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# The Product of the *fimI* Gene Is Necessary for *Escherichia coli* Type 1 Pilus Biosynthesis

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**Site-directed mutagenesis was employed to create lesions in *fimI*, a gene of uncertain function located in the chromosomal gene cluster (*fim*) involved in *Escherichia coli* type 1 pilus biosynthesis. Chromosomal *fimI* mutations produced a piliation-negative phenotype. Complementation analysis indicated that a *fimI′-kan* insertion mutation and a *fimI* frameshift mutation produced polarity-like effects not seen with an in-frame *fimI* deletion mutation. Minicell analysis associated *fimI* with a 16.4-kDa noncytoplasmic protein product (FimI). We conclude that FimI has a required role in normal pilus biosynthesis.**

Type 1 pili are filamentous proteinaceous appendages produced by many members of the *Enterobacteriaceae* (28). In *Escherichia coli*, the pili are made principally of a repeating monomer, FimA, the product of the *fimA* gene (11, 21), that is arrayed helically to form a hollow-cored fiber (5). There are at least three minor pilus proteins that are organized into structures seen on the ends of pili (10) and may also be present in the pilus fiber (24). One of these minor components, FimH (the product of the *fimH* [*pilE*] gene [15]), is the molecule that binds to mannose-containing receptors on eucaryotic cells (13).

The molecular interactions needed to construct *E. coli* type 1 pili have been examined in some detail (28). However, *fimI*, one of the nine *fim* genes clustered at centisome 98 on the *E. coli* genetic map, has not been well characterized and may have a role in the biosynthetic process. The *fimI* gene is found in both *E. coli* (12) and *Salmonella enterica* serovar Typhimurium (25). In both cases *fimI* was identified as an open reading frame located adjacent and 3′ to *fimA* and predicted to encode FimA-like proteins with mature molecular masses of ca. 17 kDa in the case of *E. coli* and 19 kDa in the case of *S. enterica*. Only recently have *E. coli* mutants been isolated with transposon insertions in *fimI* (2, 4). Fimbriation is eliminated in these mutants, suggesting a required role for the putative *fimI* product in pilus biosynthesis. However, polar effects of the insertions on the expression of downstream genes (whose products are known to be required for pilus assembly [20]) could not be ruled out (2, 4).

In this report, we employed site-directed mutagenesis to show that insertion and frameshift mutations in *fimI* indeed appeared polar. However, even when polarity was rendered undetectable by our employment of an in-frame Δ*fimI* mutant, *fimI* was still required for pilus biosynthesis. We also associated a 16.4-kDa noncytoplasmic protein with the *fimI* coding region.

**Bacterial strains, plasmids, and growth conditions.** Bacterial strains, all *E. coli* K-12 derivatives, along with the plasmids used are listed in Table 1. Media consisted of L broth and L agar (17) except where otherwise noted. Antibiotic concentrations were as described previously (21).

**Site-directed mutagenesis of *fimI*.** The steps necessary for the *fimI′-kan* insertion mutation and the *Pme*I linker insertion to generate a frameshift mutation are summarized in Fig. 1. Briefly, plasmid pORN308 containing the *fimI* gene flanked by *Eco*RI and *Xho*I restriction endonuclease sites was obtained from plasmid pORN140. The *Ase*I restriction endonuclease site in *fimI* was modified by *Pme*I linker mutagenesis following the elimination of a vector-borne *Ase*I site by end-filling and religation (22). The resulting pORN309 plasmid was then used in combination with the original pORN140 plasmid to create pORN310, a plasmid identical to the starting pORN140 plasmid except for the *Pme*I lesion in *fimI*. Subsequent addition of the kanamycin resistance cassette from Tn*903* (7) into the site created by *Pme*I linker mutagenesis produced plasmid pORN311. The transcriptional orientation in the cassette was the same as that of the *fim* cluster. Plasmid pORN315 carrying the in-frame *fimI* deletion mutation (Δ*fimI*) was generated by means of PCR amplification of the *Pme*I-linearized plasmid pORN309. The amplicons, each having the beginning of *fimI* on one tip and the end of *fimI* on the other, were digested with *Bgl*II (a site present in the oligonucleotide primers) (Table 1) and ligated, and a representative clone was obtained by standard methods.

**Introduction of the mutant *fimI* alleles into the chromosome.** The *fimI′-kan* insertion mutation was introduced into the *E. coli* chromosome via linear recombination (26) to create strain ORN220. We subsequently replaced the insertion mutation with the unmarked *fimI* alleles by using the streptomycin counter-selection technique devised by Skorupski and Taylor (27) as illustrated for the *fimI′-Pme*I allele (Fig. 2). After chromosomal introduction, PCR amplicons of both unmarked mutant alleles were completely sequenced (University of North Carolina Automated DNA Sequencing Facility, Chapel Hill). The *fimI′-Pme*I allele was identical to the parental allele except that it contained an insertion in the central portion of the gene at the former *Ase*I site of seven tandem *Pme*I sites [$(GTTTAAAC)_7$], producing stop codons in all three reading frames and truncating the predicted product (normally, 160 amino acids long) after 118 amino acids. The Δ*fimI* mutation

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TABLE 1. Bacterial strains, phage, and plasmids used in the study

| Strain, phage, or plasmid | Description | Source or reference |
|---|---|---|
| *E. coli* | | |
| ORN103 | *thr-1 leu-6 thi-1* Δ(*argF-lac*)*U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 minA minB recA13* Δ(*fimEAICDFGH*) | 23 |
| ORN178 | *thr-1 leuB thi-1* Δ(*argF-lac*)*U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44 pilG1*, *tetR* inserted ca. 200 bp 3′ to the end of *fimH*, $Tet^r\lambda^r$ $Pil^+$ (does not exhibit phase variation of piliation) | 26 |
| ORN172 | *thr-1 leuB thi-1* Δ(*argF-lac*)*U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44 pilG1* $\lambda^r$ Δ(*fimBEAICDFGH*) | 29 |
| ORN220 | ORN178 except that the *fimI′-kan* allele from pORN311 is in the chromosome and the strain is nalidixic acid resistant | This study[a] |
| ORN221 | ORN220 except that *fimI′-Pme*I from pORN313 is in the chromosome | This study |
| ORN228 | ORN220 except Δ*fimI* from pORN316 is in the chromosome | This study |
| Bacteriophage | | |
| P1 | *vir* | Laboratory collection |
| Plasmids | | |
| pACYC184 | P15A $Cm^r$ $Tc^r$ | 6 |
| pBK-CMV | $Kan^r$ | Stratagene |
| pKAS32 | *oriR*6K *oriT rpsL* $Ap^r$ | 27 |
| pSH2 | pACYC184 *fim*(*BEAICDFGH*) $Cm^r$ | 8 |
| pORN113 | pSH2 *fimC*::Tn*5* (*pilB*::Tn*5*) | 21 |
| pORN140 | pSH2 except that the *Pst*I site in *fimA* is replaced by an in-frame *Xho*I site, $Pil^+$ | This study[b] |
| pORN308 | pBK-CMV *fimI* | This study |
| pORN315 | pBK-CMV Δ*fimI* (an in-frame deletion) | This study[c] |
| pORN309 | pBK-CMV *fimI* with the *Ase*I site eliminated and modified by a *Pme*I linker insertion | This study |
| pORN310 | pORN140 with the *fimI′-Pme*I lesion | This study |
| pORN311 | pORN310 with a *fimI′-kan* insertion replacing the *fimI′-Pme*I lesion | This study[d] |
| pRN2010 | ColE1 $Sp^r$ $Tc^r$ | 21 |
| pORN104 | pRN2010 *fimBEAICDFGH* $Sp^r$ | 21 |
| pORN312 | pRN2010 *fimBEACDFGH fimI′-Pme*I $Sp^r$ | This study[e] |
| pORN313 | pKAS32 with the *fimI′-Pme*I allele from pORN309 carried on a ca. 1.5-kb *Xho*I-*Eco*RI fragment | This study |
| pORN316 | pKAS32 with the Δ*fimI* allele from pORN315 carried on a ca. 0.8-kb *Xho*I-*Eco*RI fragment | This study |

[a] Strain ORN220 was constructed by performing a linear transformation of *Eco*RI-digested pORN311 using the methods of Russell and Orndorff (26). P1 phage was grown on $Kan^r$ $Tet^s$ recombinants, and the lysate was used to introduce the *fimI′-kan* insertion mutation into strain ORN178 by transduction (26). A spontaneous nalidixic acid-resistant variant of an isolated transductant was obtained by standard methods.

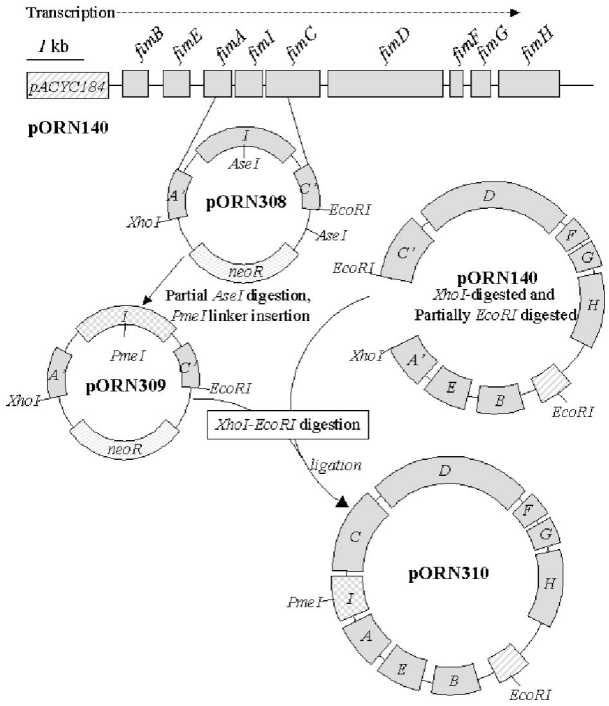


FIG. 1. Construction of the *fimI′-Pme*I mutation. The parental pORN140 *fimI* allele on an *Xho*I-*Eco*RI restriction endonuclease fragment was ligated with *Xho*I-*Eco*RI-cleaved pBK-CMV (Stratagene) to create pORN308. This ligation mixture was introduced into *E. coli* DH5α (Invitrogen) by transformation (14). Partial *Ase*I digestion and *Pme*I linker insertion followed, producing pORN309. The mutant *fimI′-Pme*I allele was then introduced into the original plasmid (pORN140) as diagrammed to create pORN310, and the *neoR* cassette was added at the unique *Pme*I site to produce pORN311. The *neoR* gene confers kanamycin resistance. The pACYC184 cloning vector confers chloramphenicol resistance. Additional details for the constructions are described in the text and in note *b* to Table 1.

removed ca. 90% of the central region of *fimI* and had the substituted *Bgl*II restriction endonuclease site carried in frame.

**Complementation.** All chromosomal *fimI* mutants were negative for piliation as indicated by the failure of the mutants to agglutinate guinea pig erythrocytes and to agglutinate in antiserum raised against purified pili (26). Further, no pili were visible upon electron microscopic examination (16) of negatively stained preparations (data not shown). Minicell analysis of the *fimI′-kan* insertion mutant strongly suggested that po-

[b] Plasmid (pORN140) contains a unique, in-frame, *Xho*I site replacing the normal *Pst*I site in the *fimA* gene. The steps in the construction were the same as those described by Orndorff and Falkow (22). The mutation created by the insertion is silent in this in-frame context.

[c] Oligonucleotide primers (P1, 5′GCGCTTTTAGATCTCAGGCCTGGTTC TCTTTAACC, and P2, 5′GCGCTTTTAGATCTGATACTGAACCTTGAAG GTCGC) were used to generate pORN315 from PCR amplification of pORN309 as described in the text.

[d] The kanamycin cassette from pACYC177 (Invitrogen) was obtained following *Pst*I digestion; the ca. 1-kb fragment was blunt ended with DNA polymerase I and ligated into *Pme*I-digested pORN310. The ligation mixture was introduced into *E. coli* DH5α by transformation with selection for kanamycin and chloramphenicol.

[e] Plasmid pORN312 was constructed by cloning the 11.2-kb *Sal*I fragment from pORN310 containing the *fim* genes with the *fimI′-PmeI* lesion into *Sal*I-digested pRN2010.

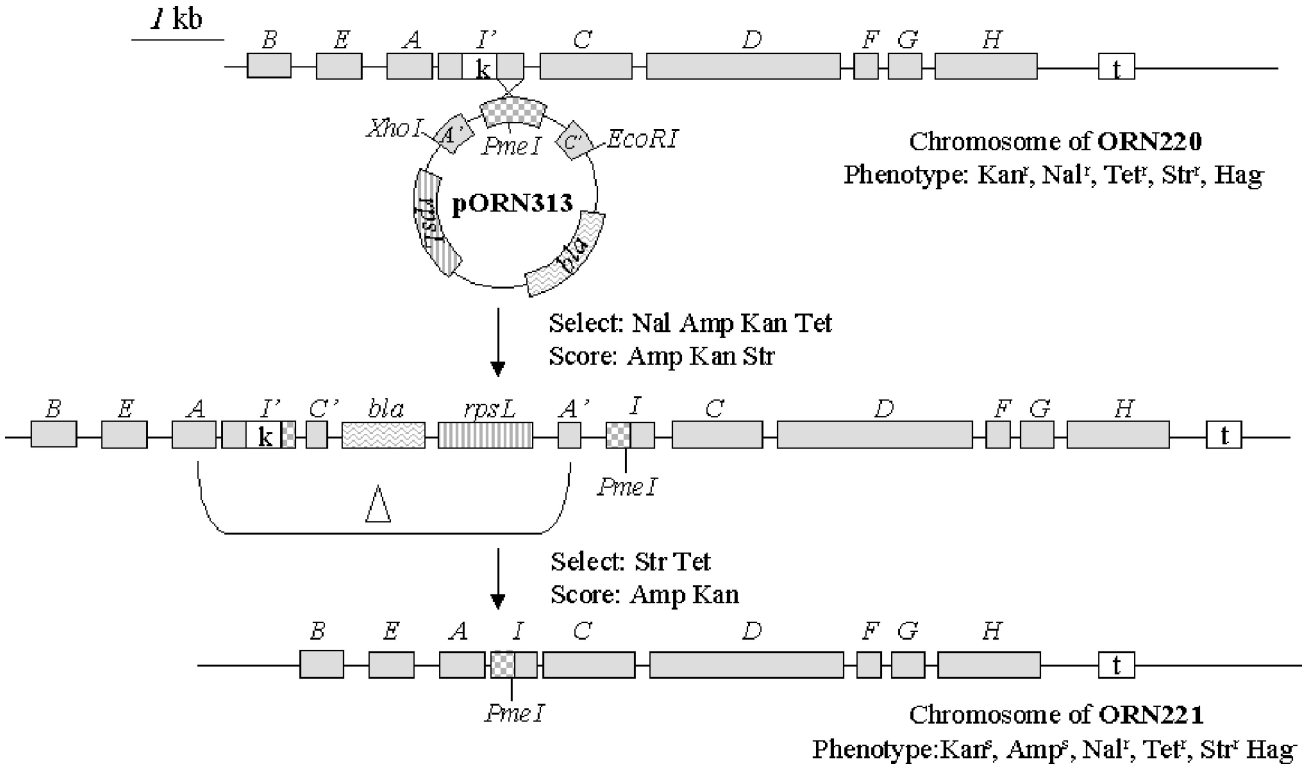


FIG. 2. Introduction of the *fimI'-Pme*I allele on pORN313 into the chromosome of ORN220. Mating and allelic exchange were performed according to the methods of Harris et al. (9). Capital letters indicate corresponding *fim* genes; *rpsL* and *bla* are genes for streptomycin resistance and ampicillin resistance, respectively. Phenotypic designations denote sensitivity (s) or resistance (r) to the following antibiotics: kanamycin (Kan), tetracycline (Tet), ampicillin (Amp), streptomycin (Str), and nalidixic acid (Nal). Hemagglutination is designated phenotypically as Hag. A delta (Δ) denotes a deletion. The *neoR* gene inserted into *fimI* and the *tetR* gene inserted adjacent to the *fim* cluster are denoted by boxes labeled with k and t, respectively. The *neoR* and *tetR* genes confer resistance to kanamycin and tetracycline, respectively.

larity could be a factor in reducing the normal expression levels of two downstream genes (*fimC* and *fimD*) (data not shown). To test whether polarity was a factor in the failure of the *fimI* mutants to produce pili, a recombinant plasmid containing all of the *fim* genes, except for *fimC* (a gene immediately downstream from *fimI* whose expression is required for piliation), was introduced into the strains containing the chromosomal mutant *fimI* alleles, and each strain was examined for its ability to agglutinate erythrocytes. The results (Table 2) indicated that the in-frame Δ*fimI* mutant was the only one that was successfully complemented to restore hemagglutination to levels that were statistically the same as for the parental strain bearing the same plasmid (Student's *t* test, $P < 0.05$). The reason for the somewhat suppressive effect of the complementing plasmid on parental piliation levels (Table 2) is unknown, but we attribute it to suboptimal ratios of various pilus components needed for the most efficient biogenesis. Levels of piliation in the parental and Δ*fimI*-complemented strains were similar also when the strains were viewed electron microscopically (data not shown).

**Association of a 16.4-kDa protein product with *fimI*.** In order to identify the *fimI* product, the *fim* region containing the *fimI-Pme*I lesion from pORN310 was cloned into another vec-

TABLE 2. Complementation of the chromosomal *fimI* mutant alleles in *trans*[a]

| Strain | Relevant chromosomal property | Plasmid | Relevant plasmid property[b] | Erythrocyte agglutination as a % of value for parental strain[c] |
|---|---|---|---|---|
| ORN178 | Parental | pACYC184 | NA | 100 |
| ORN178 | Parental | pORN113 | *fimBEAIDFGH fimC*::Tn*5* | 66 ± 5 |
| ORN220 | *fimI'-kan* | pACYC184 | NA | 0 ± 0 |
| ORN220 | *fimI'-kan* | pORN113 | *fimBEAIDFGH fimC*::Tn*5* | 0 ± 0 |
| ORN221 | *fimI'-Pme*I | pACYC184 | NA | 0 ± 0 |
| ORN221 | *fimI'-Pme*I | pORN113 | *fimBEAIDFGH fimC*::Tn*5* | 18 ± 8 |
| ORN228 | Δ*fimI* | pACYC184 | NA | 0 ± 0 |
| ORN228 | Δ*fimI* | pORN113 | *fimBEAIDFGH fimC*::Tn*5* | 64 ± 2 |

[a] Overnight cultures were concentrated ca. 10-fold and tested for their ability to agglutinate guinea pig erythrocytes in plate agglutination assays (9).
[b] The *fimC* gene lies immediately 3′ to *fimI.* See the text for additional details. NA, not applicable.
[c] $Log_2$ values of the reciprocals of the agglutination titers were compared after normalization to the values for the parental strain (100%). Values are averages (± standard deviations) of the results of at least three separate experiments.

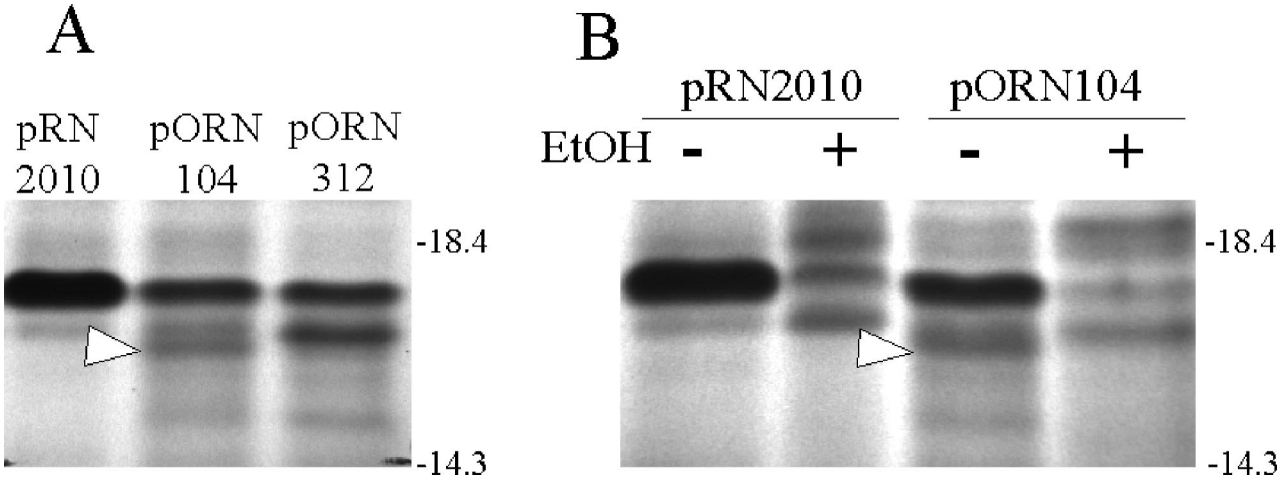


FIG. 3. (A) Transcription and translation of *fimI* in minicells. Minicells from strain ORN103 were isolated and labeled with [$^{35}$S]cysteine. Radiolabeled products were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Orndorff and Falkow (21) except that the radiolabeled products were not hydrolyzed in acid prior to solubilization in the sample buffer and the ratio of acrylamide to bis-acrylamide was adjusted to 38:1. A portion of an autoradiogram of the gel is shown. Plasmids were pRN2010 (cloning vector), pORN104 (*fimI*), and pORN312 (*fimI′-Pme*I). An arrow denotes the putative *fimI* product. (B) Minicells were obtained and incubated as described above except that 8% ethanol (EtOH) was employed (where indicated) to inhibit signal sequence processing during radiolabeling. An arrow denotes the putative *fimI* product. The positions and sizes (in kilodaltons) of molecular markers (Invitrogen) are indicated.

tor (pRN2010), creating pORN312. This plasmid was used in conjunction with an earlier construct (pORN104 [21]) that employed the parental *fim* cluster in the same vector. The change in vector was necessary in part because we anticipated that vector-encoded bands from pACYC184 would obscure the region where the *fimI* product was expected to migrate. Transcription and translation of plasmid-encoded *fimI* in minicells revealed that a band migrating with an apparent molecular mass of 16.4 kDa was absent in minicells harboring a plasmid with a *fimI′-Pme*I lesion (pORN312) but not from minicells harboring an otherwise identical plasmid with the parental *fimI* allele (pORN104) (Fig. 3A). Experiments using 8% ethanol to inhibit signal peptide processing (21) revealed that the mature 16.4-kDa FimI band was absent when ethanol was present during radiolabeling (Fig. 3B). The predicted higher-molecular-weight form of FimI was not identified (possibly because of obscuring bands). Nevertheless, the results indicated a noncytoplasmic protein. Both the extracytoplasmic location and the apparent size of the FimI band were in good agreement with DNA sequence-based predictions (3). FimI production was also noted in a minicell analysis of pORN308, which contained just the *fimI* gene. However, noticeably lower levels of FimI were seen (data not shown). Low expression of *fimI* and/or FimI instability when the protein is synthesized separately from the rest of the *fim* cluster may explain why this clone did not complement any of the chromosomal *fimI* lesions.

**Conclusions.** We compared the phenotypes of three chromosomal mutants, each bearing a site-directed mutation in *fimI*, a gene whose putative product is thought to have a role in type 1 pilus biogenesis. All lesions in *fimI* resulted in a piliation-negative phenotype. However, complementation analysis revealed that the insertion and frameshift lesions produced effects consistent with polarity on the transcriptionally downstream gene (*fimC*). Minicell analyses associated the *fimI* coding region with a noncytoplasmic protein with an apparent mature (processed) molecular mass of 16.4 kDa.

The proposed start site of *fimI* translation in *E. coli* K-12 is at nucleotide 4541188 (3). However, if both the predicted translation start site and the signal sequence cleavage site are assumed, then the predicted FimI precursor protein has an unusually long (55-amino-acid) signal sequence (a discrepancy often observed when the predicted translation initiation site is too far upstream [18]). Additionally, the proposed start site places the beginning of *fimI* translation within the preceding *fimA* coding region. In view of the foregoing, we feel that a more likely translation initiation site corresponds to the ATG codon beginning at nucleotide 4541296 (3). Such a start site eliminates translational overlap and predicts a more common 19-amino-acid signal sequence.

The role of the *fimI* product in pilus biosynthesis is unknown. Results of two recent studies in which *fimI* chromosomal insertion mutants were identified (2, 4) suggested that *fimI* is required for pilus biosynthesis. However, in both studies the authors acknowledged that insertion mutations might have polar effects and, thus, did not draw any conclusions as to the requirement for the putative *fimI* product in pilus biogenesis. Our results justify their concerns. In one of the studies (4), the authors concluded that FimH, the adhesive component of the pilus (20), could still be synthesized and become located on the cell surface in the absence of a *fimI* product and pilus. This conclusion was based upon the observation that one of their two *fimI* insertion mutants could still bind epithelial cells. Whereas our results support the idea that the *fimI* product is required for pilus biosynthesis, we found no FimH activity in any of our *fimI* mutants. However, we cannot rule out the possibility that under different assay conditions, *fimI* mutants might produce a more equivocal phenotype with regard to piliation and/or FimH expression.

Our observation that FimI did not have the characteristics of a cytoplasmic protein suggested that FimI has a direct role in pilus biosynthesis (as opposed to an indirect regulatory role). Regarding the possible presence of FimI in the pilus, biochemical evidence from purified type 1 pili (amino acid composition studies, N-terminal sequencing [19]) suggests that FimI cannot constitute a major portion of purified pili. (This assumes that FimI is not specifically lost during the pilus purification process.) However, FimI may be a minor component of the pilus or simply be required for some currently unappreciated phase of the assembly process (20).

One previous report (25) speculated that FimI could function analogously to PapH, a protein described by Baga et al. (1), whose loss through mutation produces mutants with long and cell-dissociated pyelonephritis-associated pili. This phenotype suggested to Baga et al. that PapH was involved in membrane anchoring and pilus length modulation. Our studies do not support such a role for FimI in that no long or dissociated pili were noted in any of the *fimI* mutants. Further work will be required to discern how FimI fits into the already-complicated picture of pilus biosynthesis (28).

**Nucleotide sequence accession numbers.** The parental *fimI* allele (from strain ORN178) was identical to the corresponding gene of *E. coli* K-12 (3) (accession number AE000502). The *fimI′-PmeI* and Δ*fimI* genes have accession numbers AF424784 and AY255626, respectively.

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