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Characterization of *Shigella* Type 1 Fimbriae: Expression, FimA Sequence, and Phase Variation

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This study documents the presence of type 1 fimbriae on *Shigella* and confirms these mannose-sensitive adherence structures to be bona fide components of the *Shigella* surface. While laboratory-passaged *Shigella* strains and lyophilized clinical isolates failed to express type 1 fimbriae, 6 of 20 recent clinical isolates, including 4 *Shigella flexneri* strains, 1 *Shigella boydii* strain, and 1 *Shigella dysenteriae* strain, produced type 1 fimbriae as detected by mannose-sensitive hemagglutination (MSHA) and electron microscopy. Optimal production of a predominantly Fim$^+$ population required serial passage every 48 to 72 h in unshaken brain heart infusion broth at 37°C. Fim$^+$ *Shigella* cultures were capable of reversibly switching to a non-MSHA, afimbriated phase during serial aerobic cultivation on tryptic soy agar plates. The amino acid sequence of *S. flexneri* type 1 FimA contained 18 substitutions compared to that of *Escherichia coli* fimbrillin. Indirect immunoelectron microscopy suggested the presence of both shared and unique epitopes on *E. coli* and *S. flexneri* type 1 fimbriae. Random phase variation between fimbriated and afimbriated states in *Shigella* was accompanied by the genomic rearrangement associated with phase variation in *E. coli*.

Although closely related genetically, *Escherichia coli* and *Shigella* differ in both pathogenicity and host and tissue specificity (33). *E. coli* is a normal inhabitant of the gastrointestinal tract (GIT) in humans and colonizes a wide range of hosts to produce a variety of intestinal and extraintestinal diseases. On the other hand, all four serogroups of *Shigella* exhibit a high degree of host and tissue specificity, infecting only the colonic epithelial cells of humans and higher primates to cause bacillary dysentery. The route of *Shigella* transmission is fecal-oral, with many intervening intermediaries. *Shigella* can survive for more than 6 months in water at room temperature (33), and there is circumstantial evidence that *Shigella* survives for extended periods in natural aquatic environments, since surface water sources have been implicated in outbreaks of shigellosis (30). Little is known about mechanisms that enable *Shigella* to survive either within the GIT or outside in the environment. It seems likely that an adhesin-receptor interaction is required for the organism to survive mechanical cleansing at the mucosal surface, particularly when one considers that the infectious dose of *Shigella* is relatively low (10 to 100 organisms).

Fimbrial appendages, such as type 1 fimbriae, confer on pathogenic *E. coli* a selective advantage in colonizing eukaryotic hosts (3–5, 18–20). These fimbriae are prevalent among the *Enterobacteriaceae*, and 70% of fecal *E. coli* strains isolated contain type 1 fimbriae (5). With the exception of an early work from one laboratory indicating the expression of type 1 fimbriae in some strains of *Shigella flexneri* (11, 12), other laboratories have not reported similar adhesive structures to be present in this bacterium. Thus, present-day discussions of virulence and pathogenesis in *Shigella* spp. discount the presence of fimbriae in these organisms (16, 28). This study dem-

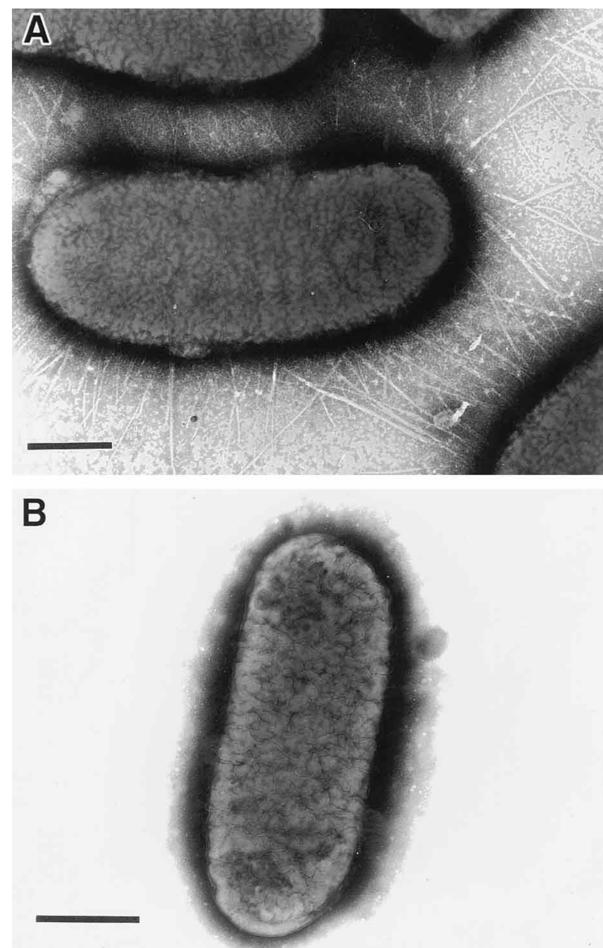

FIG. 1. Transmission electron photomicrograph of MSHA$^+$ (A) and MSHA$^-$ (B) F1a1. Bars, 0.5 μm.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Hybridization result[b]	Reference or source
Strains			
E. coli			
AAEC185	Δ*recA* Δ*fim*, MSHA$^-$, afimbriated	−	6
DH5α	MSHA$^+$, predominantly fimbriated	+	M. Wolf
HB101	MSHA$^-$	+	WRAIR
S. flexneri			
M9OT-W	Serotype 5a, MSHA$^-$, afimbriated	+	WRAIR
M9OT-55	Invasion plasmid-cured derivative of M9OT-W	+	WRAIR
F1a1	Serotype 1a, MSHA$^+$, fimbriated	+	D. C. Old (11)
F2a8	Serotype 2a, MSHA$^+$, fimbriated		D. C. Old (11)
F2b1	Serotype 2b, MSHA$^+$, fimbriated		D. C. Old (11)
F3a103	Serotype 3a, MSHA$^+$, fimbriated		D. C. Old (11)
F4a6	Serotype 4a, MSHA$^+$, fimbriated		D. C. Old (11)
F5a12	Seroytpe 5a, MSHA$^+$, fimbriated		D. C. Old (11)
187	Clinical isolate, MSHA$^+$, fimbriated		C. Prada
15CD34	Clinical isolate, MSHA$^+$, fimbriated		C. Prada
1NC101	Clinical isolate, MSHA$^+$, fimbriated		C. Prada
1NC103	Clinical isolate, MSHA$^+$, fimbriated		C. Prada
229	Clinical isolate, MSHA$^-$, afimbriated		C. Prada
S. boydii			
24NC103	Clinical isolate, MSHA$^+$, fimbriated		C. Prada
CG1159	MSHA$^-$	+	WRAIR
S. sonnei 53GI	Laboratory strain, MSHA$^-$	+	WRAIR
S. dysenteriae			
CG2572[c]	Serotype 1, MSHA$^-$	−	WRAIR
CG1535	Serotype 2, MSHA$^-$	+	WRAIR
H105-181	Serotype 3, MSHA$^-$	+	WRAIR
8CD201	Clinical isolate, MSHA$^+$, fimbriated	+	C. Prada
Plasmids			
pSH2	pACYC184 with an 11.2-kb fragment containing an *E. coli* type 1 fimbrial gene cluster	+	P. Orndorff (24)
pNS10-1	pHC79 cosmid clone containing an *S. flexneri* type 1 fimbrial gene cluster	+	This study
pNS11	Cosmid clone overlapping with pNS10-1	+	This study
pNSpil8	pUC18 clone with a 7.5-kb *Sal*I-*Bam*HI fragment from pNS11 containing the *fimA* gene	+	This study

[a] MSHA$^+$ and MSHA$^-$ refer to MSHA status after pellicle selection. All strains were MSHA$^-$ before pellicle selection. Fimbriated or afimbriated indicates presence or absence of fimbriae as shown by electron microscopy.
[b] With radiolabeled insert DNA from pSH2.
[c] Eleven other *S. dysenteriae* strains in this series, not shown here, also tested *fim* probe negative.

onstrates unequivocally the presence of type 1 fimbriae in *Shigella*.

Type 1 fimbriae were detected by mannose-sensitive hemmaglutination (MSHA) of guinea pig erythrocytes (Sigma) carried out as previously described (11, 12). *E. coli* DH5α and AAEC185 (Table 1) served as the positive and negative controls, respectively, in the assay. The selective outgrowth of fimbriated *Shigella* by using Duguid's strains (Table 1) (11) was accomplished by serially passaging these strains in unshaken broth cultures in 13 by 100-mm glass culture tubes. Type 1 fimbriae are highly hydrophobic, and fimbriated bacteria aggregate at the air-broth interface, where the supply of oxygen is abundant and where they eventually form a pellicle. Thus, the inoculum for each successive culture was taken from the surface or pellicle of the previous broth culture. Several media were tried, including Luria-Bertani broth, Trypticase soy broth, antibiotic medium 3, brain heart infusion (BHI) broth, and Casamino Acids-yeast extract broth supplemented with 1 mM calcium chloride (29). BHI broth was found to be the most suitable for expressing MSHA in these *Shigella* strains. Cultures grown for 48 h at 37°C prior to each subculture exhibited hemagglutination more rapidly than cultures passaged every 24 h. *S. flexneri* strains of serotypes 1a, 2a, 2b, 3a, 4a, and 5 exhibited MSHA$^+$ fimbriae after 5 to 10 serial transfers. *Shigella* strains could not be induced to express MSHA in shaking broth cultures or on agar plates. However, once a strongly MSHA$^+$ population had been obtained, these cultures continued to hemagglutinate when transferred to agar plates or grown in shaking broth cultures. Several serial passages on tryptic soy agar at 24-h intervals were required for such a culture to revert to a predominantly nonhemagglutinating population under these conditions. All six *S. flexneri* serotypes exhibited a reversible phase variation between MSHA$^+$ and MSHA$^-$ bacteria. MSHA$^-$ colonies gave rise to a predominantly MSHA$^+$ population after five to seven passages in static BHI broth.

The association of MSHA positivity with fimbriae was established by electron microscopy. Figure 1 contrasts *S. flexneri* F1a1 (MSHA$^+$ [Fig. 1A]) with its MSHA$^-$ counterpart (Fig. 1B). Similar structures were observed on MSHA$^+$ bacteria

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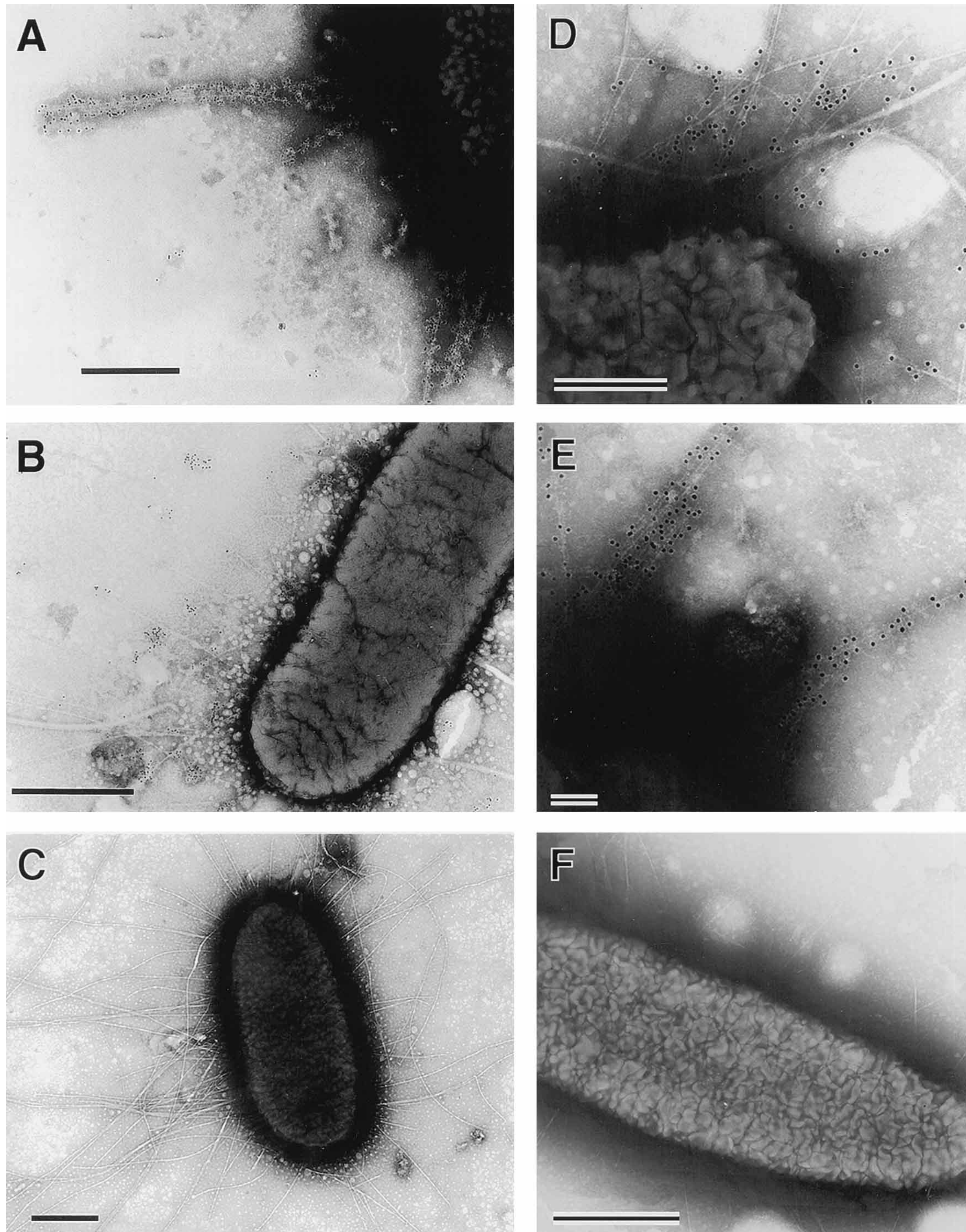

FIG. 2. Transmission electron photomicrographs of *Shigella* fimbriae. (A) MSHA$^+$ F1a1 labeled with antiserum against MSHA$^+$ F1a1 which was absorbed with MSHA$^-$ F1a1. Bar, 0.25 μm. (B) DH5α labeled with antiserum as for panel A. Bar, 0.5 μm. (C) MSHA$^+$ clinical isolate 187. Bar, 0.5 μm. (D) AAEC185(pNS10-1) labeled with antiserum against AAEC185(pNS10-1) which was absorbed with AAEC185 and cross-absorbed with AAEC185(pSH2). Bar, 0.25 μm. (E) Fim$^+$ F1a1 labeled with antiserum as for panel D. Bar, 0.25 μm. (F) DH5α labeled with antiserum as for panel D. Bar, 0.5 μm.

representing *S. flexneri* serotypes 1a, 2a, 2b, 3a, 4a, and 5 (Table 1). Fimbriae on all *S. flexneri* strains examined were morphologically similar to type 1 fimbriae, i.e., they were peritrichously distributed rigid rods ranging from 5.1 to 11.5 nm in diameter, with an average diameter of 8.9 nm. Rabbit antiserum against whole cells of Fim$^+$ F1a1, absorbed with Fim$^-$ F1a1, failed to agglutinate Fim$^-$ F1a1 but agglutinated Fim$^+$ F1a1 to a titer of 1:2,560 and *E. coli* DH5α to a titer of 1:64 in slide agglutination tests, indicating that while the serum had specificity for *Shigella*, it also contained some cross-reactive antibodies to *E. coli*. Indirect immunoelectron microscopy with this serum strongly coated Fim$^+$ F1a1 (Fig. 2A), whereas afim-

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briated F1a1 was not coated (data not shown). Fimbriae on DH5α were only weakly coated, and gold particles were predominantly clustered at the fimbrial tips (Fig. 2B). Western blots of heated saline extracts treated with the above-described antiserum showed a 17- to 18-kDa protein band for extracts of both $MSHA^+$ F1a1 and AAEC185(pSH2) (data not shown).

Strains commonly used and passaged in the laboratory, such as *S. flexneri* M9OT-W and *Shigella sonnei* 53G1 (Table 1), did not demonstrate MSHA activity during passage in BHI broth, although Southern blot analysis with a radiolabeled insert DNA probe from pSH2 extended an earlier finding (10) by showing that a homologous 16.5-kb *Sal*I fragment was present in *E. coli* HB101 and DH5α, $MSHA^+$ fimbriated F1a1, $MSHA^-$ afimbriated F1a1, M90T-W, *S. flexneri* M90T-55 (Table 1), and other *S. flexneri*, *Shigella boydii*, and *S. sonnei* strains (provided by S. B. Formal, Walter Reed Army Institute of Research [WRAIR], Washington, D.C.). The probe failed to hybridize to some strains of *Shigella dysenteriae* (Table 1), indicating that this region may undergo spontaneous deletion. Whether these deletions are gene specific or part of a larger genomic rearrangement seen in *E. coli* (7) is presently unknown. The presence of the genes in M9OT-55 confirms that the *Shigella* type 1 *fim* operon is on the chromosome, as seen in *E. coli*.

Several clinical isolates from Thailand and from the WRAIR culture collection (courtesy of P. Echeverria and S. B. Formal), which had been stored lyophilized, did not display an $MSHA^+$ reaction, indicating that the ability to express *Shigella* $MSHA^+$ fimbriae may be impaired or even lost under some growth and storage conditions. Growth conditions have been shown to influence fimbrial expression in other enteric bacteria (7, 12). M9OT-W, transformed with pSH2, expressed $MSHA^+$ fimbriae, which was confirmed by electron microscopy (data not shown). Twenty clinical isolates, which included all four *Shigella* serotypes which had been obtained from the Cayetano Heredia Hospital in Lima, Peru (Table 1), and stored on agar slab cultures, were serially passaged in BHI broth. Some of these strains, such as *S. flexneri* 187, 15CD34, 1NC101, and 1NC103, *S. boydii* 24NC103, and *S. dysenteriae* 8CD201, developed surface pellicles, exhibited $MSHA^+$ activity, and were fimbriated (Fig. 2C and Table 1).

Several overlapping $MSHA^+$ clones were obtained from a partial *Sau*3A genomic library of Fim^+ *S. flexneri* F1a1 in *Bam*HI-digested cosmid vector pHC79 and transformed into the *E. coli fim* deletion mutant, AAEC185. Of these, cosmid clone AAEC185(pNS10-1) was used in indirect immunoelectron microscopy studies described below and AAEC185 (pNS11) was used to subclone the *fimA* gene. Both these clones expressed peritrichous $MSHA^+$ fimbriae. Analysis of a 1,121-bp region that was sequenced with AAEC185(pNSpil8) as the template (Table 1) indicated that the *Shigella fimA* gene encodes a 182-amino-acid sequence. The *fimA* sequence, including the 314-bp upstream invertible regulatory element, is almost identical to the published sequence of *E. coli fimA* (21, 24). There were 8-, 42-, and 7-bp differences from *E. coli fimA* within nucleotides 1 to 140 (91% homology), 141 to 420 (86% homology), and 421 to 540 (91% homology), respectively (24). Within the same regions, *fimA* from a chicken-pathogenic *E. coli* strain (31) showed 1-bp (99.3% homology), 18-bp (94% homology), and 8-bp (93% homology) differences from *Shigella fimA*. There were 18- and 19-amino-acid differences between *Shigella* FimA and the two published sequences of *E. coli* fimbrillin (21, 24) (Fig. 3). Eleven of these differences were located within the central variable region of FimA, which is responsible for antigenicity (2, 26).

Rabbit antiserum raised against either AAEC185(pNS10-1)

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SFimA    MKIKTLAIVVLSALSLSSAAALADTTTVNG
pSH2                       TT   A A
pPKL6                      T    A A

SFimA    GTIHFKGEVVNAACAVDAGSVDQTVQLGQV
pSH2      V
pPKL6     V

SFimA     RTASLKQAGATSSAVGFNIQLNDCDTTVAT
pSH2          A E                  N   S
pPKL6         A E                  N   S

SFimA    KAAVAFLGTAIDATRTDVLALQSSAAGSAT
pSH2                 GH N
pPKL6                GH N

SFimA    NVGVQILDRTGNALTLDGATFSAQTTLNNG
pSH2               A           SE
pPKL6              A           SE

SFimA    TNTIPFQARYYAIGEATPGAANADATFKVQYQ
pSH2              F T A
pPKL6             F - A
```

FIG. 3. Comparison of *Shigella* FimA protein with *E. coli* FimA proteins. SFimA represents the amino acid sequence of the *Shigella* FimA protein derived from the nucleic acid sequence obtained in this study. *E. coli* FimA sequences in pPKL6 and pSH2 are from references 20 and 24, respectively. The positions of the variant amino acids in the two *E. coli* sequences are indicated below the SFimA sequence. The dash indicates a gap in the sequence.

or AAEC185(pSH2) was absorbed with AAEC185 to remove nonfimbrial epitopes. Then anti-serum against AAEC185 (pNS10-1) was further cross-absorbed with AAEC185(pSH2) to remove heterologous epitopes. This cross-absorbed antiserum against AAEC185(pNS10-1) strongly labeled fimbriae on AAEC185(pNS10-1) and Fim^+ F1a1, while sparse labeling was observed on DH5α fimbriae (Fig. 2D to F). Conversely, antiserum raised against AAEC185(pSH2) strongly labeled homologous fimbriae but failed to label or only faintly labeled fimbriae on F1a1 (data not shown). These results suggest that, as shown in earlier studies (11, 12), *E. coli* and *S. flexneri* type 1 fimbriae possess both shared and unique antigenic epitopes.

In *E. coli*, an asymmetric *Hin*fI restriction site within the 314-bp invertible element, upstream of the *fimA* gene, allows the determination of the orientation of the "switch" responsible for phase variation (1, 13). When amplified by PCR with one set of primer sequences upstream of the *E. coli fimA* gene and digested with *Hin*fI, DNA from predominantly Fim^+ strains, such as DH5α, showed fragments of 1,000 and 200 bp (Fig. 4A, lane 2), representing the "on" orientation, on agarose gels, while Fim^- strains, such as M9OT-W, showed fragments of 460 and 740 bp (Fig. 4A, lane 3), representing the "off" orientation of the switch. The 1,200-bp bands seen in these gels represent undigested amplified DNA. DNA from *Shigella* strains amplified with the same primers and digested with *Hin*fI indicated that cultures of Fim^- strains, such as *S. flexneri* 229, were relatively homogeneous (Fig. 4A, lane 4), while most Fim^+ cultures were composed of a mixture of Fim^+ and Fim^- cells, as shown by the presence of all four *Hin*fI bands on agarose gels (Fig. 4A, lane 5, and B, lanes 3 and 4). After PCR amplification with a different set of primers and digestion with *Hin*fI, DNA from *S. flexneri* Fim^+ F1a1, AAEC185(pSH2), and AAEC185(pNS10-1) showed bands corresponding to both orientations of the switch (Fig. 4C, lanes 2 to 4), while bands corresponding to only the off orientation were observed in Fim^- F1a1 (Fig. 4C, lane 1).

This study demonstrates definitively that *Shigella* expresses type 1 fimbrial adhesins. Recent reports have also documented the presence of type 3 and type 4 fimbrial adhesins and of afimbrial adhesins in *Shigella* strains (29, 32). The difficulty in

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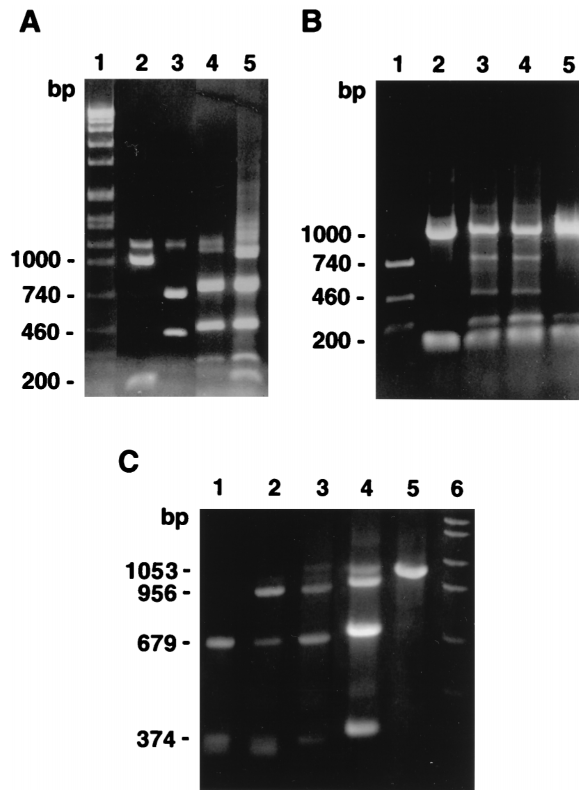

FIG. 4. Orientation of the switch regulating phase variation in *S. flexneri* and *E. coli*. (A) A 1,200-bp fragment from DH5α (lane 2), M9OT-W (lane 3), Fim$^-$ *S. flexneri* 229 (lane 4), and Fim$^+$ *S. flexneri* 15CD34 (lane 5) was amplified and digested with *Hin*fI. The digests were electrophoresed on agarose gels. Lane 1 contains DNA molecular size markers (Boehringer Mannheim DNA marker set V). (B) PCR-amplified (with the same primers as for panel A) *Hin*fI-digested DNA was obtained from M9OT-W (lane 1), DH5α (lane 2), Fim$^+$ *S. flexneri* 1NC101 (lane 3), Fim$^+$ *S. flexneri* 1NC103 (lane 4), and Fim$^+$ *S. flexneri* 187 (lane 5). The numbers to the left indicate the expected sizes of restriction fragments with this set of primers corresponding to on (1,000 and 200 bp) and off (740 and 460 bp) orientations of the invertible DNA segment. (C) PCR-amplified, *Hin*fI-digested DNA was obtained from Fim$^-$ F1a1 (lane 1), Fim$^+$ F1a1 (lane 2), AAEC185(pNS10-1) (lane 3), and AAEC185(pSH2) (lane 4). Lane 5 shows the undigested, PCR-amplified 1,053-bp DNA fragment from pSH2, and lane 6 shows a portion of the DNA marker lane (Boehringer Mannheim DNA marker set VII). The primers used for panel C were from a different location flanking the *fimA* sequence that those used for panels A and B, resulting in different-sized fragments, as expected. The 97-bp fragment, corresponding to the smaller on-phase fragment, is not visible in this photograph. The numbers to the left indicate the expected sizes of the restriction fragments.

obtaining fimbriated *Shigella* cultures could be partly explained by inappropriate growth conditions as well as by a low frequency of switching from the afimbriated to the fimbriated phase. For *E. coli* type 1 fimbriae, the rates of conversion from one state to the other are in the range of 0.1 to 1% per generation (9). For *pap* fimbrial expression, the frequency of transition from Pap$^-$ to Pap$^+$ was 2×10^{-4}/cell/generation, while the rate of the reverse transition was 3×10^{-2}/cell/generation (8). Switching frequencies have not yet been determined for *Shigella*. It is also not known why some cultures became fimbriated after a few passages and others required many serial transfers. In *E. coli*, *fimB* and *fimE* play regulatory roles in *fim* expression (17, 22). *E. coli fimE* mutants are fully fimbriated but lack MSHA expression (17). Such mutants would have been missed in the present study.

It is not known whether *Shigella* fimbriae play a direct role in pathogenesis. Most types of fimbriae, including type 1, have roles in bacterial disease (3–5, 14, 15, 18–20, 25). In vitro, *Shigella* type 1 fimbriae are not required for epithelial cell invasion, since M9OT-W is positive in these tests. However, in vitro virulence assays approximate only a fraction of the events that occur in the host and do not measure bacterial factors that are expressed only during passage through the GIT. Type 1 fimbrial adhesin (23) specifically binds mannose, which is ubiquitous in mammalian cell membranes. Thus, these structures have the potential to attach to a wide variety of host cells. It is possible that the environment within the host induces transient expression of fimbrial structures on phenotypically afimbriated strains. Fimbriae on a wide variety of saprophytes are observed only in the organisms' natural habitats or on strains freshly isolated from their natural habitats (27). Type 1 fimbriae may promote survival in aquatic environments, such as ponds and lakes, by allowing fimbriated cells to form pellicles at the water-air interface, thereby contributing to bacterial survival and outbreaks of disease.

Nucleotide sequence accession number. The nucleic acid sequence obtained in this study has been submitted to GenBank under accession no. U89135.

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