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Lesions in Two *Escherichia coli* Type 1 Pilus Genes Alter Pilus Number and Length without Affecting Receptor Binding

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We describe the characterization of two genes, *fimF* and *fimG* (also called *pilD*), that encode two minor components of type 1 pili in *Escherichia coli*. Defined, in-frame deletion mutations were generated in vitro in each of these two genes. A double mutation that had deletions identical to both single lesions was also constructed. Examination of minicell transcription and translation products of parental and mutant plasmids revealed that, as predicted from the nucleotide sequence and previous reports, the *fimF* gene product was a protein of ca. 16 kDa and that the *fimG* gene product was a protein of ca. 14 kDa. Each of the constructions was introduced, via homologous recombination, into the *E. coli* chromosome. All three of the resulting mutants produced type 1 pili and exhibited hemagglutination of guinea pig erythrocytes. The latter property was also exhibited by partially purified pili isolated from each of the mutants. Electron microscopic examination revealed that the *fimF* mutant had markedly reduced numbers of pili per cell, whereas the *fimG* mutant had very long pili. The double mutant displayed the characteristics of both single mutants. However, pili in the double mutant were even longer than those seen in the *fimG* mutant, and the numbers of pili were even fewer than those displayed by the *fimF* mutant. All three mutants could be complemented in *trans* with a single-copy-number plasmid bearing the appropriate parental gene or genes to give near-normal parental piliation. On the basis of the phenotypes exhibited by the single and double mutants, we believe that the *fimF* gene product may aid in initiating pilus assembly and that the *fimG* product may act as an inhibitor of pilus polymerization. In contrast to previous studies, we found that neither gene product was required for type 1 pilus receptor binding.

Pili (fimbriae) are filamentous, proteinaceous bacterial appendages usually associated with the capability of bacteria to bind to one or more types of eukaryotic cells. Type 1 pili are the most common of the *Escherichia coli* attachment organelles. These pili, also referred to as somatic or common pili, are approximately 7 nm wide, 1.0 to 2.0 μm long, and are composed principally of a single protein subunit (pilin) arranged helically to form a fiber with a hollow axial core (23). Type 1 pili promote binding of *E. coli* to a number of eukaryotic cells (34, 35, 64). The adherence properties of type 1-piliated *E. coli* are inhibited by the sugar D-mannose and certain mannose analogs (59), suggesting that the eukaryotic cell receptor contains mannose or a mannose-related structure (14).

Despite the adherence-conferring properties of type 1 pili, their role as a virulence factor is unclear. The structures apparently play no role in intestinal colonization (7, 18, 51). However, recent studies have indicated that they may help facilitate colonization of the lower urinary tract (1, 21, 26, 27, 31, 58) and promote colonization (7), communicability (8), and clearance of *E. coli* from other extraintestinal sites (46). In addition, type 1 pili promote binding of *E. coli* to phagocytic cells in a fashion that may actually impede the killing of piliated cells (16, 30).

The chromosomal type 1 gene cluster is located at 98 min on the *E. coli* genetic map (5). Of the eight previously described genes involved in piliation, two (*fimB* and *fimE*) encode products that regulate transcription of *fimA* (9), the gene encoding the major structural subunit (pilin) of the pilus (62). Immediately 3′ to *fimA* are two genes (*fimC* and *fimD*) whose protein products are required, in addition to that of *fimA*, for assembly and presentation of type 1 pili on the bacterial cell surface. Recent studies indicate that the *fimD* product becomes integrated into the outer membrane and may act as a scaffold or polymerization channel for pilus assembly (33). The genes encoding minor components of the type 1 system, *fimF*, *fimG*, and *fimH*, are located distally to *fimA* (32, 50, 60) and encode products predicted to be pilinlike in structure (2, 3, 22, 23). All available evidence indicates that the *fimH* product is the type 1 pilus adhesin (2, 22, 23, 49, 50, 55); that is, it is the component that interacts directly with the eukaryotic cell receptor (37).

Previous studies have suggested that either the *fimF* or *fimG* gene product is required, in conjunction with the *fimH* product, to effect normal pilus length and receptor binding, and it has been proposed that the products of these two genes may function to present the *fimH* adhesin on the surface of the pilus structure (32, 37). However, these studies employed mutations that were carried on multicopy-number plasmids, and the results were obtained with strain HB101, which has been recently shown to contain many if not all of the genes involved in type 1 piliation (9, 13).

In this study, we have attempted to determine the roles of the *fimF* and *fimG* gene products in type 1 pilus biosynthesis by generating in-frame chromosomal deletions in each of these genes singly and in combination. All three mutant strains having single or double lesions produced type 1 pili and maintained receptor-binding capability. Examination of pilus morphology revealed that *fimF* mutants had reduced numbers of pili, whereas *fimG* mutants produced pili that were longer than normal. The double mutant displayed a cumulative effect, having even fewer pili than *fimF* mutants and even longer pili than *fimG* mutants. On the basis of these

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TABLE 1. Bacteria, bacteriophages, and plasmids used for this study

Bacterium, plasmid, or bacteriophage	Description	Source or reference(s)
Bacterial strains		
LE392	F⁻ *hsdR514*(r_K^- m_K^-) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* λ^- (recipient in plasmid transformations)	57
JM101	Δ(*lac-proAB*) *supE thi*/F' *lacI*^{q}ZΔM15 *traD36 proAB*$^+$ (used for propagating M13 strains)	52
P678	*thr-1 leuB thi-1 lacY1 malA1 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44 pilG1* Pil$^+$ (does not exhibit phase variation)	28 and 29
ORN103	*thr-1 leu-6 thi-1* Δ(*argF-lac*) U169 *xyl-7 ara-13 mtl-2 gal-6 rspL tonA2 minA minB recA13* Δ(*fimEACDFGH*)	10 and 63
ORN115	*thr-1 leuB thi-1* Δ(*argF-lac*) U169 *xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44 pilG1* Pil$^+$ (does not exhibit phase variation)	63 and 69
ORN109	*thr leu proA2 lacY1 galK his argE rpsL supE mtl xyl recBC sbcB* Pil$^+$ (has Tn*10* between *hsd* and *serB*; undergoes phase variation)	63
ORN133	ORN115; *fimH'-kan*	50
ORN153	ORN115; *fimD*::Tn*5*	P1 transduction from ORN106 (49) into ORN115
ORN154	ORN109; *fimD*::Tn*5*	P1 transduction from ORN153
ORN174	ORN154; Tc^r Kn^s Pil$^+$	Constructed by linear transformation of ORN154 with *Sal*I-cut pORN149 (see Results and Fig. 4)
ORN178	ORN115; Tc^r	See Results
ORN180	ORN115; Δ*fimG* Tc^r	See Results
ORN189	ORN115; Δ*fimF* Tc^r	See Results
ORN190	ORN115; Δ*fimF* Δ*fimG* Tc^r	See Results
Bacteriophages		
P1	*vir*	Laboratory collection
M13mp18	Contains a multiple cloning site (MCS) and expresses the N-terminal portion of *lacZ* for use as a selective marker	72
M13mp19	Same as M13mp18, except that the MCS is in the opposite orientation	72
Plasmids		
pBR322	ColE1 Ap^r Tc^r	10
pORN123	pBR322 Δ*Pvu*II (ca. 50 bp)	63
pACYC184	p15A Cm^r Tc^r	11
pORN124	Mini-F plasmid pKP1013 with a unique *Eco*RI site in the chloramphenicol resistance gene; Cm^r Sp^r	29 and 48
pSH2	pACYC184 *fimBEACDFGH* Cm^r	60
pORN147	pSH2 with *Xho*I site created at the *Pvu*II site located ca. 600 bp downstream from the end of the *fimH* gene	Laboratory stock
pORN146	pSH2 containing an *Xho*I-linked central *Bgl*II fragment from Tn*10* which contains the tetracycline resistance gene (*tetR*)	Laboratory stock
pORN148	pORN123 *fimDFGH* Ap^r	See Results and Fig. 1
pORN149	pORN123 *fimDFGH* Ap^r Tc^r	See Results and Fig. 1
pORN150	pORN149 Δ*fimG* Ap^r Tc^r	See Results and Fig. 1
pORN152	pORN149 Δ*fimF* Ap^r Tc^r	See Results and Fig. 1
pORN153	pORN149 Δ*fimF* Δ*fimG* Ap^r Tc^r	See Results and Fig. 2
pORN159	pORN124 *fimDFGH* Sp^r	This study[a]
pORN160	pORN159 Δ*fimG* Sp^r	This study[a]
pORN161	pORN159 Δ*fimF* Sp^r	This study[a]
pORN162	pORN159 Δ*fimF* Δ*fimG* Sp^r	This study[a]

[a] Plasmids pORN159 through pORN162 were constructed by insertion of the *Eco*RI fragments from pORN149, pORN150, pORN152, and pORN153, respectively (the second *Eco*RI site in each of these plasmids is located in *tetR* [see Fig. 1]), into the *Eco*RI site of pORN124 and selecting for Sp^r Cm^s.

observations, we suspect that the *fimF* product aids in starting new pili and that the *fimG* product is an inhibitor of pilus polymerization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All bacterial strains used were derivatives of *E. coli* K-12. The bacterial strains, bacteriophages, and plasmids used are listed in Table 1. The media used for bacterial cell growth included L broth, L agar (54), and brain heart infusion broth (Difco Laboratories). The antibiotic concentrations used were as follows: ampicillin, 100 μg/ml; kanamycin, 40 μg/ml; tetracycline, 10 μg/ml; spectinomycin, 50 μg/ml; and chloramphenicol, 20 μg/ml.

Genetic techniques. Transformation of bacterial strains with circular plasmid DNA was as described by Lederberg and Cohen (42). Plasmid-encoded mutant alleles were introduced into the *E. coli* chromosome by recombination following transformation of a *recBC sbcB* strain of *E. coli* (71) with linearized plasmid DNA, as modified by Orndorff et al. (63).

Once they were in the chromosome of the *recBC sbcB* strain, transfer of the alleles was accomplished via P1 transduction with techniques described by Miller (54).

Recombinant DNA techniques. Plasmids used for restriction endonuclease mapping, for subcloning, and for sequencing were isolated by the method described by Birnboim and Doly (6). Plasmid DNA was routinely purified by cesium chloride density gradient centrifugation as described by Guerry et al. (19). Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Conditions for restriction enzyme digestion and ligation reactions were as specified by the manufacturer. *Taq* polymerase was purchased from Promega and used in polymerase chain reactions (PCR) as specified by Perkin-Elmer Cetus Corporation. Restriction mapping and gel electrophoresis were carried out as previously described (53). Restriction fragments for cloning procedures were separated by low-melting-point agarose gel electrophoresis and isolated with a Nacs-52 column (Bethesda Research Laboratories). Plasmid DNA used for subcloning was routinely dephosphorylated with calf intestinal phosphatase purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. End-filling reactions were done as described by Davis et al. (12). Chromosomal DNA was isolated as described previously (4). DNA hybridizations were done by the method of Southern (68), with Nytran (Schleicher & Schuell, Inc.) as the solid support.

Sequencing procedures. Sequencing of the *fimF* and *fimG* genes was accomplished by the dideoxy chain termination method with Sequenase version 2.0 purchased from United States Biochemicals Corporation (4, 67, 70). DNA fragments encompassing the *fimFG* region were generated with restriction enzyme digests and used to construct a fragment library in both M13mp18 and M13mp19 (72). Single-stranded DNA was prepared and the inserts were sequenced with the M13 universal primer (Bethesda Research Laboratories). Gel-purified PCR products were sequenced directly by the procedure described by Kusukawa et al. (40) for sequencing double-stranded PCR products.

Minicell transcription and translation of plasmid DNA. Mutant and parental plasmids were introduced into minicell-producing strain ORN103 via transformation. After 15 h of growth in 250 ml of brain heart infusion broth, the minicells were separated from the vegetative cells through successive differential and sucrose density centrifugations as described by Gill et al. (15) and as modified by Orndorff and Falkow (61). The transcription-translation products were radiolabeled by the addition of 50 μCi of [^{35}S]cysteine to minicells suspended in cysteine-labeling medium. The radiolabeled protein products were acetone precipitated and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (41).

Protein gel electrophoresis. Partially purified pilus preparations and radiolabeled protein products isolated from minicells were denatured and separated on SDS-polyacrylamide gels (15% [wt/vol] polyacrylamide) by discontinuous gel electrophoresis as described by Laemmli (41). Polyacrylamide gels were routinely stained with Coomassie brilliant blue. Following staining and destaining, radiolabeled protein gels were soaked in scintillant (En^{3}Hance; New England Nuclear Corp.) for 30 min, rinsed with distilled water, dried, and then examined by autoradiography. X-Omat R film (Eastman Kodak Co.) was exposed on radiolabeled gels for ca. 72 h prior to being developed.

Assays for piliation. Type 1 piliation was detected by bacterial agglutination in the presence of type 1-specific antiserum (a kind gift of P. O'Hanley) obtained from rabbits immunized with purified type 1 pili. The hemagglutinating activities of the bacterial strains and purified pili were detected with guinea pig erythrocytes. Slide agglutination tests were routinely employed by mixing 25 μl of an overnight broth culture or an isolated colony from a plate culture with 25 μl of antisera diluted 1:160 (for bacterial agglutination) or with 25 μl of 3% guinea pig erythrocytes suspended in phosphate-buffered saline (for hemagglutination). Average pilus length and numbers of pili per cell were determined (after examination of hundreds of individuals) with electron photomicrographs of three representative individuals from each population. Lengths were determined with a map measure and compared by using arbitrary units. The average number of pili per cell in each strain was determined by counting individual pili. The results were compared by the Student *t* test. Significantly different results were defined to have a value of $P < 0.05$.

Partial purification of type 1 pili. Partial purification of type 1 pili was accomplished through mechanical removal of pili (via a Waring blender) followed by $MgCl_2$ precipitation and centrifugation as outlined previously (49). The amount and purity of these preparations were assessed by SDS-PAGE. In all cases examined, no protein contaminants were detected after Coomassie blue staining (data not shown). After the preparations were first adjusted to equivalent protein concentrations (ca. 500 μg/ml), assays for the ability to hemagglutinate guinea pig erythrocytes were performed with slide agglutination tests. Microtiter plate hemagglutination assays were performed by dispensing 100 μl of each pilus sample into the first row of wells of a round-bottomed 96-well microtiter plate. The samples were serially diluted twofold, and then 25 μl of a 3% solution of guinea pig erythrocytes was added to each well and mixed gently. The plates were incubated overnight at 4°C, and the hemagglutination titer was recorded as the highest dilution of pili that caused agglutination of the erythrocytes.

Electron microscopy. Strains used for examination with the electron microscope were grown overnight in static broth cultures. A drop of culture was placed on a Formvar-coated 200-mesh copper grid, and the suspension was allowed to settle for 1 to 2 min before excess liquid was removed with a paper wick. A drop of 2% phosphotungstic acid (pH 7.4) was then used to stain the grid. After 1 min, excess stain was removed with a paper wick and the grid was rinsed once with distilled water. Negatively stained preparations were examined with a Philips 410 transmission electron microscope.

RESULTS

Construction of *fimF* and *fimG* parental and mutant plasmids. The overall strategy of this study was to devise a method of constructing null mutations in the *fimF* and *fimG* genes without affecting expression of genes 3′ to the introduced lesions and to assess the effect of these lesions on pilus formation. This was accomplished by creating defined in-frame deletions in each individual gene (Fig. 1) and in both genes at the same time (Fig. 2).

A plasmid containing intact *fimF* and *fimG* genes was constructed by subcloning the 3′ end of the type 1 gene cluster (Fig. 1). Introduction of a gene for tetracycline resistance (*tetR* from Tn*10* [25]) into a unique *Xho*I site 3′ to *fimH* generated the parental plasmid, pORN149, and provided a selectable marker for the adjacent genes.

DNA sequencing of the *Pvu*II-*Pvu*II fragment from pORN149 indicated that there were two open reading frames

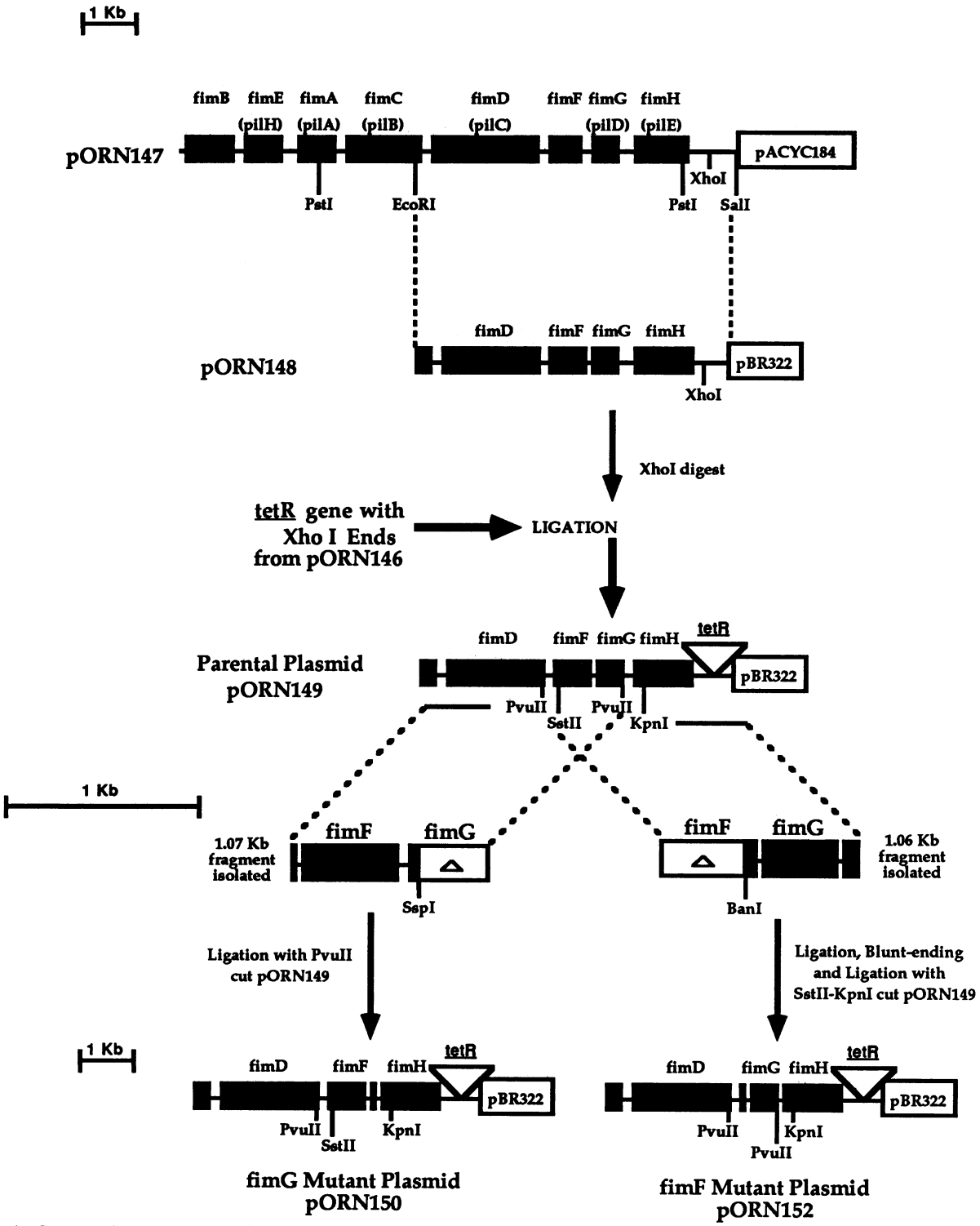

FIG. 1. Construction of the parental plasmid, pORN149, and subsequent construction of mutant plasmids pORN150 and pORN152. Plasmid pORN147 shows the genes of the *fim* region isolated from *E. coli* J96 (60). Transcription is from left to right for all of the genes (black rectangles). The upper mnemonics are those used by Bachmann (5) for *E. coli* K-12. The lower mnemonics are those of Orndorff and Falkow (60). Plasmid pORN148 was constructed by subcloning an *Eco*RI-*Sal*I fragment from pORN147 into the pBR322-derived plasmid pORN123. This fragment contained the *fimDFGH* region of the type 1 cluster and a unique *Xho*I site constructed ca. 600 bp 3′ to the *fimH* gene. This unique site allowed introduction of a *Xho*I-linked *Bgl*II fragment from Tn*10* that contains the gene encoding tetracycline resistance (*tetR*). Mutant plasmids pORN150 (Δ*fimG*) and pORN152 (Δ*fimF*) were constructed from parental plasmid pORN149 by deleting (Δ) the designated areas as described in the text.

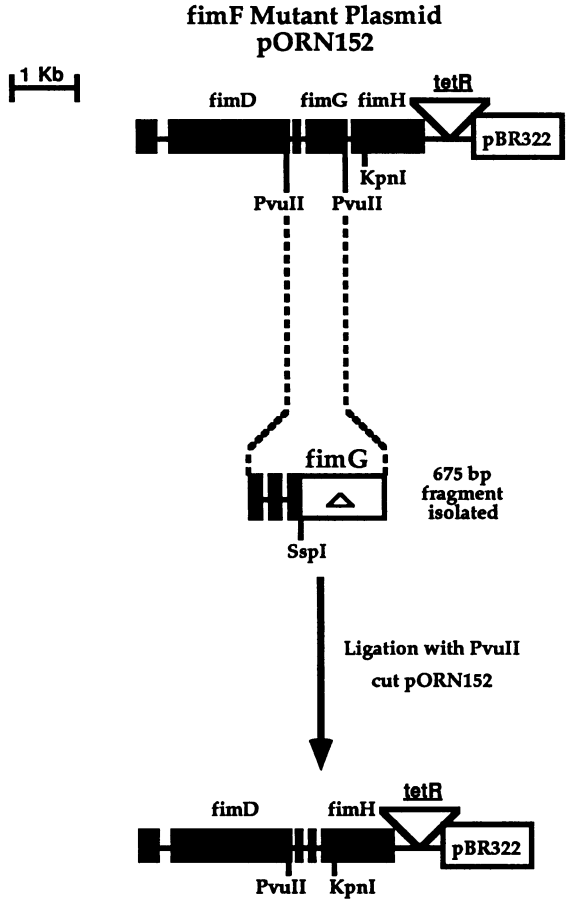

FIG. 2. Construction of the double-mutant plasmid pORN153. The *fimF* deletion plasmid, pORN152, was used as the starting material for construction of an in-frame deletion in *fimG*. Cleavage of pORN152 with *Pvu*II allowed isolation of a 675-bp fragment containing the *fimG* gene. An *Ssp*I restriction cut of this fragment resulted in removal (Δ) of 462 bp from the *fimG* gene. The remaining blunt-ended *Pvu*II-*Ssp*I fragment was introduced back into the *Pvu*II-cut pORN152 plasmid as described in the text to yield a new construct having lesions in both *fimF* and *fimG* and is designated pORN153.

(*fimF* and *fimG*), confirming what was found by Klemm and Christiansen (32) in studies of the *E. coli* K-12 type 1 gene cluster (our Fim region was isolated originally from a clinical strain of *E. coli* [28, 60]). Comparison of our DNA sequence with that reported by Klemm and Christiansen (GenBank accession no. X05672) revealed a 98% homology, and we have assumed that the few resulting differences in amino acid composition are functionally inconsequential.

Analysis of our sequencing data revealed unique restriction sites in the *fimFG* region, which allowed us to use pORN149 for the construction of, ultimately, three mutant plasmids containing deletions in this region. Restriction endonuclease digestion of the parental plasmid with either *Pvu*II or *Sst*II and *Kpn*I yielded two different fragments, each approximately 1.1 kbp in size and each containing the *fimF* and *fimG* genes (Fig. 1). Both fragments, as well as the cleaved plasmid DNA, were isolated. Further digestion of

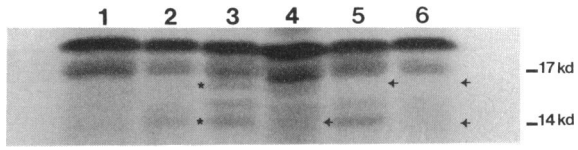

FIG. 3. Analysis of the transcription-translation products derived from parental and *fimF* and *fimG* mutant plasmid constructs with minicells. Lanes 1 and 2 are control lanes showing the background products produced by the minicell strain ORN103 and the vector plasmid pORN123, respectively. Lane 3 displays the products of parental plasmid pORN149 (*fimF*$^+$ *fimG*$^+$). Asterisks indicate the products generated by intact *fimF* (16-kDa) and *fimG* (14-kDa) genes. Lane 4 shows the products of mutant plasmid pORN150 (*fimF*$^+$ Δ*fimG*). Lane 5 shows the products of pORN152 (Δ*fimF* *fimG*$^+$). Lane 6 shows the products of pORN153 (Δ*fimF* Δ*fimG*). The arrows indicate loss of products associated with each mutation.

the isolated *Pvu*II fragment with *Ssp*I removed ca. 90% (precisely 462 bp) of the *fimG* gene. The remaining blunt-ended *Pvu*II-*Ssp*I fragment, containing an intact *fimF* gene, was isolated and ligated back into the original *Pvu*II-cut plasmid to yield a new plasmid (pORN150) containing a deletion in *fimG*. Digestion of the ca. 1.1-kb *Sst*II-*Kpn*I fragment with *Ban*I removed ca. 75% (precisely 387 bp) of the *fimF* gene. As with the previous construct, the remaining fragment, containing an intact *fimG* gene, was isolated. This *Ban*I-*Kpn*I fragment was ligated back into the original *Sst*II-*Kpn*I-cut plasmid, the remaining *Sst*II- and *Ban*I-staggered ends were blunt ended by digestion with DNA polymerase I, and then a second ligation was performed to circularize the new plasmid construct (pORN152) containing the deleted *fimF* gene.

The pORN152 plasmid was used for construction of a plasmid with deletions in both *fimF* and *fimG* (Fig. 2). By digesting pORN152 with *Pvu*II, it was possible to isolate a 675-bp fragment containing the *fimG* gene. Cleavage of this fragment with *Ssp*I resulted in removal of precisely 462 bp from the *fimG* gene in the same fashion used to construct the *fimG* deletion mutation in plasmid pORN150. The remaining blunt-ended *Pvu*II-*Ssp*I fragment was isolated and ligated back into the *Pvu*II-cut pORN152 plasmid to yield a new construct having lesions in both *fimF* and *fimG* which were the same as each individual deletion generated in plasmids pORN150 and pORN152. This double-mutant plasmid was designated pORN153.

Prior to introduction of the lesions into the *E. coli* chromosome, restriction fragment analysis, sequencing, and Southern blot analysis confirmed that the parental and three mutant plasmid constructs were as depicted (Fig. 1 and 2).

Loss of gene products due to lesions generated in the *fimFG* region. The transcription-translation products of pORN123, pORN149, pORN150, pORN152, and pORN153 were examined in minicells in order to identify the *fimF* and *fimG* gene products and to confirm loss of these products as a result of the deletions generated in the mutant plasmids. The minicell products were examined by SDS-PAGE (Fig. 3). There are two products (asterisks in lane 3) produced by the parent plasmid pORN149 (*fimF*$^+$ *fimG*$^+$) that correspond to the expected sizes of the *fimF* (16-kDa) and *fimG* (14-kDa) gene products. As predicted, mutant plasmid pORN150 (*fimF*$^+$ Δ*fimG*) is missing the 14-kDa band (Fig. 3, arrow in lane 4) but still has the 16-kDa band. Mutant plasmid pORN152 (Δ*fimF* *fimG*$^+$) is missing the 16-kDa band (arrow in lane 5) but still has the 14-kDa *fimG* product. The products of

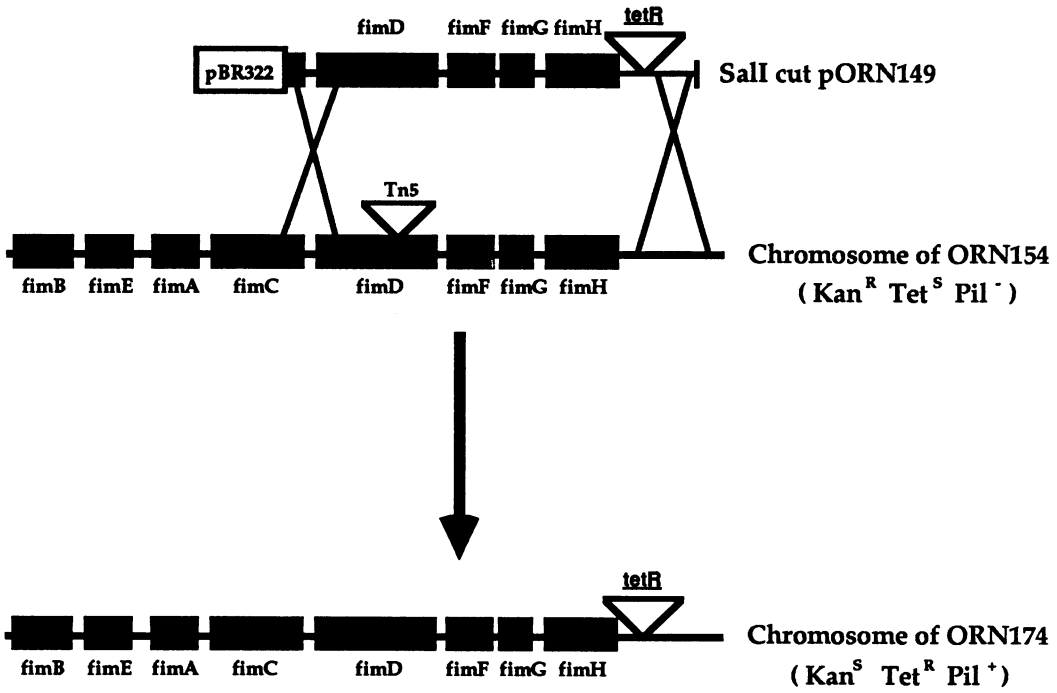

FIG. 4. Introduction of intact parental *fimF* and *fimG* genes into the chromosome of ORN154 (*fimD*::Tn*5*). *Sal*I-linearized plasmid pORN149 was introduced into ORN154 by transformation. Transformants were selected for tetracycline resistance and screened for ampicillin and kanamycin sensitivity. One such isolate, ORN174, is shown.

pORN153 (Δ*fimF* Δ*fimG*) lack both the 16- and 14-kDa bands (arrows in lane 6). The faint 14-kDa band produced by the vector (Fig. 3, lane 2) is evidently not produced after DNA is inserted, since this band is not evident in lanes 4 and 6.

Introduction of mutant alleles into the *E. coli* chromosome. In order to examine the effects of the deletion mutations under normal physiological conditions, the mutant alleles were introduced into the *E. coli* chromosome by linear transformation of plasmid DNA into a *recBC sbcB* mutant strain of *E. coli*. Such mutants are deficient in exonucleases I and V, permitting transformation of linear DNA with reduced degradation (17, 39). ORN154, a *recBC sbcB* strain of *E. coli* containing a *fimD*::Tn*5* lesion, was transformed with each of the three *Sal*I-linearized mutant plasmids and the parental plasmid, pORN149. The latter plasmid was introduced as a control to determine whether the inserted tetracycline resistance marker had any effect on pilus expression. Depicted in Fig. 4 is the linear transformation of pORN149 into strain ORN154. A double crossover restores the *fimD* gene, resulting in loss of kanamycin resistance, while the tetracycline resistance marker, located 3′ to *fimH*, is introduced into the chromosome. The *fimFGH* region located in between is thus introduced into the chromosome in Tc^r Kn^s transformants.

Isolated *recBC sbcB* transformants containing the parental allele and each of the three types of mutant alleles were transferred via P1 transduction into ORN153, a strain that does not exhibit phase variation of pilus expression (31). Since ORN153 has the same *fimD*::Tn*5* lesion found in the *recBC sbcB* strain, it was possible to select for tetracycline resistance and score kanamycin sensitivity to obtain transductants that had acquired the *fimF* and *fimG* alleles of the donor. The new strains isolated were designated as follows: parental strain ORN178 (*fimF*$^+$ *fimG*$^+$), ORN180 (*fimF*$^+$ Δ*fimG*), ORN189 (Δ*fimF* *fimG*$^+$), and ORN190 (Δ*fimF* Δ*fimG*). In addition to these strains, a prototypic (wild-type) Pil$^+$ strain, ORN115, was employed which was identical to the parental strain, except that it lacked the *tetR* gene inserted adjacent to *fimH*. This strain was used as a control to assess any effect that the adjacent *tetR* gene might have on the piliation phenotype. In none of the cases described below was there any evidence for an effect due to this insertion.

Southern blot and PCR product sequence analysis of the parental and mutant strains. Southern blot analysis supported the uncomplicated introduction of the parental and mutant *fimF* and *fimG* alleles into the chromosome (Fig. 5). That is, the numbers and sizes of the bands seen on the Southern blot correspond well with the numbers and sizes of the bands predicted to be present in the parental and mutant strains (see diagrams adjacent to blot [Fig. 5]).

Oligonucleotide primers that annealed to regions flanking the *fimFG* region were used to generate PCR products from the parental and mutant strains. Generation of these products allowed sequencing of relevant portions of the *fimFG* region in the parental and each of the three mutant strains. The results confirmed that, as expected, the mutations introduced into the chromosomes of these strains were the same as those constructed originally on the plasmids (data not shown).

Effects of *fimF* and *fimG* lesions on type 1 pilus function and morphology. The parental and all three mutant strains were piliated, as assayed by bacterial agglutination in anti-type 1 antisera, and maintained receptor-binding function (*fimH* product activity), as assayed by hemagglutination of guinea pig erythrocytes (Fig. 6). However, the double mutant displayed noticeably lower levels of bacterial and guinea pig

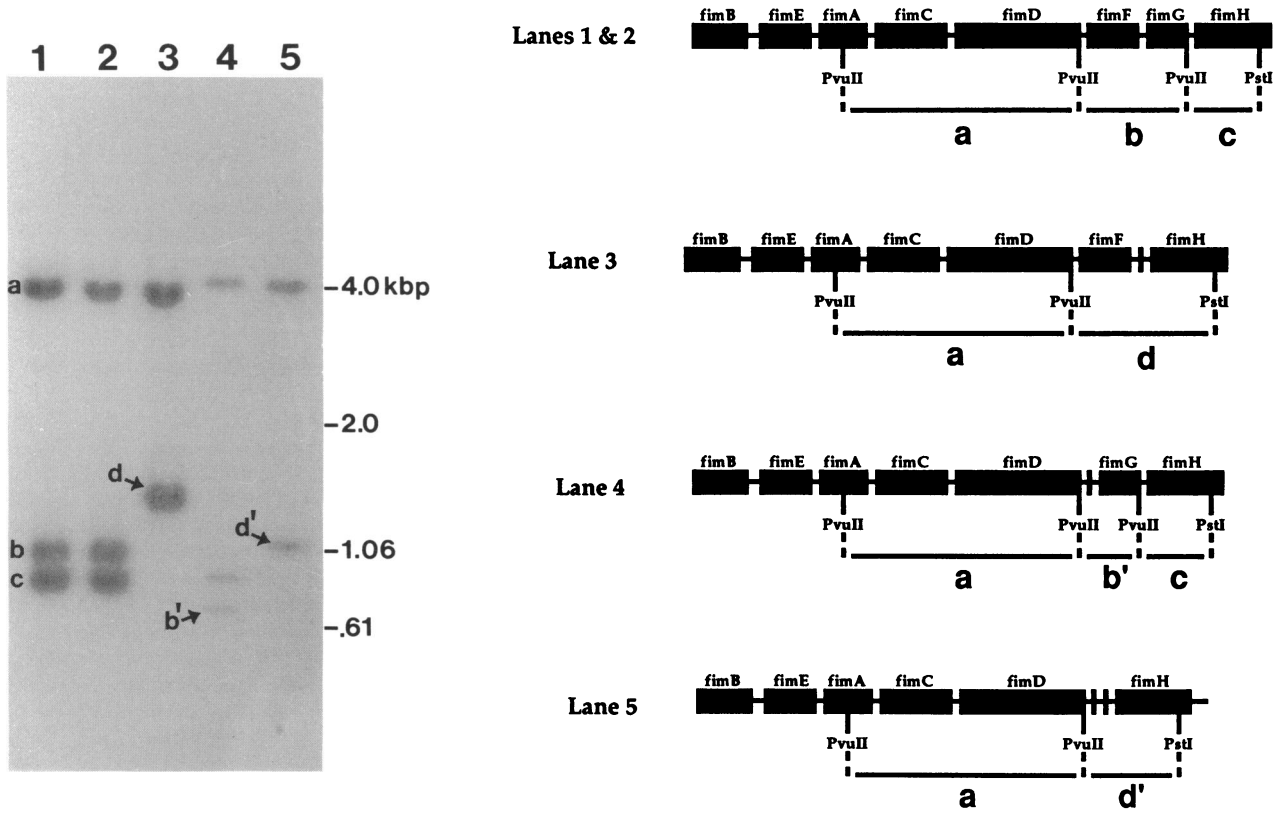

FIG. 5. Comparative Southern blot analysis of wild-type, parental, and mutant strains. Chromosomal DNA isolated from wild-type strain ORN115, parental strain ORN178, and each of the mutant strains was digested to completion with *Pvu*II and *Pst*I, separated by agarose gel electrophoresis, and transferred to Nytran. A 5.75-kbp *Pst*I fragment from plasmid pORN147 which encompassed the type 1 operon from the middle of *fimA* to almost the end of *fimH* was used as the radiolabeled probe (see Fig. 1). Fragments that hybridized (lowercase letters) corresponded well to different portions of the type 1 operon shown in the diagrams beside the blot. Size markers are designated in kilobase pairs. Lanes: 1, wild-type strain ORN115; 2, parental strain ORN178; 3, *fimG* mutant strain ORN180; 4, *fimF* mutant strain ORN189; 5, double-mutant strain ORN190.

erythrocyte agglutination when compared with the other strains (Fig. 6).

Each of the strains was subjected to electron microscopic examination. Representative individuals are shown in Fig. 7. Both the wild-type (ORN115 [Fig. 7B]) and parental (ORN178 [Fig. 7C]) strains displayed similar piliation phenotypes. All three mutants were also piliated (Fig. 7D to F). However, the mutant strains displayed distinct differences in pilus numbers or morphology from those of the parental strain. Measurement of pilus length indicated that the *fimG* mutant had pili that were significantly longer than those of the parental strain (averaging 3.6 times longer) (compare Fig. 7D with C). In the *fimF* mutant, the most obvious and significant difference was an average fourfold reduction in the number of pili (compare Fig. 7E with C). Pilus lengths in this strain were, on average, not significantly different from those of the parental strain. The double mutant (Fig. 7F) displayed the properties of both single mutants; it had fewer pili, like the *fimF* mutant, and longer pili, like the *fimG* mutant. Interestingly, the numbers of pili were even smaller and pilus lengths were significantly longer (ca. twofold) in the double mutant than in each single mutant alone (compare Fig. 7F with D and E).

Pili were partially purified from each of the strains for use in hemagglutination assays (see Materials and Methods). The recovery of pili from the double mutant was only one-fifth of that obtained from the parental strain. However, when the pilus preparations from all three mutant strains were adjusted to an equivalent protein concentration (ca. 500 μg/ml), their hemagglutination titers were identical (1:2). This evidence, combined with the consistently low yields of pili and the morphological evidence seen with the electron microscope, suggested that reduced levels of bacterial hemagglutination displayed by the double mutant (Fig. 6) resulted more from reduced numbers of pili per cell than from the production of pili defective in their ability to mediate hemagglutination. Nevertheless, this latter possibility cannot be ruled out as contributing to the weak level of hemagglutination.

Complementation studies. A complementation study of each of the three mutant strains was carried out with *fimF* and *fimG* combinations subcloned onto a single-copy-number plasmid vector, pORN124, creating plasmids pORN159 through pORN162 (Table 1). The results of these studies (Fig. 8) revealed that all three deletion mutants could be complemented to yield cells with near-parental pilus numbers and lengths, if provided with the appropriate parental genes in *trans*. However, we noticed that strains complemented with plasmids bearing the parental *fimG* gene tended to have pili that were shorter than normal. This effect can be seen by comparing the individuals shown in Fig. 8A-1 through D-1 and A-3 through D-3 with 8A.

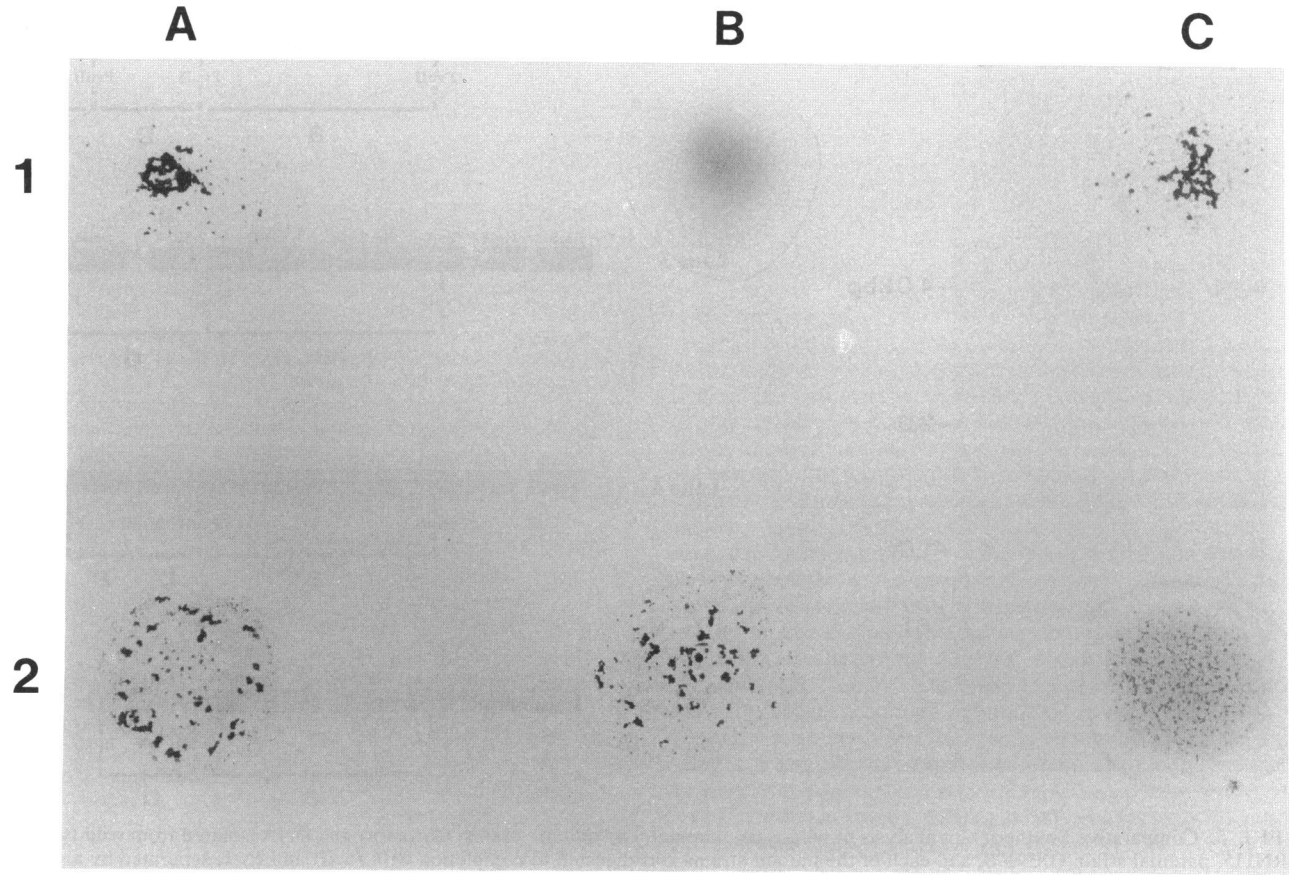

FIG. 6. Slide hemagglutination of the mutant strains. A volume of 10 μl of 3% guinea pig erythrocytes was mixed with 10 μl of each of the strains grown overnight in L broth to a constant optical density. (1A) wild-type ORN115; (1B) ORN133 (Δ*fimH*); (1C) parental strain ORN178; (2A) ORN180 (Δ*fimG*); (2B) ORN189 (Δ*fimF*); (2C) ORN190 (Δ*fimF* Δ*fimG*).

DISCUSSION

The *fimF*, *fimG*, and *fimH* genes encode products that are minor type 1 pilus components (3, 23, 32, 37). Studies of other pilus gene clusters, such as K88, K99, and Pap (pyelonephritis-associated pili), have demonstrated that minor pilus components are important for the adhesive functions of the pilus or that they are required for pilus formation to occur (20, 43–45, 56, 66). Evidence from a number of studies of the *fimH* product have indicated that it is required for the receptor-binding properties of type 1 pili (36, 49, 50) and may be incorporated at the tip and at intervals along the pilus fiber (3, 37, 65). It has been postulated that the main role of the *fimF* and *fimG* products is to work in conjunction with the *fimH* product to help mediate receptor binding (32, 37). However, up until now, defined chromosomal lesions in *fimF* and *fimG* have not been examined and no clearly defined role has been shown for these two genes or their products. The results herein presented indicate that the products of these two genes are not required for either receptor binding of type 1 pili or the actual assembly of the pilus structure but are instead needed for the normal display of pilus numbers and lengths.

In the work reported here, null mutations were generated in *fimF* and *fimG* in vitro and then introduced into the *E. coli* chromosome. This allowed us to study the role of these two genes under normal physiological conditions. All of the mutations were made in frame to reduce potential polar effects on the transcription of genes 3′ to the lesion. The following three lines of evidence indicate that this latter goal was achieved: (i) examination of transcription-translation products revealed that for each single lesion in *fimF* or *fimG*, the other product was produced, (ii) the phenotype of each single lesion could be complemented in *trans* with a single-copy-number plasmid carrying the appropriate parental gene, and (iii) activity of the product of the *fimH* gene (located 3′ to the lesions) was intact in all mutants tested. The *fimH* product not only appeared to be active but, to a first approximation, was properly located (i.e., partially purified pili from each mutant still agglutinated guinea pig erythrocytes, indicating that the *fimH* product was incorporated into pili). However, the actual amount of the *fimH* protein produced by each mutant was not determined.

The phenotypes exhibited by each single mutant and their combined effects in the double mutant suggested a way in which these two minor pilus components might act in effecting normal pilus assembly. In the parental or normal situation, the *fimF* product may be part of an envelope complex required for efficient initiation of new pili. Loss of the *fimF* product may destabilize this complex, resulting in fewer pilus initiation events. The *fimG* product could serve as an inhibitor of pilus growth (type 1 pili grow from the base [47]), perhaps by inserting into the growing pilus fiber and preventing further addition of pilin monomers. Alternatively, the *fimG* product could create a weak point in the

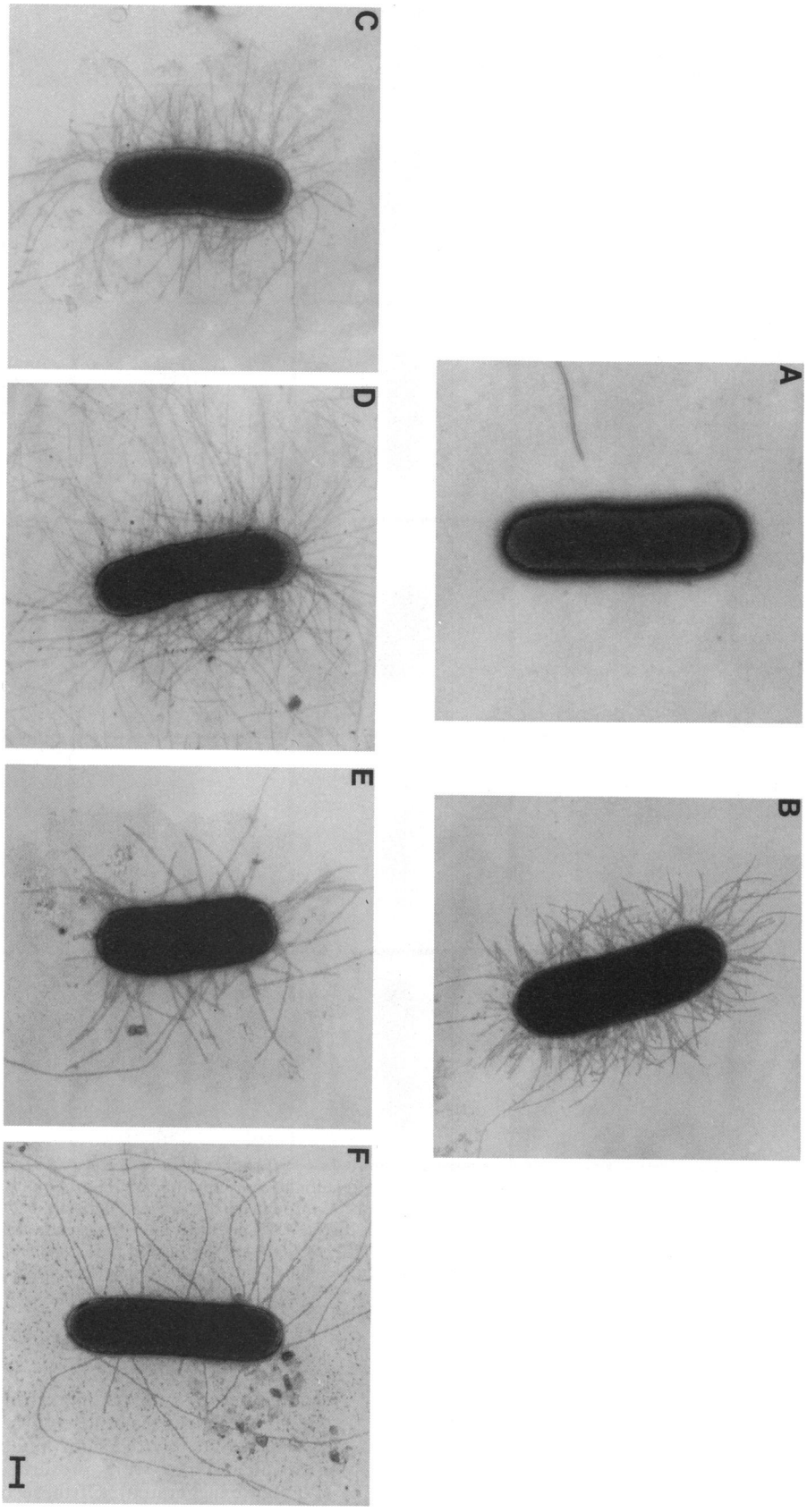

FIG. 7. Electron micrographs showing the different type 1 pilus phenotypes of the parental and mutant strains. (A) Nonpiliated strain ORN153 (*fimD*::Tn*5*); (B) wild-type strain ORN115; (C) parental strain ORN178 (ORN115 with *tetR* adjacent to *fimH*); (D) *fimG* mutant strain ORN180; (E) *fimF* mutant strain ORN189; (F) double-mutant strain ORN190. The bar in panel F equals 0.5 μm.

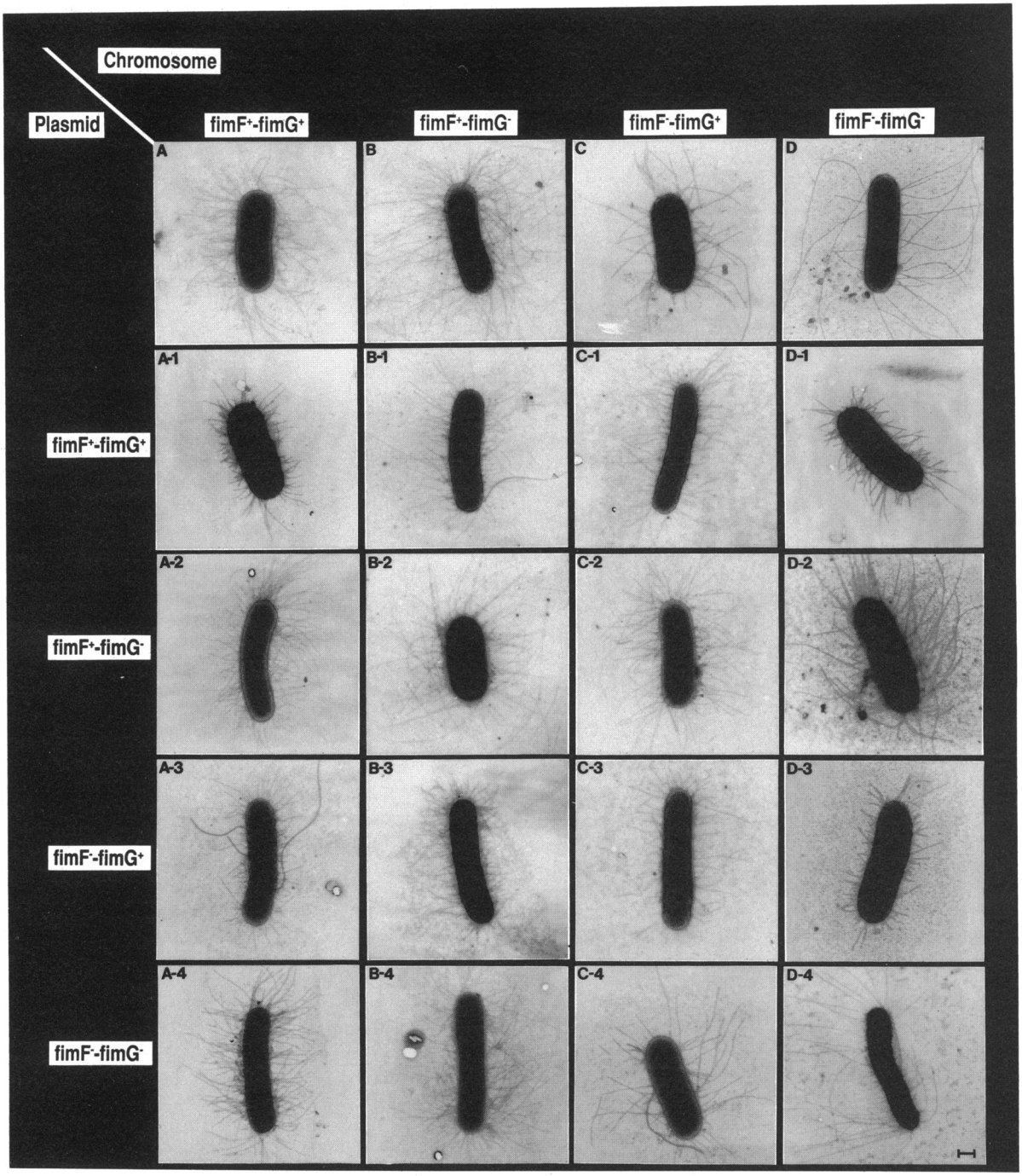

FIG. 8. Electron micrographs showing representative individuals resulting from complementation of the parental strain and the three mutant strains with plasmids bearing different parental genes. Panels A through D display parental strain ORN178, *fimG* mutant strain ORN180, *fimF* mutant strain ORN189, and the double-mutant strain ORN190, respectively. Above each column is a label designating the chromosomal genotype with respect to *fimF* and *fimG*. Deletions of *fimF* or *fimG* are designated $fimF^-$ or $fimG^-$. Panels A-1 through D-1, A-2 through D-2, A-3 through D-3, and A-4 through D-4 display the complementation of each strain with a single-copy-number vector plasmid (pORN124) containing both *fimF* and *fimG* (pORN159), a *fimG* deletion derivative (pORN160), a *fimF* deletion derivative (pORN161), and a *fimF fimG* deletion derivative (pORN162), respectively. The bar in panel D-4 equals ca. 0.5 μm.

pilus so that its addition, while not precluding further addition of pilin, would act to regulate length by generating sites for breakage.

Three key observations that support the picture described above are as follows: (i) *fimF* mutants have dramatically fewer pili per cell, but those pili are, on average, the same length as those of the parental strain; (ii) *fimG* mutants have pili that are longer than those of the parental strain, but they have approximately the same number per cell; and (iii) both single phenotypes were preserved in the double mutant (although exaggerated). Of these observations, the third is most important, because it indicates the independence of the

processes carried out by each gene product. The exaggerated effect displayed in the double mutant also reinforces the notion of separate functions since, according to the picture described above, the few pili that are started by the double mutant it cannot stop.

Biochemical lines of evidence supporting our suggested roles for the *fimF* and *fimG* products in normal pilus biogenesis may be difficult to obtain, and attempts to specifically locate these products within the pilus immunologically have been unsuccessful (3, 37). However, some predictions involve fairly straightforward consequences. For example, pilus length should be dependent on the pilin/*fimG* product ratio, which could be adjusted experimentally by using artificial promoters. In fact, the results of *fimG* complementation experiments, herein presented, and of previous work in which pilin was overproduced (61) tend to support the hypothesized role for the *fimG* product. Pilus initiation may prove to be a more complex problem to address in a straightforward manner.

A clue to the general complexity of the process of establishing proper pilus numbers and lengths at the molecular level may be illustrated by two additional observations: (i) certain point lesions in *fimH* (which encodes the adhesin) result in strains that, depending on the lesion, resemble either the *fimG* mutant or the double mutant (24); and (ii) *fimH* mutants with insertion lesions in the 5′ end, but not the 3′ end, of *fimH* produce long pili reminiscent of those of the *fimG* mutant (50). Thus, the adhesin encoded by *fimH* may also be involved in effecting normal pilus number and morphology, perhaps by interacting with the *fimF* and *fimG* products. Although this hypothetical interaction is evidently not required for incorporation of functional *fimH* product into the pilus, it may be necessary for the proper functioning of the *fimF* and *fimG* products and could be the ultimate reason for the changes in pilus morphology and numbers observed in certain *fimH* mutant strains (24, 50).

Previous models of type 1 pilus assembly have compared *fimF* and *fimG* with *papE* and *papF* (32, 37). The latter two genes are located at a similar position in the *pap* (pyelonephritis-associated pili) gene cluster and are approximately the same size as *fimF* and *fimG* (20, 32, 44). Thus, our suggested roles for the *fimF* and *fimG* products may be usefully compared and contrasted with the suspected roles of the *papE* and *papF* products. The *papE* and *papF* products are minor *pap* pilus components involved in properly locating and presenting the *papG* product (the adhesin) at the tip of the pilus (43–45). Loss of *papF* results in loss of receptor-binding functions and a drastic reduction in pilus numbers. The latter property may suggest an initiating function for the *papF* product (43, 45). Such an initiating role would be similar to our suggested role for the *fimF* gene product in this study. However, in contrast to *papF*, we found that *fimF* was not required for the proper adherence of type 1 pili. Loss of the other Pap gene, *papE*, causes lowered binding capacity due perhaps to an altered tip structure (38), but *papE* mutants have normal pilus numbers and, except for the tip, normal morphology (38, 43). This differs dramatically from the phenotype displayed by our *fimG* mutant, which has greatly increased pilus lengths but normal adherence properties. Most recent evidence on *pap* pilus structure suggests a higher-order structure for the tip (38). Such a tip structure has not been noted for type 1 pili.

In conclusion, our results demonstrate that the receptor-binding function of type 1 pili is independent of the *fimF* and *fimG* products. However, these products do appear to be involved in proper pilus assembly, as shown by the aberrant lengths and numbers of pili displayed by our mutant strains. Previous findings which suggest a role for *fimF* or *fimG* in receptor binding (32, 37) may be due to the drastically different approach used to construct the mutations in the *fimF* and *fimG* genes.

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