specific antibodies is currently under investigation. In this connection, it is interesting to mention that the hypervariable regions which appear suitable for insertion of other epitopes contain the natural P-fimbrial antigenic determinants (35). Therefore, it may be expected that other sequences inserted into this region are effective antigenic determinants.

ACKNOWLEDGMENTS

We thank Marja Agterberg for the gift of synthetic FMDV-oligonucleotide and Simon Barteling for the gift of the MAb MA18.

This project was supported by the Netherlands Technology Foundation.

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