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Type 1 Fimbriation and *fimE* Mutants of *Escherichia coli* K-12

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We reexamined the influence of *fimE*, also referred to as *hyp*, on type 1 fimbriation in *Escherichia coli* K-12. We found that one strain used previously and extensively in the analysis of type 1 fimbriation, strain CSH50, is in fact a *fimE* mutant; the *fimE* gene of CSH50 contains a copy of the insertion sequence IS*1*. Using a recently described allelic exchange procedure, we transferred the *fimE*::IS*1* allele from CSH50 to our present wild-type strain, MG1655. Characterization of this IS*1*-containing strain (AAEC137), together with another *fimE* mutant of MG1655 (AAEC143), led to two conclusions about the role of *fimE*. First, the formation of phase variant colony types, reported widely in strains of *E. coli*, depends on mutation of *fimE*, at least in K-12 strain MG1655. Here we showed that this phenomenon reflects the ability of *fimE* to stimulate the rapid inversion of the *fim* invertible element from on to off when the bacteria are grown on agar. Second, our analysis of *fimE* mutants, which is limited to chromosomal constructs, provided no evidence that they are hyperfimbriate. We believe that these results, which are at odds with a previous study using *fim*-containing multicopy plasmids, reflect differences in gene copy number.

The expression of type 1 fimbriae in *Escherichia coli* is phase variable (metastable), with cells oscillating between fimbriate and afimbriate states (8, 13). Phase variation is apparent when cells are examined in the electron microscope or by immunofluorescence microscopy and is associated with the inversion of a 314-bp DNA element (1). The invertible element, which probably contains a promoter, influences the transcription of the major fimbrial subunit gene, *fimA* (30, 43). Mutants that contain the invertible element frozen in one orientation (phase-locked mutants) have been isolated (11, 16, 18). In these strains, *fimA* is transcribed when the element is locked in one (the "on") but not the other (the "off") orientation. Thus, it is presumed that the orientation of the invertible element determines *fimA* transcription in wild-type cells.

Genetic control of the inversion is influenced by *fimB* and *fimE* (*hyp*) (30, 31, 44, 45), genes that map adjacent to the invertible element, as well as other unlinked loci. In the absence of both *fimB* and *fimE*, the invertible element becomes locked in either the on or off orientation (46). According to one model, *fimB* and *fimE* possess antagonistic activities, with *fimB* stimulating inversion from the off to on orientation, while *fimE* promotes the opposite reaction (31, 46). *fimE* also acts to repress *fimA* transcription directly (45). Phase variation at wild-type frequencies also requires integration host factor, since mutation of either the *himA* or *himD* (*hip*) genes markedly reduces inversion frequencies (11, 16). In contrast, mutants with lesions in the structural gene for H1, termed *osmZ* (25, 26) and, independently, *pilG* (42, 55), display elevated inversion frequencies.

Although the proposed opposing activities of *fimB* and *fimE* have suggested that the inversion is influenced by environmental conditions, it has become widely accepted that wild-type *E. coli* K-12 strains switch phase at random. Thus, although the presence of fimbriae can provide a selective advantage in some conditions (growth in static liquid culture) but reduce growth in others (on agar) (12, 15, 24, 32, 41), the frequency of inversion is believed to be unaffected by the environment. Despite the growth advantage of afimbriate cells on agar, the different phases produce distinct (phase variant) colony types that are easily recognized (8, 45, 48, 56), colonies composed mainly of fimbriate cells being smaller and smoother than those consisting largely of afimbriate bacteria. The ability to distinguish, and therefore count, the proportion of phase variant colonies has allowed measurement of the frequency of variation (between 10^{-4} and 10^{-3} per cell per generation) (8, 45). More recently, these values have been confirmed by studying operon fusion strains in which the *lac* genes are coupled to *fimA* (13, 45). As expected, the *lac* fusion strains show phase variation in the ability to metabolize lactose rather than in fimbriation.

In contrast to the data outlined above, early studies of *E. coli* K-12 (32), together with the more recent characterization of clinical isolates (28), identified strains that are largely afimbriate on agar. The basis of this phenomenon, and whether it is distinct from the selective outgrowth of afimbriate cells on agar, is the focus of this report. We showed that the production of phase variant colonies can be associated with the presence of a mutated *fimE* gene. The suppression of type 1 fimbria synthesis seen in a wild-type strain, MG1655, was mediated by the influence of *fimE* on the invertible element. We also showed that strain CSH50, which produces phase variant colonies and which has been used extensively in the analysis of fimbrial phase variation, contains a copy of the insertion sequence IS*1* (17, 27) in the carboxy-terminal region of *fimE*. This lesion appeared mainly to account for observed differences between MG1655 and CSH50 since transfer of the IS*1* sequence from CSH50 to MG1655 produced a mutant that closely resembled CSH50. Despite our finding that *fimE* promotes inversion from on to off, *fimE* mutants still undergo phase variation; the invertible element does not become locked in the on orientation as predicted by a current model (31, 46). Our data suggest that *fimB* is capable of promoting inversion in both directions, whereas at least in the presence of *fimB*, *fimE* stimulates inversion from on to off.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference or construction
E. coli		
CSH50	λ⁻ F⁻ *ara* Δ(*lac-pro*) *rpsL thi fimE*::IS*1*	36
MG1655	λ⁻ F⁻ Fim⁺	21
AAEC064	MG1655 Δ*fimB-fimA*, insert *sacB*-Neo^r	7
AAEC100	MG1655 Δ*lacZYA*	7
AAEC112	MG1655 Δ3′*fimE-5′fimA*, insert *sacB*-Neo^r	*sacB*-Neo^r from pIB331 into MG1655
AAEC135 and -136	MG1655; reconstructed wild type	Exchange of *sacB*-Neo^r of AAEC112 for wild-type sequences of pIB315
AAEC137 to -142	MG1655; *fimE*::IS*1* (CSH50) into AAEC112	Exchange of *sacB*-Neo^r of AAEC112 for *fimE*::IS*1* of pIB328
AAEC143 and -144	MG1655; *fimE-Xba*I linker in *Eco*O1091 site	Exchange of *sacB*-Neo^r of AAEC112 for *fimE-Xba*I of pIB330
AAEC185	MM294 λ⁻ F⁻ *supE44 hsdR17 mcrA mcrB endA1 thi-1* Δ*fimB-fimH* Δ*recA*	6
AAEC187	AAEC100 Δ3′*fimE-5′fimA*, insert *sacB*-Neo^r	P1 transduction of *sacB*-Neo^r from AAEC112 to AAEC100
AAEC193	AAEC100 Δ*fimB-fimA*, insert *sacB*-Neo^r	P1 transduction of *sacB*-Neo^r from AAEC064 to AAEC100
AAEC198 and -202	AAEC193 *fimA-lacZYA*	Exchange of *sacB*-Neo^r of AAEC193 for *fimA-lacZYA* of pIB333
AAEC203 and -204	AAEC187 *fimA-lacZYA fimE*	Exchange of *sacB*-Neo^r of AAEC187 for *fimA-lacZYA fimE* of pIB338
Plasmids		
pRS415	Amp^r *lacZYA*	53
pKE005	Amp^r pRS415 *lacZYA* flanked by *Xba*I translation-terminator linkers in *Sma*I (upstream) and *Stu*I (downstream) sites	This work
pIB306	Cm^r; temperature-sensitive pSC101 vector	7
pIB308	Cm^r *Hin*dIII-*Bam*HI *fimA-D′* of pIB238 into pIB306	7
pIB225	Amp^r pBR322 derivative containing *fimB-fimA* region of strain CSH50	This work
pIB254	Amp^r pBR322 derivative; pIB248 (5) containing the entire *fim* region (*fimB-fimH*)	This work
pIB315	Cm^r pIB308, Δ*fimB*, insert *Hin*dIII linker	This work
pIB322	Cm^r pIB308, *Xba*I translation-termination linker inserted in *Hpa*I site of *fimA*	This work
pIB328	Cm^r pIB315, 28-bp *Sac*II-*Stu*I fragment replaced by 0.7-kbp *Sac*II-*Stu*I (IS*1*) fragment of pIB225	This work
pIB330	Cm^r pIB315, *Eco*O1091 site (*fimE*), filled in; *Xba*I translation-termination linker inserted	This work
pIB331	Cm^r pIB315, Δ*Eco*O1091 (*fimE*)-*Hpa*I (*fimA*), insert *sacB*-Neo^r cassette of pIB279 (7)	This work
pIB333	Cm^r pIB322, *lacZYA* subcloned onto *fimA* on *Xba*I fragment of pKE005	This work
pIB338	Cm^r pIB333 *fimE*; Replacement of 3.3-kbp *Hin*dIII-*Sac*II (*fimB fimE*) with 0.7-kbp *Hin*dIII-*Sac*II *fimE-Xba*I allele of pIB330	This work

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Bacterial strains were all derivatives of *E. coli* K-12. Strains and plasmids used in this study are described in Table 1. Media included L broth, which contained 5 g of sodium chloride, 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), and 10 g of tryptone (Difco) per liter, and L agar, which was L broth containing 1.5% agar (BBL, Cockeysville, Md.). Sucrose agar, used to select recombinant bacteria (7), is L agar supplemented with 6% sucrose (J. T. Baker Chemical Co., Phillipsburg, N.J.) but lacking sodium chloride. Both static and aerated cultures comprised 15 ml of L broth in a 125-ml Erlenmeyer flask. When appropriate, media were supplemented with ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), or kanamycin (25 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) as recommended (33).

Molecular biological techniques. Generalized transduction was done as described by Silhavy et al. (52) with P1 *vir*. Chromosomal DNA was isolated as described previously (4), except that incubation with sodium dodecyl sulfate and proteinase K was at 65°C for 20 min rather than at 37°C for 60 min. Plasmid DNA was isolated by the modified alkaline lysis procedure; large-scale preparations were purified by centrifugation in cesium chloride (33). Restriction endonuclease digestions were done according to the specific manufacturers' recommendations (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.). DNA labeling was done with the Multiprime kit supplied by Amersham Corp. (Arlington Heights, Ill.). Other molecular genetic procedures and recombinant DNA techniques (agarose gel electrophoresis, isolation of restriction fragments, ligation and transformation of plasmid DNA, Southern hybridization) were done by standard procedures (4, 33). DNA sequencing by the dide-

oxy technique of Sanger et al. (50) employed the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio).

Quantitation of the *fim* switch. The accurate assessment of the orientation of the *fim* switch element (on versus off) is a primary requisite for a complete understanding of the role of this element in the expression of type 1 fimbriae. Optical quantitation of autoradiograms is severely limited by a variety of parameters, e.g., type of X-ray film, level of saturation of film, etc. (57). Therefore, we chose to use a method involving direct counting of radiolabeled DNA fragments that have hybridized to filter-bound *fim* switch DNA.

Quantitation of the orientation (percentage on versus percentage off) of the *fim* switch was achieved as follows. Chromosomal DNA samples were digested with the restriction enzymes *Ava*I and *Sna*BI, electrophoresed through a 1.8% agarose gel, and transferred to Zetabind nylon membranes according to the manufacturer's recommendations (AMF Cuno, Meriden, Conn.), with modifications (47). Southern hybridization was done as described above with an *Aat*II-*Stu*I *fim* fragment encompassing the invertible region as the probe. Radioactivity was quantitated by measurement in a Betascope 603 Blot Analyzer (Betagen Corp., Waltham, Mass.), as has been used previously for the analysis of DNA restriction fragments (3). All filters were scanned for 12 h. A control scan, run in triplicate, revealed a range of less than 2% between the highest and lowest values, indicating that there was a high degree of precision associated with this means of measurement.

Allelic exchange procedure. The exchange of chromosomal for plasmid DNA was as described before (7). The *sacB*-Neo^r-containing intermediate strain used here, AAEC112, was constructed essentially as described for similar strains, AAEC162 and AAEC164 (6). Thus, the *sacB*-Neo^r cassette was transferred from plasmid pIB331 into the chromosome of strain MG1655, taking advantage of the properties of the temperature-sensitive vector pMAK705 (22), on which pIB331 is based. Plasmid integrates of the *sacB*-Neo^r intermediate strain, AAEC112, were isolated at 42°C on chloramphenicol agar, inoculated directly into prewarmed broth, and then grown to the late log phase with rapid aeration at 42°C. The desired recombinants were finally selected on sucrose agar at 30°C.

Immunological techniques. Quantitation of fimbriae by enzyme-linked immunosorbent assay (ELISA) has been described previously (10). Polyclonal antiserum was produced by injecting purified fimbriae (14) into rabbits. Monoclonal antibody FG6 (14) was used as the tissue culture supernatant. Indirect immunofluorescence labeling was done in the liquid phase as described previously (23), using monoclonal antibody FG6 and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Organon Teknika Corp., West Chester, Pa.). Labeled cells were combined with low-gelling-temperature agarose (SeaPlaque; FMC Corp., Rockland, Maine) before being spread on glass slides. The addition of agarose (0.5% final concentration) helped to prevent cell clumping and ensured their uniform distribution. Samples were air dried briefly, covered in mounting fluid (Difco), and examined by phase-contrast and fluorescence microscopy with a Leitz orthoplane UV fluorescence microscope (100× objective). The proportion of fimbriate to afimbriate cells was determined by counting a minimum of 200 (individual colonies) or 400 (other tests) cells per sample. Each reported value represents the average obtained by counting cells on duplicate slides. We observed a maximum discrepancy between duplicates of 10%; however, in most experiments this difference was much lower (average = 2.8%).

RESULTS

Identification of IS*1* element in *fimE* gene of CSH50. Strain CSH50 (36) has been used widely in the analysis of phase variation of type 1 fimbriae (1, 2, 11, 13, 15, 16, 18, 19). Like many other *E. coli* K-12 and B strains, CSH50 produces readily distinguishable phase variant colony types (8, 13, 45, 56). In contrast to CSH50, strain MG1655 (21), although fimbriate in static broth culture, inevitably produces colonies that resemble the afimbriate phase variants of CSH50 (see below). Southern hybridization analysis of the *fim* region of CSH50, MG1655, and the two *E. coli* K-12 wild-type strains, EMG2 and WG1, revealed that although MG1655 was indistinguishable from the wild-type strains, CSH50 contained an insertion of approximately 0.8 kbp in the *fimE* gene (Fig. 1). We have recently cloned the *fimB-A* region (30, 31, 43) from CSH50 and the entire *fim* region from MG1655 (data not shown). DNA sequence analysis of the cloned CSH50 DNA demonstrated the presence of the insertion sequence IS*1* (17, 27) in the carboxy-terminal region of *fimE* (data not shown). The IS*1* element is located between known *Sac*II and *Stu*I restriction endonuclease sites (31), in the orientation shown in Fig. 3.

Initial characterization of strain MG1655. In part because of our finding that strain CSH50 is mutant in *fimE*, we chose to use MG1655 as an alternative standard strain for studies of type 1 fimbriation. This strain provides an attractive host background for genetic studies; MG1655 has undergone comparatively little manipulation but has been cured of bacteriophage lambda and the F$^-$ factor plasmid. In addition, it lacks any known nonsense suppressor and has been used to host an extensive collection of drug resistance markers situated around the chromosome (54).

We initially confirmed, by electron microscopy and by reaction with an anti-type 1 fimbria monoclonal antibody (14), that strain MG1655 produces type 1 fimbriae. Subsequently, we cloned the entire *fim* region from MG1655 and showed that the cloned DNA (plasmid pIB254) effects the synthesis of type 1 fimbriae in strain VL584 (data not shown), which is Δ*fim* (6, 18). As expected, strains MG1655 and VL584(pIB254) agglutinated guinea pig erythrocytes and mannan-containing yeast cells (38, 48). In line with previous results, agglutination was inhibited either by mannose or by α-methyl mannopyranoside. Moreover, we found that the type 1 fimbriae produced by MG1655 were antigenically indistinguishable from those of strain CSH50; anti-MG1655 fimbria sera and anti-CSH50 fimbria sera produced equivalent results in cross-competitive ELISAs regardless of the target reagent (either purified MG1655 fimbriae or CSH50 fimbriae) or the competitor (either MG1655 cells or CSH50 cells) used (data not shown).

A striking difference between MG1655 and CSH50 became apparent, however, when colonies of the two strains were compared after growth on agar (Fig. 2). Whereas CSH50 gave rise to a mixture of small, convex colonies (which mainly contain fimbriate bacteria) and large, flat colonies (which mainly contain afimbriate bacteria), colonies of MG1655 inevitably resembled the afimbriate phase of CSH50, suggesting that fimbriation is largely suppressed in MG1655 during growth on agar. This observation was confirmed and extended by using a hemagglutination test with guinea pig erythrocytes (12) and a bacterial agglutination test with the anti-type 1 fimbria monoclonal antibody FG6 (14).

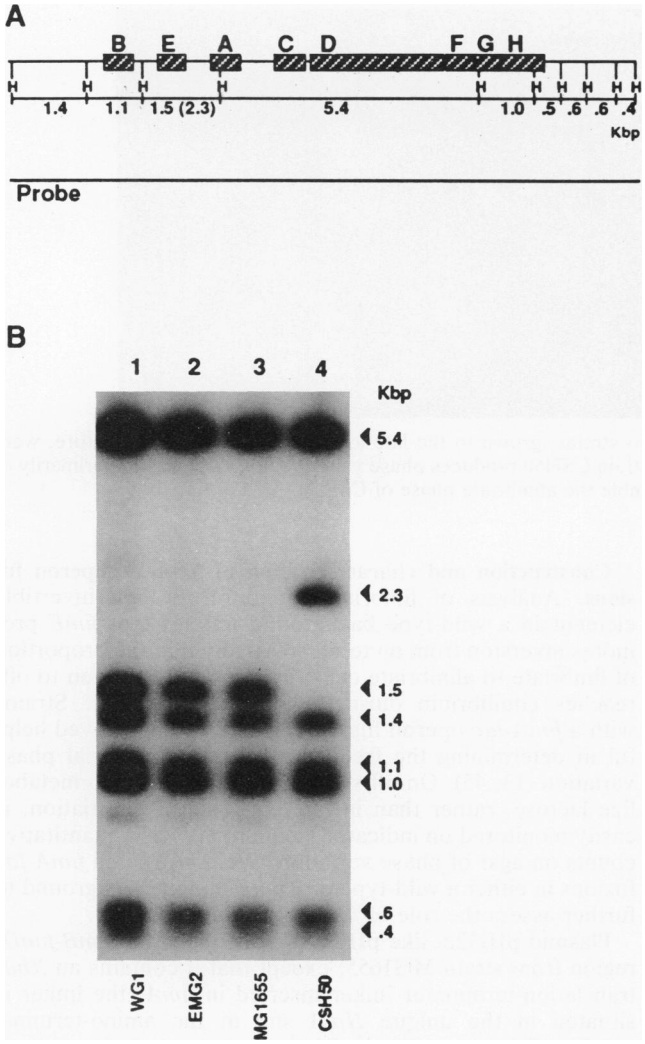

FIG. 1. Southern hybridization analysis of the *fim* region of strains WG1, EMG2, MG1655, and CSH50. (A) Physical and genetic organization of the *fim* genes in the various strains, showing the position of *Hin*cII restriction endonuclease sites (H) and the sizes of corresponding fragments. The *Hin*dIII-*Sal*I *fim* probe used in the hybridization is also indicated. (B) Chromosomal DNA isolated from the indicated strains was digested with *Hin*cII, separated on a 1% agarose gel, and blotted to Zetabind nylon membranes. Hybridizations were done at high stringency (33). Although MG1655 is indistinguishable from strains WG1 and EMG2, CSH50 contains a 0.8-kbp insert in the *fimE-A* region.

We found that colonies of MG1655, like those of the off-phase variants of CSH50, invariably produced a weak agglutination reaction.

Construction of isogenic *fimE* mutants of MG1655. Since the only known genetic difference in the *fim* genes between CSH50 and MG1655 is that CSH50 contains an IS*1* element in *fimE*, we next constructed isogenic *fimE* mutants of MG1655 to determine whether *fimE* is responsible for the phenotypic differences. By manipulating the cloned type 1 fimbria genes in vitro and using a recently described allelic exchange procedure (7), we constructed two series of *fimE* mutant strains, AAEC137 to -142 and AAEC143 and -144, of MG1655. Strains AAEC143 and -144 contain an *Xba*I translation-terminator linker in the amino-terminal region of *fimE*. Strains AAEC137 to -142 include the copy of IS*1* from the *fimE* gene of CSH50; the IS*1* element was positioned in the same site in the MG1655 *fimE* gene as found in the CSH50 *fimE* gene.

Plasmid pIB315, a derivative of the temperature-sensitive pSC101 vector pMAK705 (34), contains the *fimE-fimC* genes derived from MG1655. An *Xba*I linker containing stop codons in each reading frame was introduced into the unique *Eco*O1091 site of pIB315 after treatment of the linearized plasmid with Klenow enzyme to produce the *fimE* mutant plasmid, pIB330. The IS*1* element situated in the *fimE* gene of CSH50 is conveniently located between known *Sac*II and *Stu*I restriction endonuclease sites (31). Accordingly, the 28-bp *Sac*II-*Stu*I *fimE* fragment of pIB315 was replaced by the corresponding 0.8-kbp *fimE*-IS*1* fragment from pIB225, a plasmid that contains the *fimB-fimA* region cloned from CSH50, to yield pIB328.

The mutant *fimE* genes were transferred into the chromosome of AAEC112 by allelic exchange (Fig. 3). As a control, we also reconstructed the wild-type *fim* organization by transferring the unaltered *fimE* allele of plasmid pIB315 into strain AAEC112. Strain AAEC112 is a derivative of MG1655 that contains a *sacB*-Neo^r cassette replacing *fimE* and the amino-terminal portion of *fimA*. The allelic exchange was completed in three steps, following exactly the same procedure that we have described before (7). In the first step, plasmid integrate derivatives of AAEC112 were selected on chloramphenicol at 42°C, the nonpermissive temperature for plasmid replication (34). Next, the plasmid integrates were grown at 42°C in the absence of antibiotics to enrich for cells that had first excised, and later cured, the plasmid. Finally, since *sacB* confers sucrose sensitivity, the cells were plated onto sucrose agar to select the desired recombinants that had both excised and cured the *sacB*-Neo^r cassette. Confirmation that the allelic exchange had been successful was provided by Southern hybridization analysis (Fig. 4). Moreover, the Southern blot of DNA from AAEC137 to -142 was probed with IS*1* to confirm that each strain had acquired a single additional copy of the insertion element only. This was indeed true for all the strains except AAEC141, which contained two extra copies of IS*1* (data not shown). Strains AAEC137 (*fimE*::IS*1*) and AAEC143 (*fimE-Xba*I translation termination linker) were used in all subsequent studies of fimbriation. Strains AAEC135 and -136, produced by reconstructing the wild-type chromosomal *fim* region in AAEC112, closely resembled the wild-type parent, MG1655.

Comparison of isogenic MG1655 strains and CSH50. When examined by immunofluorescence microscopy, samples of MG1655, AAEC137, and AAEC143, like CSH50, contained both fimbriate and afimbriate cells. Phase variant cells were apparent in all the growth conditions tested (static or shaking LB broth or LB agar at 37°C), although the proportion of fimbriate to afimbriate cells was influenced by growth conditions. The proportion of fimbriate cells present in liquid cultures of each strain, starting with an inoculum containing a majority of afimbriate bacteria, was strongly influenced by aeration (Table 2); the proportion of fimbriate bacteria was greater when the cells were grown in static broth culture (15 ml of LB broth in a 125-ml Erlenmeyer flask) than in shaking broth culture. These results duplicated previous studies (15, 32, 40, 41) and are explicable by the strong selective advantage of fimbriate cells in static culture; in poorly aerated conditions, fimbriate cells outgrow afimbriate bacteria after formation of a surface pellicle at the oxygen-rich air-liquid interface (40, 41).

When samples of the various strains were inoculated onto

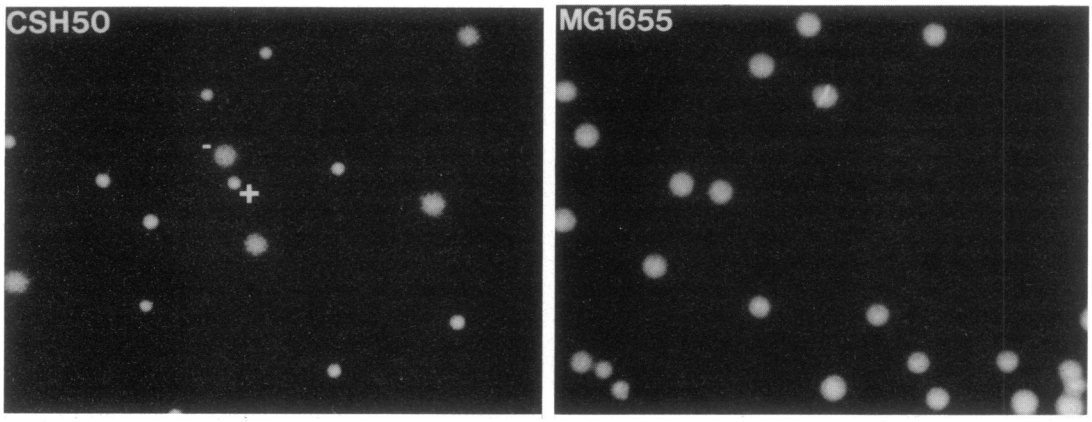

FIG. 2. Colonies of strains MG1655 and CSH50. Cultures of the two strains, grown to the late log phase in static broth culture, were inoculated onto L agar plates and incubated for 14 h at 37°C. Although strain CSH50 produces phase variant colonies consisting primarily of either fimbriate cells (+) or afimbriate cells (−), those of MG1655 resemble the afimbriate phase of CSH50.

agar, we found that, like CSH50, both of the *fimE* mutants of MG1655 produced phase variant colony types (data not shown). The proportion of the fimbriate-phase colonies of the *fimE* mutants was strongly influenced by the prior growth conditions; cells inoculated from static broth culture produced many more fimbriate-phase colonies than those grown previously in shaking broth (data not shown). As expected, this difference was reflected in the proportion of fimbriate bacteria counted by immunofluorescence analysis of pooled colonies (Table 3). In contrast, we found that, although a small proportion of MG1655 cells were fimbriate, the ratio of fimbriate to afimbriate cells present in pooled colonies was unaffected by prior growth conditions (Table 3).

Previously published results have demonstrated that the orientation of the *fim* invertible element determines *fimA* transcription and therefore fimbriation (1). As expected, analysis of chromosomal DNA samples isolated both from MG1655 and from the *fimE* mutants showed reasonable agreement between the orientation of the invertible element and the proportion of fimbriate cells (Tables 2 and 3).

All colonies of MG1655 appeared to resemble the afimbriate phase of the *fimE* mutant strains. To test this idea directly, we compared individual colonies of MG1655 with those of the *fimE* mutant AAEC143 to determine both the proportion of fimbriate cells and the orientation of the invertible element. Samples of the two strains were grown to the late log phase in static broth culture and diluted onto agar. At the time of inoculation, we also isolated chromosomal DNA to allow quantitation of the orientation of the invertible element. Sixteen colonies each of MG1655 and of AAEC143 were picked at random and processed as outlined above. As expected, we observed good agreement between percent fimbriation and the orientation of the invertible element. Each of the 16 MG1655 colonies consisted mainly (>85%) of afimbriate bacteria and contained the invertible element predominantly in the off orientation (Fig. 5). In contrast, only two colonies of AAEC143 consisted primarily (>85%) of afimbriate bacteria; the rest were fimbriate phase variants. Our analysis of the state of the invertible element in the inoculum used to seed the agar plates allowed us to estimate that 35% of MG1655 and 68% of AAEC143 cells contained the switch in the on orientation. Thus, it is very unlikely that all the colonies of MG1655 that we picked arose from afimbriate-phase cells by chance ($P = 0.65^{16} = 0.001$, from the binomial distribution).

Construction and characterization of *fimA-lac* operon fusions. Analysis of fimbriation and of the *fim* invertible element in a wild-type background implies that *fimE* promotes inversion from on to off so rapidly that the proportion of fimbriate to afimbriate cells (invertible element on to off) reaches equilibrium during colony development. Strains with a *fimA-lac* operon fusion have previously proved helpful in determining the frequency of type 1 fimbrial phase variation (13, 45). On-off switching in the ability to metabolize lactose, rather than in the synthesis of fimbriation, is easily monitored on indicator medium, allowing quantitative counts on agar of phase variation. We constructed *fimA-lac* fusions in either a wild-type or a *fimE* mutant background to further assess the role of *fimE* in phase variation.

Plasmid pIB322, like pIB308 (7), includes the *fimB-fimD'* region from strain MG1655, except that it contains an *Xba*I translation-terminator linker inserted in *fimA*; the linker is situated in the unique *Hpa*I site in the amino-terminal portion of the gene. The *lacZYA* genes were subcloned from pKE005, a descendant of pRS415 (53), in which *lacZYA* are flanked by inserted *Xba*I linkers. Plasmid pIB333 contains the *lac* genes in the same transcriptional orientation as *fimA*. A derivative of pIB333, plasmid pIB338, that contains a mutant allele of *fimE* was constructed by replacing the 3.3-kbp *Hin*dIII-*Sac*II (*fimB-fimE'*) fragment of pIB333 with a 0.7-kbp *Hin*dIII-*Sac*II fragment from pIB330.

The *fimA-lac* operon fusion constructs, together with *fimE*, were transferred into the chromosome of strain AAEC187 or AAEC193, *lac* deletion derivatives of MG1655 that contain a *sacB*-Neo^r cassette replacing *fimA* and *fimE*, using allelic exchange as described above. Since the *fim* deletion in the intermediate strains includes *fimE* and extends beyond the *Hpa*I site in *fimA*, recombinant strains acquire both the plasmid-borne *fimA-lac* fusion and *fimE*. Confirmation that the recombinant strains contain the expected chromosomal structure was provided by Southern hybridization (data not shown).

As predicted by our prior analyses of fimbriation and of the invertible element, the *fimA-lac* derivative of MG1655 (AAEC198, AAEC202) invariable produced a single phase-negative colony type on lactose-MacConkey indicator media. In sharp contrast, the *fimE* mutant strains (AAEC203, AAEC204) produced two colony types: light pink phase-negative colonies, which closely resembled those of the parent strain, and deep red phase-positive variants (data not

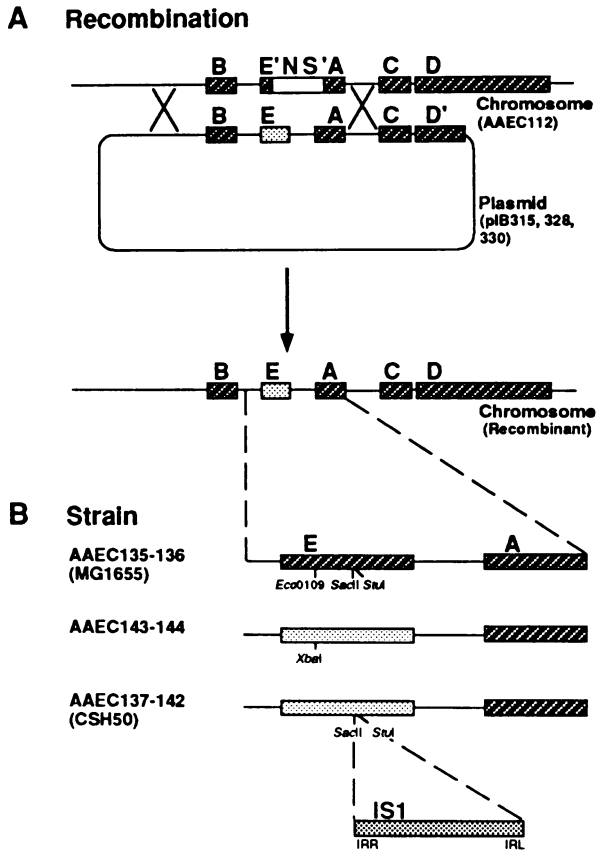

FIG. 3. Construction of *fimE* mutants. (A) Recombination between AAEC112, the intermediate strain containing the *sacB*-Neo^r cassette (NS), and the temperature-sensitive plasmid pIB315 (wild-type control), pIB328 (*fimE*::IS*1*), or pIB330 (*fimE-Xba*I translation-terminator linker) regenerates the wild-type strain (AAEC135 and -136) or *fimE* mutants (AAEC137 to -144), respectively. The *sacB*-Neo^r cassette replaces the region encompassing the 3′ section of *fimE* to the 5′ end of *fimA*. Plasmid-located *fimE* is represented by the lightly shaded box. (B) Organization of the *fimE-A* region of the recombinant strains. Wild-type (darker shading) or mutant (lighter shading) *fimE* alleles together with relevant restriction endonuclease sites (*Eco*O1091, *Sac*II, *Stu*I, and *Xba*I) are shown. Strains AAEC143 and -144 contain a 14-bp *Xba*I translation-terminator linker inserted in the *Eco*O1091 site of the wild-type gene. Strains AAEC137 to -142, like CSH50, contain IS*1* inserted between the *Sac*II and *Stu*I sites. The orientation of the IS*1* element, shown by the inverted repeats IRR and IRL, is as defined previously (39).

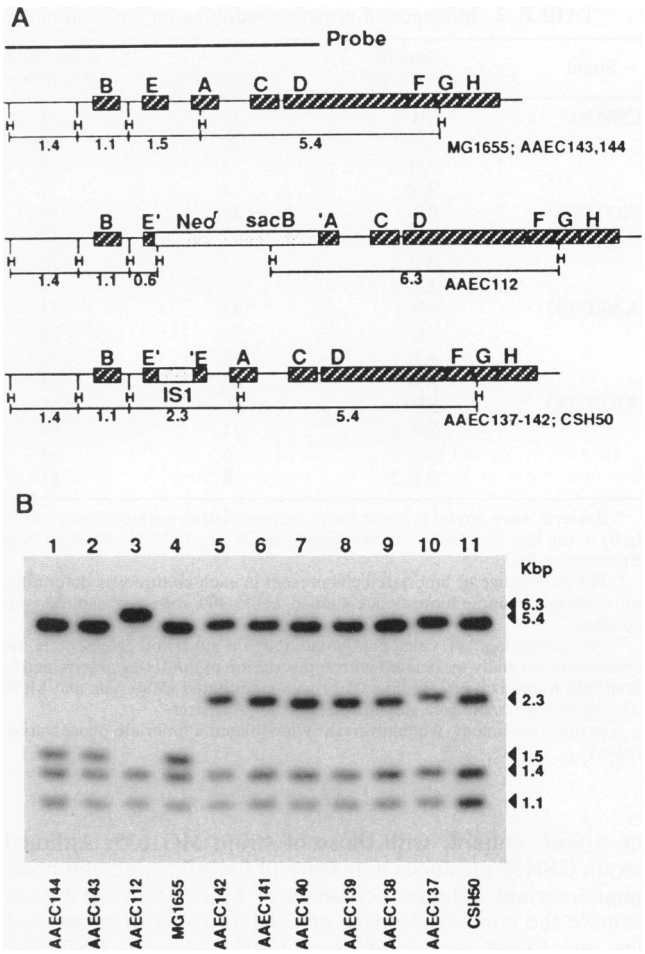

FIG. 4. Southern hybridization analysis of wild-type (MG1655), intermediate (AAEC112), and *fimE* mutant (AAEC137 to -144, CSH50) strains. (A) Physical and genetic organization of relevant strains, showing the position of pertinent *Hin*cII restriction endonuclease sites (H) and the sizes of corresponding fragments (in kilobase pairs). The *Hin*dIII-*Bam*HI *fim* probe used in the hybridization is also indicated. (B) Chromosomal DNA isolated from the indicated strains was digested with *Hin*cII, separated on a 1% agarose gel, and blotted to nitrocellulose. Hybridizations were done at high stringency (33). As expected, recombinant strains AAEC143 and -144, containing an *Xba*I translation-terminator linker in the *Eco*O1091 site of *fimE* (lanes 1 and 2), are indistinguishable from the wild-type strain, MG1655 (lane 4). Strains AAEC137 to AAEC142, with the *fimE*::IS*1* allele from CSH50 (lanes 5 to 10), resemble CSH50 (lane 11). None of the recombinants resemble the intermediate strain, AAEC112 (lane 3), their immediate parent.

shown). Thus, in a wild-type strain background, counts of the *fimA-lac* fusion strain on indicator media cannot be used to measure the frequency of inversion.

Fimbriation in wild type and *fimE* mutants. Previous work has suggested that *fimE* (*hyp*) mutants are hyperfimbriate (44). Thus, we were particularly interested to assess the extent of fimbriation in the various *fimE* mutants described here. Accordingly, we estimated the amount of fimbrial antigen present in cultures of MG1655 and in the various *fimE* mutants by ELISA inhibition, as described previously (10). The cultures, grown to the late log phase in static broth culture at 37°C, were also examined by immunofluorescence microscopy to determine the proportion of fimbriate cells. We found that whereas strain CSH50 appeared to be somewhat hyperfimbriate in comparison with MG1655, the isogenic *fimE* mutants of MG1655 seemed no more fimbriate. Indeed, when we estimated the amount of fimbriae per fimbriate cell, the isogenic *fimE* mutants were actually less fimbriate than their wild-type parent (Fig. 6).

DISCUSSION

The study of type 1 fimbrial phase variation has been greatly facilitated by the ability to distinguish phase variant colony types on agar. Here, however, we show that the appearance of fimbriate phase variant colonies can, at least in the strain background used in this study, depend on mutation of the *fimE* gene. We first suspected this association when we compared colonies of strain CSH50, found to

TABLE 2. Influence of growth conditions on fimbriation

Strain	Growth conditions[a]	% Fimbriate[b]	% Invertible element on[c]
CSH50	SB 1	45	33
	SB 2	30	37
	AB 1	3	4
	AB 2	4	7
MG1655	SB 1	44	36
	SB 2	47	37
	AB 1	5	10
	AB 2	2	4
AAEC137	SB 1	41	51
	SB 2	78	76
	AB 1	4	12
	AB 2[d]	82	74
AAEC143	SB 1	66	66
	SB 2	71	69
	AB 1	6	8
	AB 2	8	11

[a] Bacteria were grown in static broth culture (SB) or aerated broth culture (AB) to the late log phase. Cultures consisted of 15 ml of L broth in a 125-ml Erlenmeyer flask.

[b] The percentage of fimbriate cells present in each culture was determined by counting immunofluorescence-stained cells. 400 cells per sample were counted.

[c] The percentage of cells containing the *fim* invertible element in the orientation normally associated with transcription of *fimA* was determined by Southern hybridization. Radioactivity was quantitated (Materials and Methods) by measurement in a Betascope 603 Blot Analyzer.

[d] In this experiment, we inadvertently inoculated a fimbriate phase variant colony.

be a *fimE* mutant, with those of strain MG1655. Although strain CSH50 produces a mixture of fimbriate and afimbriate phase variant colonies, colonies of MG1655 invariably resemble the afimbriate phase of CSH50. In subsequent studies, we found that transfer of *fimE*::IS*1* from CSH50 to MG1655 by allelic exchange or construction of a *fimE* mutant by introducing a translational stop in the gene produced strains that acquired the phase variation phenotype of CSH50. We believe that strains MG1655 and CSH50 are otherwise isogenic in the *fim* gene cluster; type 1 fimbriae isolated from the two strains are antigenically indistinguishable and the DNA sequence of the invertible region of the two strains is identical (data not shown). Therefore, the *fimE*::IS*1* allele present in CSH50 probably accounts for the differences in fimbrial expression between this strain and MG1655.

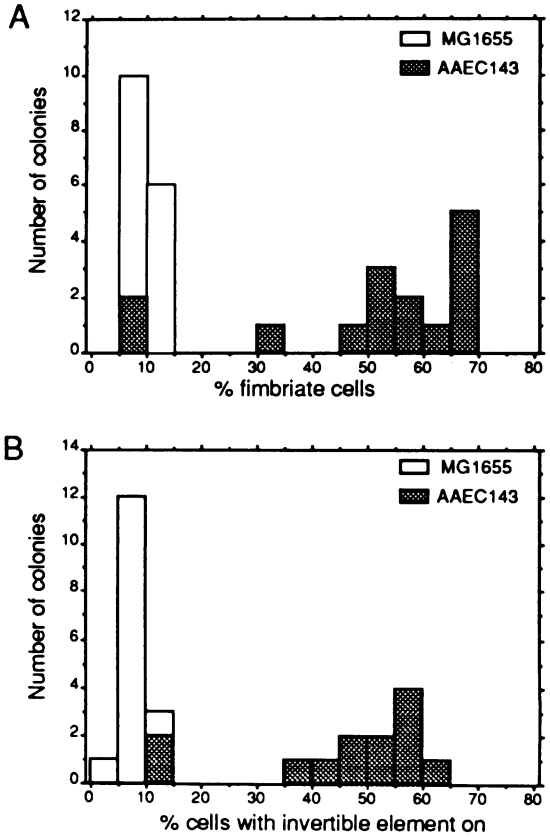

FIG. 5. Fimbriation in individual colonies of strain MG1655 and the *fimE* mutant AAEC143. Cells grown in static broth culture were inoculated onto L agar and incubated for 16 h at 37°C. Chromosomal DNA, isolated at the time of inoculation onto agar, was later examined to determine the orientation of the invertible element. Colonies were picked at random, and the constituent cells were processed to determine the proportion of fimbriate cells by immunofluorescence microscopy (A) and the state of the invertible element by Southern hybridization analysis and quantitative radioactive counting (B).

TABLE 3. Fimbriation in agar-grown bacteria

Strain	Prior growth conditions[a]	% Fimbriate[b]	% Invertible element on[c]
MG1655	SB	5	13
	AB	5	NT[d]
CSH50	SB	32	37
	AB	6	NT
AAEC137	SB	33	34
	AB	11	NT
AAEC143	SB	41	45
	AB	11	NT

[a] Bacteria were grown in static broth culture (SB) or aerated broth culture (AB) to the late log phase, diluted onto L agar, and incubated at 37°C for 16 h.

[b] Colonies from each agar plate were resuspended in 2 ml of L broth (100 to 200 colonies). The percentage of fimbriate cells present in each sample was determined by counting immunofluorescence-stained cells. 400 cells per sample were counted.

[c] The percentage of cells containing the *fim* invertible element in the orientation normally associated with transcription of *fimA* was determined by Southern hybridization analysis. Radioactivity was quantitated (Materials and Methods) by measurement in a Betascope 603 Blot Analyzer.

[d] NT, not tested.

In all strains examined, the extent of fimbriation was associated with the orientation of the *fim* DNA invertible element. The invertible element is mainly off in colonies of strain MG1655 but either mainly on, in the phase-on colonies, or mainly off, in the phase-off colonies, of the various *fimE* mutants. Although MG1655 clearly undergoes phase variation, the process is phenotypically cryptic when assessed by the classical indicator, colony variation. These observations, together with our recent studies of plasmid recombination (35), show that *fimE* promotes inversion of the *fim* phase switch from on to off.

Inversion from on to off in the wild-type background is so rapid that the proportion of fimbriate to afimbriate cells (invertible element on to off) reaches equilibrium during colony development. Thus, measurement of the frequency of phase variation cannot be made by counting colony types as it has in the past (13, 45); nevertheless, a quantitative

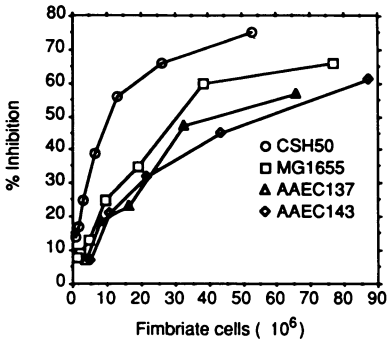

FIG. 6. ELISA inhibition studies of MG1655 and *fimE* mutants CSH50, AAEC137, and AAEC143. The strains were grown to the late log phase in static broth culture at 37°C. ELISA values were determined as described previously (10). Samples of the culture were examined by immunofluorescence microscopy (Materials and Methods) to determine the proportion of fimbriate cells. Values shown are corrected for the differences in this proportion, allowing determination of the average amount of fimbriae per fimbriate cell.

estimate can be made by analyzing chromosomal DNA isolated from single colonies. Using this approach, we observed little difference in the apparent frequency of inversion from off to on between wild-type and the isogenic *fimE* mutant strains, with values of approximately 10^{-3} per cell per generation. These values are close to those reported before for derivatives of CSH50 (13). The frequencies of inversion from on to off are $>10^{-2}$ and approximately 10^{-3} per cell per generation for the wild-type and *fimE* mutant strains, respectively. The wild-type value represents a minimum, since colonies reach equilibrium before sampling.

Although the presence of fimbriate phase variant colonies has been widely reported in both *E. coli* B and *E. coli* K-12, our present studies of MG1655 confirm an early report by Maccacaro and Hayes (32) that fimbriation in wild-type *E. coli* K-12 is suppressed by growth on agar. These workers also isolated mutants, termed sigma, that displayed many of the properties of CSH50 and of the *fimE* mutants of MG1655. Other investigators have separated clinical isolates into different categories depending on whether or not they produce type 1 fimbriae during growth on agar (20, 28, 29, 49, 51). We suspect that this distinction, and the observations noted above, may reflect the high prevalence of *fimE* mutants in both laboratory and clinically isolated collections of strains. Our present study of fimbrial phase variation in a wild-type background, compared with that in isogenic *fimE* mutants, is somewhat at odds with a previous study (45). However, it has since been shown that the strain used as wild type in this earlier work already contained an insertion in or near *fimE* (*hyp*). The lesion in this strain appears to alter either the level or activity of *fimE* (42), presumably accounting for the discrepancy between this earlier work and our present study.

In agreement with previous workers, we also observed a clear association between colony morphology and fimbriation; colonies mainly composed of fimbriate bacteria are smaller and smoother than those consisting primarily of afimbriate cells (8, 45, 48, 56). Nevertheless, we also noticed variation in colony morphology both in MG1655 and in the *fimE* mutants of this strain that could not be attributed to differences in fimbriation (data not shown). This difference apparently corresponds to the variation in cell agglutination (fluffing) described previously in other *E. coli* K-12 derivatives (9). Contrary to this previous report, however, we failed to detect any correlation between variations in fimbriation and cell fluffing (data not shown).

According to a previous model, *fimB* and *fimE* influence the orientation of the *fim* invertible element by displaying opposing activities (31, 46). Thus, it has been suggested that *fimB* promotes inversion from the off to on direction, whereas *fimE* stimulates the opposite reaction. One clear prediction of this model, that *fimE* mutants contain the invertible region locked in the on orientation, is supported neither by our previous observations of CSH50 (1, 2, 11, 13, 15, 16, 18, 19) nor by the data presented here. In addition, we note that another *fimE* (*hyp*) mutant constructed independently also undergoes phase variation associated with the *fim* inversion (45). We have recently demonstrated that *fimB* is capable of promoting inversion in both directions (35).

In contrast to a previous study (44), we found no evidence to suggest that *fimE* mutants are hyperfimbriate. Indeed, the average amount of fimbriae per fimbriate cell was somewhat lower in the isogenic *fimE* mutants of MG1655 than in their wild-type parent. We believe that both our present results and those reported previously are explicable by the fact, as shown here and elsewhere (31, 35, 46), that *fimE* promotes inversion of the invertible element from on to off. The effect of *fimE* applies to both plasmid-borne and chromosomally located invertible elements. Thus, extra copies of plasmids containing the *fim* invertible element in the on orientation, as occurs in the absence of *fimE*, should result in the elevated levels of fimbriation that were previously reported (44).

In separate studies, workers have characterized mutants that show a high frequency of *fim* phase variation. These mutants, termed *pilG* (55) and, independently, *osmZ* (25), are alleles of the structural gene for the abundant DNA-binding protein H1 (26, 42, 55). The influence of the *osmZ* allele on phase variation was originally investigated in a *fimA-lac* operon fusion derivative of CSH50, called VL386. Strain VL386 also contains the IS*1* insert in *fimE* (data not shown). Moreover, the strains used to assess the activity of the *pilG* allele on fimbrial phase variation also contain an insert in or near *fimE* that appears to alter its activity or function (42). Thus, the influence of H1 in fimbrial phase variation and expression in a wild-type background has yet to be determined. We have recently initiated studies to address these questions using MG1655 and *fimE* mutant derivatives of this strain.

Our finding that the *fimE* gene of CSH50 contains IS*1* has led us to an explanation of why multiple hybridization signals were detected in one (1), but not other (2, 55), studies of *fim*. The *fim* DNA probe used in the first study was derived from CSH50 and included the IS*1* element. Thus, the hybridizations in this study detected sequences corresponding to both the *fim* invertible region and the multiple copies of IS*1* present in the chromosome of *E. coli* K-12 strains (37).

Like others, we also believe that the enrichment of fimbriate bacteria observed during growth in static culture reflects their selective advantage in these conditions (15, 32, 40, 41). This conclusion is supported by the substantially reduced growth of a *fim* deletion mutant of MG1655 (6) in these conditions (data not shown). In addition, we also confirm that afimbriate cells tend to outgrow their fimbriate counterparts during growth on agar (data not shown). Although previous studies have concluded that the phase variation of type 1 fimbriation is a random process, our finding that strains used previously are *fimE* mutants, coupled to the discovery that *fimB* is capable of promoting inversion in both directions, suggests to us that phase

variation is subject to environmental control in a wild-type background. We are currently investigating this possibility.

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