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Inversion-Independent Phase Variation of Type 1 Fimbriae in *Escherichia coli*

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The roles of *fimB* and *fimE* in the phase-variable expression of type 1 fimbriae in *Escherichia coli* were examined. A method was developed to study the effects of *fimB* and *fimE* on both recombination of the *fim* invertible element and fimbrial expression. The method used an allelic exchange procedure consisting of two steps. The first step, construction of intermediate strains, deleted *fimB* and *fimE*. This step locked the invertible element in either the on or the off orientation. The second step of the exchange procedure introduced either wild-type or mutant alleles of *fimB* and/or *fimE* into the chromosome of the intermediate strains. Analysis of the resulting strains supported the current, plasmid-based model of recombination. Unexpectedly, strains in which the invertible element was locked in the on orientation (either by mutation of both *fimB* and *fimE* or, in a control strain, by mutation of the left inverted repeat sequence of the invertible element) continued to exhibit phase-variable expression of type 1 fimbriae. A strain in which *fimA* was transcribed from the *tac* promoter continued to exhibit phase-variable fimbrial expression, suggesting that inversion-independent phase variation cannot be explained by variable transcription initiation of *fimA*.

The expression of type 1 fimbriae in *Escherichia coli* exhibits on-off phase variation. This phase variation correlates with the orientation of a short, invertible DNA sequence located immediately upstream of *fimA*, the gene encoding the major fimbrial subunit (1, 15, 18). The promoter for *fimA* is believed to reside within this invertible element and to direct the transcription of *fimA* when the element is in one orientation ("on") but not when the element is in the alternate orientation ("off"). Furthermore, the orientation of the *fim* invertible element correlates with the Fim phenotype (8, 18).

Two genes, *fimB* and *fimE*, map immediately adjacent to the *fim* invertible element and are believed to encode site-specific recombinases (8, 24, 29, 35, 36, 38). The current model of the roles of *fimB* and *fimE* in inversion suggests that *fimB* promotes recombination of the *fim* invertible element in both directions, whereas *fimE* promotes recombination primarily from on to off (29). However, this model is based on a recombinant plasmid-based assay with which fimbrial expression cannot be examined (29).

In the present study, the roles of *fimB* and *fimE* in the phase-variable expression of type 1 fimbriae were examined by use of a method that preserved the native location, organization, stoichiometry, and topology of the recombination elements. Thus, in contrast to the plasmid-based assay (29), in this assay both recombination and fimbrial expression could be examined. Analysis of the recombination results supported the current, plasmid-based model of the roles of *fimB* and *fimE*. Additionally, examination of fimbrial production indicated that the on orientation of the *fim* invertible element was necessary but not sufficient for fimbrial expression; strains in which the invertible element was locked in the on orientation continued to exhibit phase-variable expression of type 1 fimbriae.

MATERIALS AND METHODS

Bacterial and bacteriophage strains and media. The bacterial strains are all derivatives of *E. coli* K-12 and are listed, with genotype and source, in Table 1. Bacteria were grown in Luria-Bertani (LB) medium (Bacto Tryptone and Bacto Yeast Extract were purchased from Difco Laboratories, Detroit, Mich.) at 37°C with aeration for the purposes of plasmid construction and propagation. Cultures were grown in morpholinepropanesulfonic acid (MOPS)-buffered rich defined medium (31) at 37°C with aeration to determine the orientation of the invertible element and the Fim phenotype. For plating medium, agar (BBL, Cockeysville, Md.) was added to 1.5%. Sucrose agar medium (9) consisted of 5 g of yeast extract per liter, 10 g of tryptone per liter, 6% sucrose, and 1.5% agar. Media were supplemented with ampicillin (40 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (30 μg/ml) as appropriate (Sigma Chemical Co., St. Louis, Mo.).

Recombinant DNA techniques. Restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Boerhinger Mannheim Biochemicals, Indianapolis, Ind.; or New England Biolabs, Beverly, Mass.), T4 DNA ligase, and the Klenow enzyme (Bethesda Research Laboratories) were used in accordance with the manufacturers' recommendations. Oligonucleotides were synthesized at the University of Michigan Biomedical Research Core Facilities. Site-specific mutagenesis was performed essentially as described previously (4, 22, 25). DNA sequencing was performed with Sequenase II (United States Biochemical Corp., Cleveland, Ohio). DNA labelling was performed with a Multiprime kit purchased from Amersham Corp. (Arlington Heights, Ill.). All other molecular genetic procedures were performed essentially as described previously (4). Recombinant plasmids generated in this study are described in Table 2 and were constructed by use of *fim* deletion strain AAEC185 or AAEC189 (6).

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TABLE 1. Bacterial strains

Strain	Genotype[a]	Reference or source
MG1655	K-12 F$^-$ λ$^-$	Our stocks (8, 20)
AAEC078	MG1655 Δ(*fimBCDEFGH*) Δ*recA*	6
AAEC185	F$^-$ λ$^-$ *supE44 hsdR17 mcrA mcrB endA1 thi-1* Δ(*fimBEACDFGH*) Δ*recA*	6
AAEC187	MG1655 Δ*lacZYA* Δ(*fimE-fimA*)-*sacB* Neo^r	8
AAEC189	F$^-$ λ$^-$ Δ(*argF-lac*)*205*(*U169*) *supE44 hsdR17 endA1 thi-1* Δ(*fimBEACDFGH*) Δ*recA*	6
AAEC198A	MG1655 *fimA-lacZYA*	5
AAEC278 and AAEC279	MG1655 Δ(*fimBE*)-*sacB* Neo^r; invertible element on	This study
AAEC280 and AAEC281	MG1655 Δ(*fimBE*)-*sacB* Neo^r; invertible element off	This study
AAEC282A and AAEC283A	AAEC198A Δ(*fimBE*)-*sacB* Neo^r; invertible element on	This study
AAEC284A and AAEC285A	AAEC198A Δ(*fimBE*)-*sacB* Neo^r; invertible element off	This study
AAEC344 and AAEC345	Reconstruction of the wild type (from AAEC278)	This study
AAEC346 and AAEC347	Reconstruction of the wild type (from AAEC280)	This study
AAEC348 and AAEC349	MG1655 *fimB-am6* (from AAEC278)	This study
AAEC350 and AAEC351	MG1655 *fimB-am6* (from AAEC280)	This study
AAEC352 and AAEC353	MG1655 *fimE-am18* (from AAEC278)	This study
AAEC354 and AAEC355	MG1655 *fimE-am18* (from AAEC280)	This study
AAEC356 and AAEC357	MG1655 *fimB-am6 fimE-am18* (from AAEC278)	This study
AAEC358 and AAEC359	MG1655 *fimB-am6 fimE-am18* (from AAEC280)	This study
AAEC374A	AAEC198A *fimB-am6 fimE-am18* (from AAEC278)	5
AAEC376A	AAEC198A *fimB-am6 fimE-am18* (from AAEC280)	5
AAEC548 and AAEC549	AAEC356 (*sacB*-Neo^r) in the IRL	This study
AAEC550 and AAEC551	*fimB-am6 fimE-am18 fimL1*; invertible element on	This study
AAEC552 and AAEC553	AAEC550 Δ(*fimBE*)-*sacB* Neo^r	This study
AAEC554 and AAEC555	*fimB*$^+$ *fimE*$^+$ *fimL1*; invertible element on	This study
AAEC560 and AAEC561	P*tac-fimA*	This study
AAEC562 and AAEC563	P*tac-fimA-lac*	This study
P678-54	F$^-$ λ$^-$ *thr-1 leuB6 azi-8 tonA2 lacY1 minA1 supE44? gal-6 minB2 rfbD1 mgl-50 galP63 rpsL135 malA1 xyl-7 mtl-2 thi-1*	B. Bachmann (CGSC 4928) (3)
K4336	*dut ung*	D. Friedman

[a] invertible element on = invertible element in the on orientation; invertible element off = invertible element in the off orientation.

Site-directed mutagenesis of the IRL. The polymerase chain reaction was used to change the sequence of the left inverted repeat (IRL) (1, 22) from 5′-TTGGGGCCA to 5′-GTGCTAGCG. The mutated polymerase chain reaction product was used to generate pMM108. The complete nucleotide sequence of the insert was determined to confirm the mutation of the IRL and to ensure that no additional mutations were present. This mutation was designated *fimL1*.

TABLE 2. Plasmids

Plasmid	Description
pMM36	Invertible element cloned in the on orientation (29)
pMM39	*fimB fimE* (29)
pMM69	*fimB fimE* from pMM39 (29) cloned into pIB307 (9)
pMM71	*Eco*O109I *fim* fragment from pMM69 replaced by the *sacB* Neo^r cassette from pIB279 (9)
pMM74	*Bgl*II-*Cla*I fragment (including the 5′ end of *fimB*) from pIB238 (29) cloned into *Bam*HI-*Cla*I-restricted pSK$^-$ (Stratagene, La Jolla, Calif.)
pMM76	*Hpa*I-*Sac*II fragment (including the 5′ end of *fimE*) from pIB238 cloned into *Sma*I-*Sac*II-restricted pKS$^-$
pMM78	*fimB-am6* derivative of pMM74 isolated by site-directed mutagenesis
pMM80	*fimE-am18* derivative of pMM76 isolated by site-directed mutagenesis
pMM82	*fimB-am6* mutation from pMM78 cloned into pMM69
pMM86	*fimE-am18* mutation from pMM80 cloned into pMM69
pMM87	*fimB-am6* mutation from pMM82 cloned into pMM86
pMM91	*Xba*I site of pIB307 (9) removed
pMM93	*Bgl*II-*Bam*HI *fim* fragment from pIB238 (29) cloned into pMM91
pMM95	Deletion of *fimB*, *fimE*, and the *fim* invertible element from pMM93
pMM97	*trpA* terminator-*tac* promoter cassette cloned into pMM95
pMM99	*Xba*I site of pMM97 removed
pMM106	Deletion of 3′ *fimA* through 5′ *fimD* from plasmid pMM99
pMM108	*Sph*I-*Hpa*I *fim* fragment with the invertible element in the on orientation was cloned into pIB307 (9); the sequence of the IRL was changed from 5′-TTGGGGCCA to 5′-GTGCTAGCG by site-directed mutagenesis
pMM110	*sacB* Neo^r cassette from pIB279 (9) cloned into the *Nhe*I site of pMM108
pKE7	*fimB-am6 fimE* from pMM82 cloned into pMM39 (29)
pKE9	*fimB fimE-am18* from pMM86 cloned into pMM39 (29)
pKE11	*fimB-am6 fimE-am18* from pMM87 cloned into pMM39 (29)

Allelic exchange (9) between AAEC356 and pMM110 resulted in intermediate strains AAEC548 and AAEC549, in which the *sacB*-Neo^r gene cassette interrupted the IRL. Exchange between AAEC548 and pMM108 (carrying the mutation of the IRL) resulted in strains AAEC550 and AAEC551. Allelic exchange between AAEC550 and pMM71 produced intermediate strains AAEC552 and AAEC553. Exchange between AAEC552 and pMM69 produced strains AAEC554 and AAEC555.

Construction of *tac-fimA* strains. The polymerase chain reaction was used to generate a deletion of *fim* DNA in pMM93 from an *Eco*O109I site upstream of *fimB* to a position 93 bp upstream of *fimA*, generating pMM95. This deletion resulted in the insertion of unique *Xba*I and *Xho*I restriction sites. The polymerase chain reaction was used to synthesize a 132-bp DNA fragment including the *trpA* transcription terminator (11) followed by the *tac* promoter (13). This fragment was cloned into pMM95 to generate pMM97. DNA sequencing confirmed the sequence of the terminator-promoter cassette on pMM97.

Allelic exchange between intermediate strain AAEC187 (8) and pMM99 produced strain AAEC560, and that between AAEC282A and pMM106 produced strain AAEC562.

Immunological techniques. Fimbrial expression among colonies grown on nitrocellulose filters was determined by use of a dot blot assay with a monoclonal antibody essentially as described previously (16, 18).

The number of fimbriate and afimbriate bacteria within a given sample of bacteria was determined by indirect immunofluorescence in the liquid phase with a monoclonal antibody as described previously (8, 16).

Enrichment for afimbriate bacteria. A 2-liter overnight culture of Fleischmann's RapidRise yeast grown in YPG medium (10 g of yeast extract, 20 g of peptone, 20 g of glucose per liter) was pelleted, washed, and resuspended in 50 ml of medium. A 100-ml culture of bacteria grown overnight with aeration in MOPS-buffered rich defined medium was pelleted and resuspended in 10 ml of medium. To this bacterial suspension 5 ml of the washed yeast cells was added, and the mixture was rocked gently at 4°C for 20 min. The yeast cells and bound fimbriate bacteria were pelleted at 500 × *g* for 5 min. The supernatant was pipetted to a new tube, to which 5 ml of the washed yeast cells was added. Extraction with yeast cells continued (three or four times) until the bacterial supernatant failed to agglutinate yeast cells. The bacteria in the supernatant were pelleted and resuspended in 10 ml of medium. This bacterial suspension was applied to a 1.5-ml column of D-mannose-conjugated agarose beads (Sigma). The eluate represented the afimbriate bacterial fraction.

Plating efficiency. The total number of bacteria within a culture was determined with a Petroff-Hausser counting chamber (Thomas Scientific, Swedesboro, N.J.). The number of viable bacteria (i.e., CFU) was determined following plating of culture dilutions onto LB agar plates and growth at 37°C. The plating efficiency was calculated by dividing the number of CFU per milliliter by the total number of bacteria per milliliter.

Electron microscopy. Bacterial samples were pelleted and resuspended in Hank's balanced salt solution. The resuspended bacteria were applied to 300-mesh carbon-coated copper specimen grids and negatively stained with NH_4MoO_4. The stained cells were examined in a Philips EM-400 transmission electron microscope.

Analysis of plasmid-encoded proteins. Plasmid-encoded proteins were specifically labelled with [^{35}S]methionine (Amersham) by minicell analysis essentially as described previously (12) with *E. coli* P678-54 (3). Minicells were labelled in Difco methionine assay medium. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described previously (27). An autoradiograph of the gel revealed the plasmid-encoded proteins.

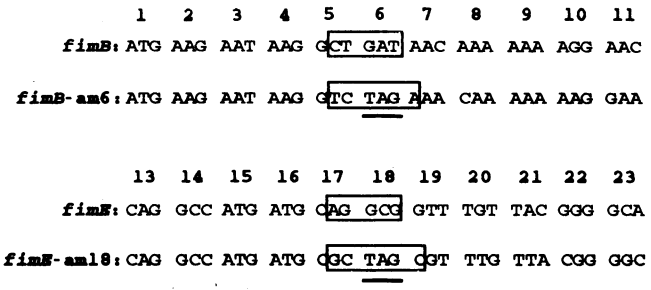

FIG. 1. Partial DNA sequences of the wild-type *fimB* and *fimE* alleles shown in alignment with the partial sequences of the *fimB-am6* and *fimE-am18* alleles. The numbers above the aligned sequences indicate the predicted codon positions. The nucleotides deleted from the wild type and inserted to generate the mutant alleles are enclosed in boxes. The in-frame amber nonsense codons are underlined. Note that the reading frames downstream of the nonsense codons have been shifted.

RESULTS

Construction of *fimB-am6* and *fimE-am18* mutations. Small mutations (5 bp of *fim* DNA were replaced by 6 bp of non-*fim* DNA) were introduced into cloned copies of both *fimB* and *fimE* by an oligonucleotide-directed approach (4, 25). Each mutation placed an in-frame amber nonsense codon very near the 5′ end of the respective open reading frame; translation initiation codons for *fimB* and *fimE* have been predicted from nucleic acid sequence data (24). Additionally, each mutation disrupted the reading frame and was recognized by a restriction enzyme that does not otherwise cut in *fim* (*Xba*I or *Nhe*I; Fig. 1) (7, 24). For *fimB*, the amber codon was inserted at the 6th codon, whereas that for *fimE* was inserted further into the gene, at the 18th codon. Although the translation of FimE probably initiates at a GTG codon (24), it is possible that translation initiates at either of two ATG codons (codons 15 and 16; Fig. 1). Thus, insertion of the nonsense codon beyond these methionine codons ensured mutation of *fimE*. Following the mutagenesis procedure, the mutations were confirmed by DNA sequence analysis. The mutant *fimB* and *fimE* alleles were designated *fimB-am6* and *fimE-am18*, respectively.

We confirmed that the mutations disrupted the respective open reading frames by transforming minicell-producing strain P678-54 (3) with one of the following plasmids: pMM39 (*fimB fimE*), pKE7 (*fimB-am6 fimE*), pKE9 (*fimB fimE-am18*), and pKE11 (*fimB-am6 fimE-am18*). A subsequent analysis of the plasmid-encoded proteins by SDS-PAGE revealed no detectable product from either mutant allele, whereas the wild-type alleles yielded proteins of the expected mobilities (24; data not shown).

We then assessed the ability of the mutant alleles to promote the inversion of a cloned copy of the *fim* invertible element (29). Strain AAEC078(pMM36) was transformed with pMM39, pKE7, pKE9, or pKE11. Plasmid DNA isolated from the transformants was treated with appropriate restriction endonucleases to determine the orientation of the

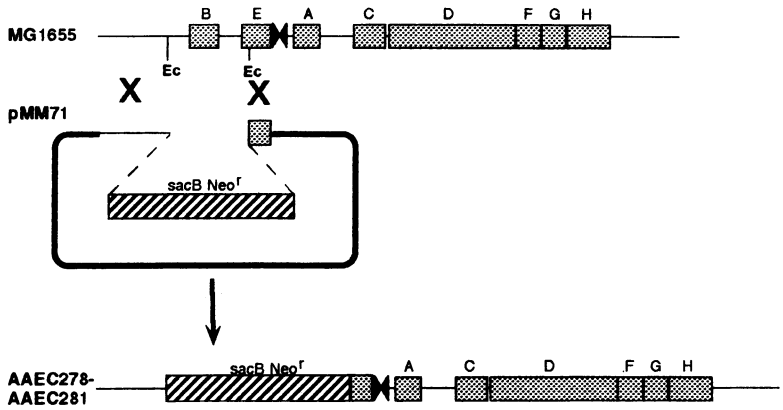

FIG. 2. Construction of the allelic exchange intermediate strains AAEC278 to AAEC281. At the top of the figure is shown the arrangement of the genes (A to H) within the *fim* locus of MG1655. In plasmid pMM71, the *Eco*O109I (Ec) *fim* fragment has been replaced by the *sacB* Neo^r gene cassette. Following a pair of homologous recombination events (indicated by the X's) between the chromosome of MG1655 and pMM71, intermediate strains AAEC278 to AAEC281 are produced. Note that the *fim* invertible element (indicated by the opposing arrowheads) is present only on the chromosome.

fim invertible element carried by pMM36 (29). Neither *fimB-am6* nor *fimE-am18* was able to promote inversion in this genetic background (data not shown).

Allelic exchange of mutant alleles into the chromosome. The *fimB-am6* and/or *fimE-am18* mutations were introduced into the chromosome of *fim*$^+$ strain MG1655 by a two-step allelic exchange procedure essentially as described previously (9). In the first step (construction of intermediate strains; Fig. 2), *fimB* and part of *fimE* were deleted from the chromosome of MG1655 and replaced with the *sacB* Neo^r genes carried by pMM71. As a result, we isolated intermediate strains (strains AAEC278 and AAEC279 and strains AAEC280 and AAEC281; Fig. 3) in which the *fim* invertible element was in either the on or the off orientation, respectively. This approach was also used to make corresponding intermediate strains (strains AAEC282A and AAEC283A and strains AAEC284A and AAEC285A, respectively) of *fimA-lac* fusion strain AAEC198A (5) (data not shown).

The second step of the allelic exchange replaced the chromosomal *sacB* Neo^r genes in intermediate strains AAEC278 and AAEC280 with the *fimB* and *fimE* alleles of interest (Fig. 4). These alleles were provided by the following plasmids: pMM69 (*fimB fimE*), pMM82 (*fimB-am6 fimE*), pMM86 (*fimB fimE-am18*), and pMM87 (*fimB-am6 fimE-am18*). Southern hybridization analysis confirmed the allelic exchange (Fig. 5). The resulting strains, AAEC344 to AAEC359, are listed in Table 1.

Roles of *fimB* and *fimE* in recombination. MG1655 and each of the even-numbered strains from AAEC344 to AAEC358 were maintained in steady-state growth with shaking in rich defined medium essentially as described previously (31). Aliquots of each culture were taken during the mid-log phase, and genomic DNA was isolated from a portion of each sample for Southern hybridization analysis (the remainder of each aliquot was analyzed by immunofluorescence microscopy to determine the Fim phenotype). Using a

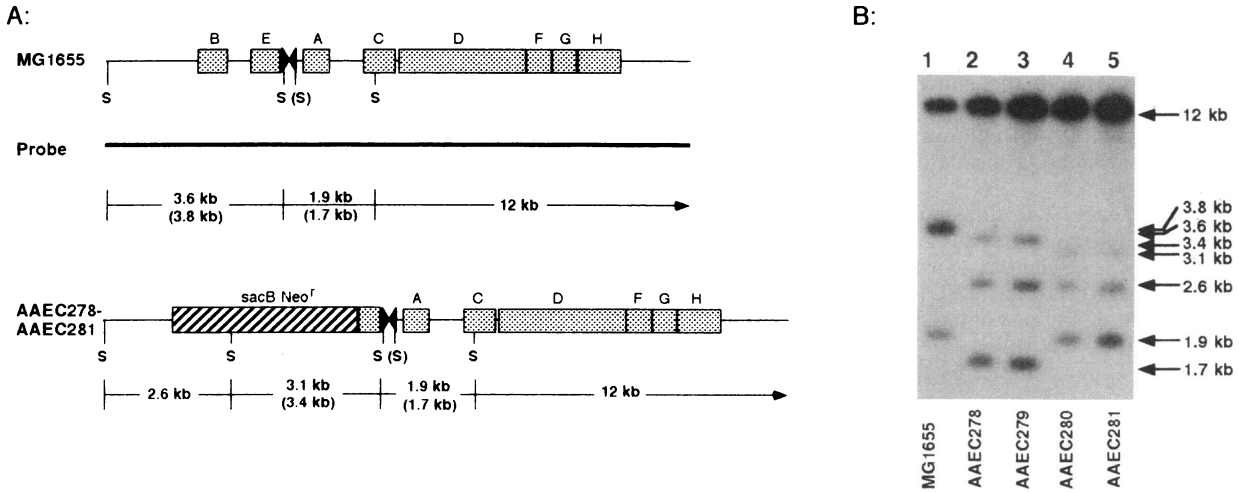

FIG. 3. Southern hybridization analysis confirming the construction of the intermediate strains and indicating the orientation of the *fim* invertible element. (A) The arrangement of genes within the *fim* locus of the indicated strains is shown along with the relevant *Sna*BI (S) restriction sites and fragments. Restriction sites and fragment sizes enclosed in parentheses are for the on orientation of the *fim* invertible element. The invertible element is indicated by the opposing arrowheads. The probe used is represented by the thick line. (B) Southern hybridization analysis of *Sna*BI-restricted genomic DNA isolated from the indicated strains.

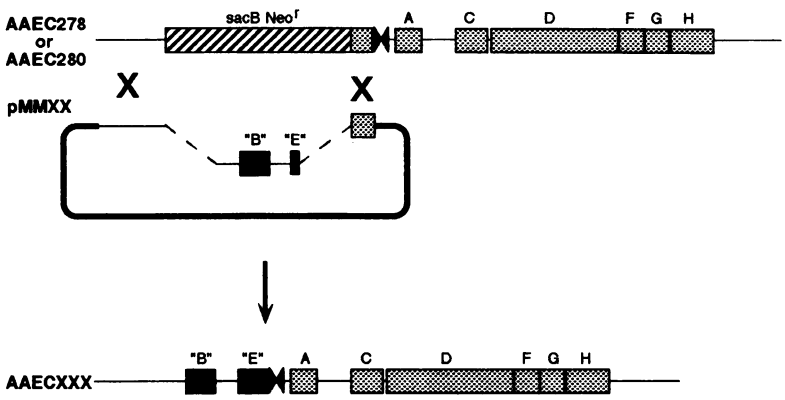

FIG. 4. Completion of the allelic exchange. The arrangement of genes within the *fim* locus of AAEC278 and AAEC280 is shown at the top of the figure. Plasmid pMMXX represents pMM69, pMM82, pMM86, or pMM87. Following a pair of homologous recombination events (indicated by the X's) between the chromosome of AAEC278 (or AAEC280) and pMMXX, the *sacB* Neo^r genes are replaced by the *fimB* and *fimE* alleles carried on pMMXX to produce strains AAEC344 to AAEC359 (AAECXXX). Note that the *fim* invertible element (indicated by the opposing arrowheads) is present only on the chromosome.

fim-specific probe, we were able to determine the orientation of the element in each strain and thus infer the activities of *fimB* and *fimE*. The fact that strains AAEC344 to AAEC358 were derived from intermediate strains that contained the *fim* invertible element in either the on or the off orientation (AAEC278 or AAEC280, respectively) allowed us to determine the abilities of both *fimB* and *fimE* to promote inversion in either the on-to-off or the off-to-on direction.

The results of this analysis (Fig. 6 and Table 3) were, for the most part, as expected (29). The invertible element in *fimB fimE* mutant strains AAEC356 and AAEC358 appeared to be locked in either the on or the off orientation, respectively, depending on the state of the invertible element in the parental intermediate strains. In contrast, the invertible element in strain MG1655, as well as in the reconstructed wild-type strains AAEC344 and AAEC346, existed primarily in the off orientation; the growth conditions used favored the off orientation (7). Both orientations of the invertible element were observed in *fimE* mutant strains AAEC352 and AAEC354. These latter results confirm that *fimB* promotes inversion in both directions. As previously observed (8), the percentage of *fimE* mutant cells with the invertible element in the on orientation varied greatly from culture to culture (data not shown).

In contrast to the result obtained in our plasmid-based assay (29), the invertible element in *fimB* mutant strains AAEC348 and AAEC350 appeared to be locked in the off orientation. This result supports the notion that *fimE*, in the absence of *fimB*, promotes inversion from the on to the off orientation. In addition, we saw no evidence that *fimE* promotes recombination in the alternate direction. It is possible that *fimE* promotes recombination from the off to the on orientation inefficiently and that this activity was masked by the off-orientational bias conferred by the growth conditions.

Role of the invertible element in fimbrial production. The

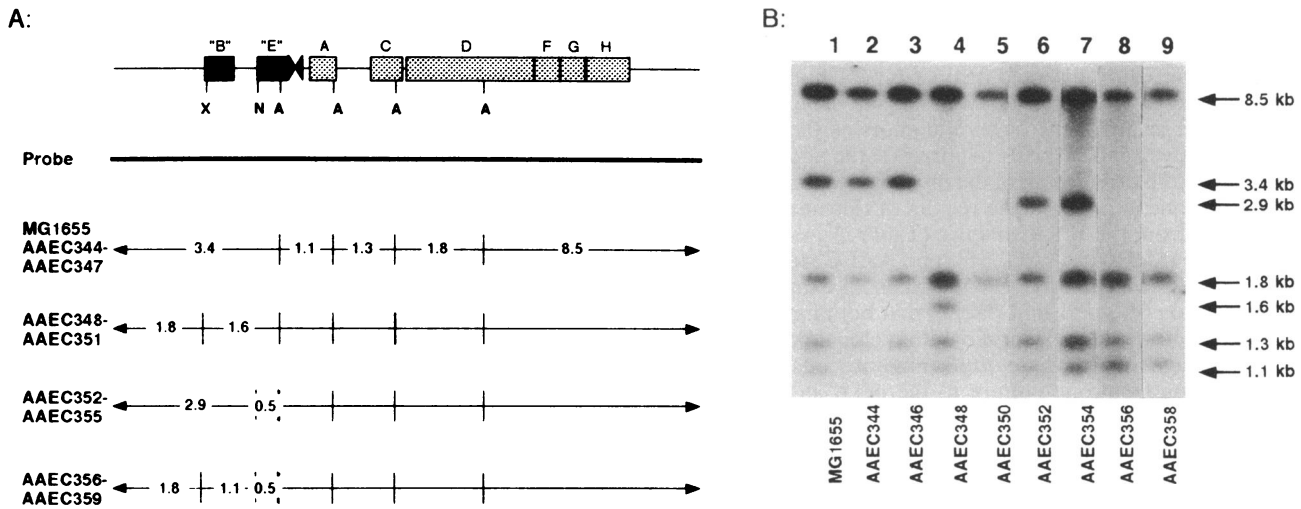

FIG. 5. Southern hybridization analysis confirming the allelic exchange. (A) The arrangement of genes within the *fim* locus is shown along with the relevant *Ava*I (A), *Nhe*I (N), and *Xba*I (X) restriction sites and fragments. Note that the *Nhe*I and/or *Xba*I restriction sites are present only in the mutant strains. The invertible element is indicated by the opposing arrowheads. The probe used is represented by the thick line. (B) Southern hybridization analysis of *Ava*I-, *Nhe*I-, and *Xba*I-restricted genomic DNA isolated from the indicated strains.

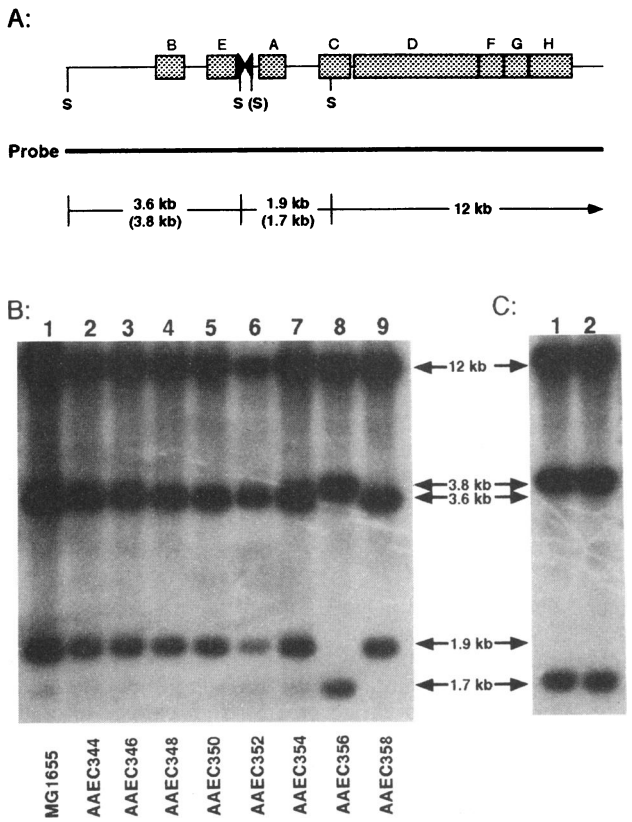

FIG. 6. Southern hybridization analysis indicating the orientation of the *fim* invertible element. (A) Arrangement of *Sna*BI (S) restriction sites and fragment sizes in and near the *fim* invertible element. The restriction site and fragment sizes enclosed in parentheses are for the on orientation of the *fim* invertible element. The probe used is represented by the thick line. (B) Southern hybridization analysis of *Sna*BI-restricted genomic DNA isolated from the indicated strains. (C) Southern hybridization analysis of *Sna*BI-restricted genomic DNA isolated from AAEC554 either from the starting sample (lane 1) or from the fraction enriched for Fim$^-$ cells (lane 2).

Fim phenotype of each strain was determined by use of a monoclonal antibody directed against type 1 fimbriae in an immunofluorescence microscopy assay (8). The cells analyzed came from the same culture aliquots as those used in our DNA analysis, allowing us to correlate the appearance of fimbriae with the orientation of the invertible element in each strain. With one exception, the results of this analysis were as predicted from the DNA analysis (Table 3). As expected, the wild-type (MG1655, AAEC344, and AAEC346) and *fimE* mutant (AAEC352 and AAEC354) strains all exhibited variable expression of type 1 fimbriae, in that both fimbriate and afimbriate bacteria were observed within clonal populations. The percentage of fimbriate *fimE* mutant cells exhibited much variation from one culture to the next, as previously observed (8; data not shown). In addition, *fimB* mutant strains AAEC348 and AAEC350 and the *fimB fimE* mutant strain with the invertible element locked in the off orientation, AAEC358, appeared completely afimbriate (Table 3). In each of these cases, the percentage of fimbriate cells agreed with the percentage of cells containing the invertible element in the on orientation.

Surprisingly, only 87% of the AAEC356 cells (the *fimB fimE* double mutant strain with the invertible element locked in the on orientation) appeared fimbriate (Table 3). Similar results were found when independently isolated AAEC357 was examined (data not shown). Mixtures of fimbriate and afimbriate cells were not due to mixtures of the orientation of the *fim* invertible element; using Southern hybridization analysis and densitometry, we were unable to detect any copies of the invertible element in the off orientation in these cells (Table 3; data not shown).

TABLE 3. Fimbriation in *fimB* and *fimE* mutant strains

Strain	Relevant genotype	% with invertible element in the on orientation[a]	% Fim$^+$[b]
MG1655	B$^+$E$^+$	0.97 ± 0.33	0.40 ± 0.55
AAEC344	B$^+$E$^+$	0.92 ± 0.36	0.25 ± 0.50
AAEC346	B$^+$E$^+$	0.98 ± 0.46	0.40 ± 0.55
AAEC348	B$^-$E$^+$	0.00[c]	0.00[d]
AAEC350	B$^-$E$^+$	0.00[c]	0.00[d]
AAEC352	B$^+$E$^-$	3.4 ± 1.1	1.2 ± 0.86
AAEC354	B$^+$E$^-$	2.8 ± 1.3	1.6 ± 1.5
AAEC356	B$^-$E$^-$	100.00[c]	87 ± 3.9
AAEC358	B$^-$E$^-$	0.00[c]	0.00[d]

[a] Values represent the mean percentage of cells with the invertible element in the on orientation plus or minus 1 standard deviation, as determined by densitometry. Densitometry was performed with a Gilford response spectrophotometer (Ciba-Corning, Medfield, Mass.) equipped with a gel scanning accessory. Analyses of autoradiograms (including that used in Fig. 6B) were performed in two dimensions, down individual lanes as well as across all lanes. The ratio of the integrated area corresponding to the on orientation to the sum of the areas for the off and on orientations was used to calculate the percentage of cells with the invertible element in the on orientation.

[b] Values represent the mean number of fimbriate cells plus or minus 1 standard deviation, as determined by immunofluorescence microscopy. Cells were grown in aerated rich defined medium and maintained in steady-state logarithmic growth by dilution. A minimum of four samples of 100 cells each were counted.

[c] The optical densities corresponding to the on orientation (or off orientation in the case of AAEC356) could not be distinguished from the background.

[d] No fimbriate cells were detected among a minimum of 500 cells counted.

As a control, we constructed a strain (AAEC554) with the invertible element locked in the on orientation by mutation of the IRL to examine fimbrial expression in such a strain with wild-type alleles of *fimB* and *fimE* (see Materials and Methods). Southern hybridization analysis confirmed that the invertible element in AAEC554 remained locked in the on orientation, even in the presence of wild-type *fimB* and *fimE* (data not shown). Despite the invertible element being locked in the on orientation, immunofluorescence microscopy indicated that only 74% of the cells from a mid-log-phase culture were fimbriate.

Inversion-independent phase variation. The afimbriate cells present in AAEC356, AAEC357, and AAEC554 were not afimbriate mutants, as none of these strains produced afimbriate colonies (data not shown). Moreover, both fimbriate and afimbriate bacteria were invariably observed when these colonies were cultured in broth. Thus, whereas AAEC356, AAEC357, and AAEC554 were incapable of reorientating their invertible elements (as expected), they unexpectedly exhibited variable (and reversible) expression of type 1 fimbriae.

For further examination of this phenomenon, Fim$^+$ bacteria were selectively removed from the AAEC554 population by a combination of yeast cell agglutination and affinity chromatography (see Materials and Methods). When carried out in the absence of yeast cells and with omission of the affinity chromatography step, the various manipulations of

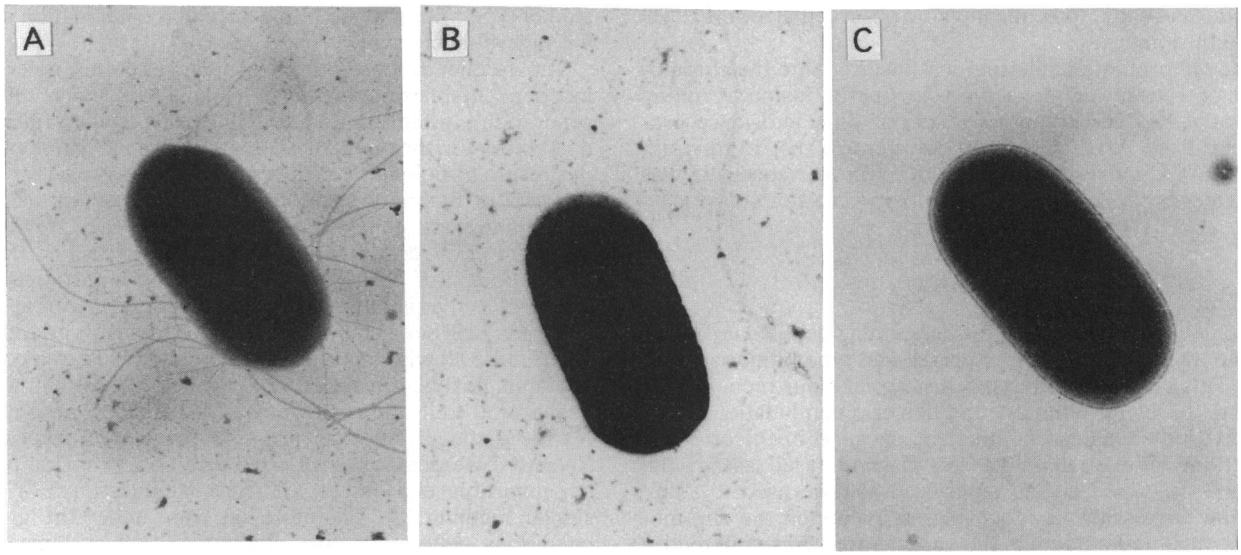

FIG. 7. Electron micrographs showing representative cells of strain AAEC554. (A) Type 1 fimbriate cell from unfractionated AAEC554. (B) Afimbriate cell from unfractionated AAEC554. (C) Afimbriate cell from the AAEC554 fraction enriched for afimbriate cells.

this procedure did not alter the percentage of fimbriate cells in the bacterial population, as determined by immunofluorescence microscopy (data not shown). The cells remaining following the fractionation, more than ninefold enriched for Fim$^-$ bacteria, as determined by immunofluorescence microscopy, were analyzed by several additional methods.

We first isolated genomic DNA from both the starting sample and the fraction enriched for Fim$^-$ bacteria. Southern hybridization analysis with a *fim*-specific probe confirmed that the *fim* invertible element was exclusively in the on orientation in both samples (Fig. 6C).

We next performed a qualitative analysis of fimbriation by using electron microscopy, a more sensitive assay for fimbriae than immunofluorescence microscopy. Afimbriate bacteria were observed in both the starting sample and the fraction enriched for Fim$^-$ bacteria (Fig. 7). The presence of these afimbriate bacteria indicated that the on orientation was necessary but not sufficient for fimbrial expression.

Lastly, we grew the cells within the fraction enriched for Fim$^-$ bacteria on solid medium, permitting the analysis of progeny colonies. The plating efficiency (Materials and Methods) of the fraction enriched for Fim$^-$ bacteria was equivalent to that of the initial population, thereby demonstrating the viability of the afimbriate cells. All colonies from the Fim$^-$ fraction reacted with an antifimbrial antibody, as determined by a dot blot assay (see Materials and Methods), and representative colonies were found to contain 87% Fim$^+$ cells, as determined by immunofluorescence microscopy; representative colonies from the unfractionated AAEC554 sample exhibited 88% Fim$^+$ cells. Taken together, these results demonstrate that the expression of type 1 fimbriae continues to vary in the absence of recombination of the *fim* invertible element.

Inversion-independent phase variation in a *tac-fimA* strain. Inversion-independent phase variation could result from phase-variable transcription initiation from the *fimA* promoter (believed to be located within the invertible element). For testing this possibility, *fimA* was placed under the transcriptional control of the isopropyl-β-D-galactopyranoside (IPTG)-inducible *tac* promoter (see Materials and Methods). This well-characterized promoter is not known to exhibit phase-variable transcription initiation. The *tac* promoter was used to replace the *fim* invertible element (and the putative *fimA* promoter) on a recombinant plasmid, such that the transcription start site of the *tac* promoter coincided with the normal 5′ end of the *fimA* mRNA (39). This construct was then introduced into the MG1655 chromosome by allelic exchange.

The resulting strain, AAEC560, exhibited IPTG-inducible fimbrial expression, as determined by immunofluorescence microscopy (Fig. 8). Fim$^-$ bacteria were observed at all IPTG concentrations examined; the highest concentration

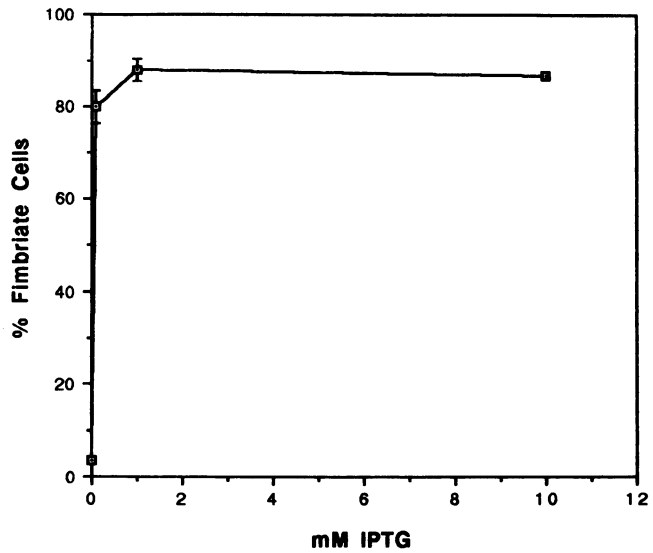

FIG. 8. Immunofluorescence microscopy of strain AAEC560. Cultures were grown in MOPS-buffered rich defined medium (31) at 37°C with aeration in the presence of 0, 0.1, 1.0, or 10 mM IPTG. Values represent the mean plus or minus 1 standard deviation. A minimum of four samples of 100 cells each were counted for each of the cultures.

tested was 10 mM. The mean value observed at 1 mM IPTG was 88% Fim$^+$.

The *tac* promoter construct was inserted into the chromosome of a strain carrying a *fimA-lac* operon fusion, resulting in strain AAEC562. Upon induction, β-galactosidase production from AAEC562 was equivalent to that from strain AAEC374A, indicating that this *tac* promoter construct was as strong as the native *fimA* promoter (data not shown).

DISCUSSION

We developed a method for performing detailed examinations of the phase-variable expression of type 1 fimbriae in *E. coli*. This chromosome-based system, like our recombinant plasmid-based system (29), can detect recombination of the *fim* invertible element in either the on-to-off or the off-to-on direction. However, unlike the plasmid-based assay, the method described in this report can also be used to study fimbrial expression. By focusing on events on the chromosome, this method avoids the generic problems suffered by plasmid-based systems, namely, alterations in both gene dosage and DNA supercoiling. In addition to its use in this study, the chromosome-based assay has proven helpful in studying the effects of Lrp on phase variation (5) and should similarly help determine the roles of other factors that affect *fim*.

Using this chromosome-based assay, we obtained substantial evidence supporting the current, plasmid-based model of the roles of *fimB* and *fimE* in recombination. *fimB* promotes recombination in both directions, whereas *fimE* promotes recombination from the on to the off orientation. In contrast to the results of our previous, plasmid-based study (29), in this study we found no evidence that *fimE* additionally promotes recombination from the off to the on orientation. It is possible that *fimE* promotes recombination from the off to the on orientation inefficiently. Thus, with this caveat, plasmids may be used to explore the recombination event in greater detail.

This study and other studies demonstrated that FimB and FimE have different specificities (8, 24, 29, 38). The basis for the different reaction specificities remains unclear. Presumably, there must be some means by which the proteins distinguish between the on and off orientations. Perhaps the alternate substrate orientations assume different topologies that are recognized by FimB and FimE. An additional possibility is that the arrangement of the various *trans*-active accessory factors (IHF, H1, and Lrp) is asymmetrical and that FimB and FimE are sensitive to this asymmetry. To date, there is no evidence for a differential requirement for an accessory factor such as is found in lambda integration and excision (i.e., lambda Xis is required only for the excision event) (2).

Although results from the recombination assay were not surprising, those from the fimbriation assay were, to some degree. Whereas strains in which the invertible element was locked in the off orientation were completely afimbriate, strains in which this element was locked in the on orientation were not 100% fimbriate, as determined both by immunofluorescence and by electron microscopy. Isolation and subsequent growth of afimbriate bacteria from strains in which the invertible element was locked in the on orientation resulted in mixtures of fimbriate and afimbriate cells. This result indicates that the on orientation of the *fim* invertible element is necessary but not sufficient for fimbrial expression. Rather, *E. coli* continues to exhibit phase-variable expression of type 1 fimbriae in the absence of recombination of the *fim* invertible element.

The frequency of off-to-on switching of this inversion-independent phase variation is rapid ($>10^{-2}$ per cell per generation); when afimbriate cells with the invertible element locked in the on orientation are grown on agar medium, the resultant colonies reach an equilibrium consisting of a majority of fimbriate bacteria. These colonies are indistinguishable from colonies resulting from the growth of phase-on cells (cells with the invertible element in the on orientation). For production of colonies of uniform composition, phase variation must occur at a frequency of at least 10^{-2} per cell per generation (8). Were phase variation to occur more slowly, a phase-on cell would result in a colony containing a higher proportion of phase-on cells than would a phase-off cell and vice versa. Such colonies would be readily distinguishable as phase-variant colony types (8). Recently, we demonstrated rapid on-to-off inversion of the *fim* invertible element in wild-type bacteria (*fimE*$^+$) on a similar medium (8). Therefore, at least under the growth conditions tested, the two phase variation systems have different equilibrium points. Whereas the *fim* invertible element is predominantly locked in the off orientation under these conditions, inversion-independent phase variation favors fimbriation.

The mechanism of inversion-independent phase variation remains unclear; it is possible that the variation has a genetic basis. Southern hybridization analyses detected no additional DNA inversions in *fim*. We also detected no evidence within *fim* of DNA insertions or deletions or recombination of pseudogenes, all of which are associated with other phase variation systems (37, 41). Possible explanations include variations in the number of nucleotide repeats (30), either inside or outside *fim*, DNA rearrangements outside *fim*, and regulatory events following *fimA* transcription initiation (e.g., translation of *fimA* or transcription-translation of the other fimbrial structural genes). Inversion-independent phase variation is observed when *fimA* is transcribed from the recombinant *tac* promoter, suggesting that inversion-independent phase variation cannot be explained by variable transcription initiation of the major subunit gene, as has been found for the phase variation of Pap fimbriae (10). However, it is possible that *cis*-acting regulatory elements downstream of the *fimA* promoter (this region is retained in the *tac-fimA* constructs) influence transcription.

Alternatively, inversion-independent phase variation may be epigenetic as opposed to genetic. Little is known about either the development of fimbriae from the constituent subunits or the events involved in the loss of fimbriae from the cell surface. Failure to properly assemble the subunits into fimbriae or an increased loss of fimbriae from the cell surface could account for the afimbriate subpopulation.

The type 1 fimbria system of *E. coli* is not unique in involving two phase variation events. Phase variation of *Haemophilus influenzae* lipopolysaccharide involves variable numbers of CAAT repeats in the first open reading frame of each of three loci (43); an additional, uncharacterized phase variation event also regulates expression at one of these loci (42). Phase-variable expression of pili in *Neisseria gonorrhoeae* involves the nonreciprocal recombination of silent gene loci into active genes (antigenic variation) and phase-variable expression of an accessory protein involved in the surface expression of pili (23, 41).

One reason for dual phase variation events in the expression of type 1 fimbriae may be the fact that fimbriae can be both beneficial and detrimental to the bacterium. In vitro,

fimbriae may be positively selected for by growth in static broth, whereas fimbriate cells grow more slowly than their afimbriate counterparts in aerated broth or on agar plates (7, 8, 14, 17, 21, 28, 34). In vivo, fimbriae may promote bacterial colonization, which in turn may be beneficial for bacterial growth (33, 44), or they may promote killing of the bacteria (32) or clearance of the bacteria from the host (26). The two mechanisms of phase variation may cooperate to avoid the inappropriate expression of fimbriae that could result in dire consequences for the cell.

Inversion-independent phase variation in *E. coli* might be related to the mechanism of type 1 fimbrial phase variation in *Klebsiella pneumoniae* and *Salmonella typhimurium*. The mechanism of phase variation in *K. pneumoniae* and *S. typhimurium* is unclear. Whereas DNA sequence analysis suggests that an invertible element may reside immediately upstream of *fimA* in each of these bacteria (40), recombination of these putative invertible elements has not been observed (19, 40).

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