Growth Conditions Mediate Differential Transcription of *fim* Genes Involved in Phase Variation of Type 1 Pili

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Type 1 pili in *Escherichia coli* undergo phase variation in which individual cells in a population reversibly switch between piliated (Pil^+) and nonpiliated (Pil^-) states. The switching process is mediated by an invertible DNA fragment which contains the promoter for *fimA*, the gene encoding the major structural subunit of type 1 pili. Although type 1 pili randomly phase vary in broth cultures, many clinical isolates of *E. coli* do not express type 1 pili when cultured on agar media. We investigated the role of the invertible element and the upstream genes, *fimB* and *fimE*, in the agar-mediated suppression of pili in an agar-negative clinical isolate, strain 149. Southern hybridization and polymerase chain reaction analyses of the *fimA* promoter region in broth-grown 149 cells indicated that the invertible element was present in orientations corresponding to both Pil^+ and Pil^- phenotypes. In contrast, only one orientation of the invertible element, corresponding to the Pil^- phenotype, was observed in strain 149 cells cultured on agar. A second clinical isolate, strain 2-7, which expresses type 1 pili on agar was also examined; the invertible element was found in both the Pil^+ and Pil^- orientations during growth of this strain on agar as well as in broth. The introduction of the *fim* gene cluster from strain J96 on a multicopy plasmid into agar-negative strain 149 resulted in the production of both J96 and 149 pili during growth on agar. Experiments with subclones of the J96 genes indicated that the presence of an intact *fimB* gene allowed strain 149 pili to be produced on agar. Northern (RNA) hybridizations showed that transcripts of *fimB* and *fimA*, as well as an antisense transcript of *fimE*, were present in broth-grown cells of strain 149, but not in agar-grown cells. Only *fimE* transcripts were present in agar-grown cells, but they appeared to be degraded and were not consistently observed. Transcripts of *fimA*, *fimB*, *fimE*, and antisense *fimE* were detected in both broth- and agar-grown cells of phase-variable strain 2-7. The results suggest that in agar-negative strain 149, the *fimA* promoter element is phase locked in an off orientation on agar. Differences in pilus production between agar and broth cultures appear to be the result of differential transcription of *fimB* and *fimE* under the two growth conditions. In contrast, the pattern of expression of these genes in agar phase-variable strain 2-7 did not differ between broth- and agar-grown cells.

The ability to adhere to epithelial cells is an important step in the pathogenesis of many *Escherichia coli* infections (11, 50). In a number of instances, pili (fimbriae), long filamentous structures on the bacterial cell surface, have been implicated in the adherence process. Type 1 pili recognize mannose-containing receptors and are characterized by their ability to produce mannose-sensitive hemagglutination (MSHA) of erythrocytes as well as mannose-sensitive binding of piliated bacteria to other types of cells (12, 40, 44). Eight genes (*fimA-H*) composing a gene cluster are involved in synthesis, assembly, and regulation of type 1 pili (29-32, 34, 42).

The expression of type 1 pili involves a process known as random phase variation in which individual cells in a population alternate between piliated (Pil^+) and nonpiliated (Pil^-) states (5). Phase variation is due to the inversion of a 314-bp invertible DNA segment containing the promoter for *fimA*, the structural gene for the major subunit of type 1 pili (1). The inversion of this segment alters the orientation of the *fimA* promoter and allows individual cells to switch between piliated and nonpiliated phases (1, 30). Two additional genes, *fimB* and *fimE*, are involved in the inversion of the *fimA* promoter element (30); recent evidence indicates that *fimB* mediates inversion of the invertible element in both directions (35), whereas *fimE* primarily rearranges DNA in the off

position (3, 35). The FimB and FimE proteins are thought to act as site-specific recombinases (10, 21, 30) which effect the inversion of the *fimA* promoter element.

In addition to *fimB* and *fimE*, two histonelike factors affect phase variation (49). The *pilG* locus codes for the bacterial histonelike protein H1 (27). Mutations in *pilG* alter the frequency of inversion of the invertible element that controls expression of the *fimA* gene, increasing the switching approximately 100-fold (28). Integration host factor is also required for phase variation. Mutations in either *himA* or *himD* lock the invertible element in either the on or off orientation (10, 14). Thus, DNA topology may be important in *fim* expression as defined by the integration host factor and H1 mutants.

Growth conditions also affect expression of type 1 pili in *E. coli* (11). The piliated phenotype is favored when cultures are passaged in broth; this appears to be due to the selective outgrowth of piliated versus nonpiliated cells (41). In contrast, the nonpiliated phenotype predominates when strains are grown on agar (11). In studies of type 1-piliated *E. coli* isolates from patients with urinary tract infections, we identified three groups of organisms which differed in their ability to produce pili on agar (25, 26). The strains in one group (agar phase-locked) became nonpiliated and MSHA negative after growth on agar, regardless of medium composition or environmental condition. The strains in a second group (conditional agar phase-locked) were nonpiliated on blood and MacConkey agar but produced pili at low levels

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Reference or source
<i>Escherichia coli</i>		
HB101	F ⁻ <i>lacY1 recA13 Δfim</i>	4
DH5α MCR	F ⁻ <i>recA1 Δ(argF-lac) φ80 ΔlacZΔM15</i>	Bethesda Research Laboratories
RDP146	F ⁻ <i>Δ(lac-pro) recA1</i>	48
149	Agar phase-locked <i>fim</i>	24
149 Sm	Agar phase-locked <i>fim</i> Sm ^r	24
J96	Agar phase-locked <i>fim</i>	23
2-7	Agar phase-variable <i>fim</i>	25
2-14	Agar phase-variable <i>fim</i>	25
Plasmids		
pSH2	pACYC184 J96 <i>fimB-H</i> Cm ^r	42
pRK212.1	RK2 derivative, Ap ^r	17
pKK3535	<i>rmb</i> operon Ap ^r Tc ^r	6
pTJS82	<i>oriT</i> Ap ^r	Don Guiney, Jr.
pHSS22	<i>oriT</i> Km ^r	This study
pMMB91	<i>oriT</i> Km ^r	18
pPR274	Mini-F Cm ^r	38
pUC9	<i>olacZ</i> Ap ^r	51
pGEM4Z	Sp6 and T7 start sites, <i>olacZ</i> Ap ^r	Promega
pWRS1-17	pHSS22 J96 <i>fimH-B</i> Km ^r	This study
pWRS1-23	pHSS22 J96 <i>fimA</i> Km ^r	This study
pWRS2-15	pHSS22 J96 <i>fimB-H</i> Km ^r	This study
pWRS2-12	pUC9 J96 <i>fimB</i> Ap ^r	This study
pWRS2-10	pHSS22 J96 <i>fimBEA</i> Km ^r	This study
pWRS4-1	pHSS22 J96 <i>fimB</i> Km ^r	This study
pWRS5-2	pHSS22 J96 <i>fimB-5'</i> Km ^r	This study
pWRS6-1	pHSS22 J96 <i>fimB-3'</i> Km ^r	This study
pWRS8-25	pMMB91 J96 <i>fimH-B</i> Km ^r	This study
pWRS9-2	pHSS22 J96 <i>fimEA</i> Km ^r	This study
pWRS12-27	pPR274 J96 <i>fimB-H</i> Cm ^r	This study
pWRS12-46	pPR274 J96 <i>fimH-B</i> Cm ^r	This study
pWRS15-4Z14	pGEM4Z J96 <i>fimB</i> Ap ^r	This study
pWRS16-4Z11	pGEM4Z J96 <i>fimA</i> Ap ^r	This study
pWRS17-4Z7	pGEM4Z J96 <i>fimE</i> Ap ^r	This study

on other types of agar media and/or on blood agar incubated anaerobically. The cells in a third group (agar phase variable) expressed type 1 pili in a phase-variable manner on all types of agar media. The differential ability of various *E. coli* strains to express pili on agar suggests that growth conditions affect the phase variation process. This report describes our analysis of the switch region and the genes that are thought to regulate the switch (*fimB*, *fimE*) in clinical isolates of *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. The bacteria were grown on Luria-Bertani agar (LA) and in Luria-Bertani broth (LB) (37). Broth-grown cells were analyzed on cultures that had been passaged five or more times in LB incubated without shaking. These broth cultures had hemagglutination titers of 128 to 256; previous electron microscopy studies have shown that >50% of the cells are piliated in such cultures (unpublished observations). Similar broth cultures were used to inoculate agar media. Agar-grown cells were analyzed after five or more consecutive passages on LA.

Plasmids. The plasmids used in this study are described in Table 1. Plasmid pSH2 contains the *E. coli* J96 *fim* gene

cluster (*fimB-H*) flanked by *SalI* sites on pACYC184 (23, 42). pHSS22, a derivative of pHSS6 (47), contains an RK2 *oriT* site from pTJS82 in the *SmaI* site, a polylinker region, and a kanamycin resistance gene. Single-copy-number mini-F plasmid pPR274 (38) and the RSF1010-derived low-copy-number plasmid pMMB91 (18) were used to determine single- and low-copy-number effects of the *fim* gene cluster on pilus expression. The pGEM4Z vector (Promega, Madison, Wis.) was used in the preparation of strand-specific RNA of *fim* genes.

Piliation assays. Type 1 piliation was detected with guinea pig erythrocytes in a microtiter plate hemagglutination assay (25) or with antibodies against type 1 pili in a bacterial concentration fluorescence immunoassay (BCFIA) (46). A monoclonal antibody (3C3D3) raised against strain 149 type 1 pili was used to detect 149 pili in the BCFIA (46). This antibody did not react with strain J96 type 1 pili. Polyclonal antiserum to J96 type 1 pili was prepared by immunizing New Zealand White rabbits (24) with pili purified from *E. coli* HB101/pSH2 (15). The serum was absorbed with HB101 cells as described previously (25) and used in the BCFIA at a 1:500 dilution that produced no reactivity with strain 149 type 1 pili. Equivalent optical density readings and dilutions of the bacterial cells were used when comparing bacterial populations.

Recombinant DNA techniques. Plasmid DNA was isolated by the method of Clewell and Helinski (7). *E. coli* strains were transformed by the CaCl₂ procedure (9). DNA probes for Southern hybridizations were prepared by isolating appropriate restriction fragments on low-melting-point agarose gels. The gels (0.8 to 3%) were prepared in Tris-acetate-EDTA buffer and electrophoresed to separate the restriction fragments, and agarose slices that displayed the correct restriction fragment size were cut from the gel. The slices were placed in microcentrifuge tubes, suspended in 3 volumes of H₂O, and heated at 65°C to melt the agarose. This DNA was labeled for Southern hybridizations as described below. Each sample was placed at -20°C until needed.

PCR. The polymerase chain reaction (PCR) was performed by the method of Coen (8). Briefly, 300 ng of chromosomal DNA from strain 149, 2-14, or 2-7 broth- or agar-grown cells was used as the template for amplification. The amplification was done in a Programmable Thermal Cycler (MJ Research, Inc., Watertown, Mass.) for 28 cycles: primary denaturation at 94°C, 1 min; primer annealing at 55°C, 1 min; and primer extension at 72°C, 2 min. Fifty picomoles of each primer and 2 U of *Taq* DNA polymerase (Promega) were used in each reaction.

The three primers for the PCR were prepared with an automated synthesizer (model 380B; Applied Biosystems, Foster City, Calif.). The primers were as follows: INV, 23-mer, 5' GAGGTGATGTGAAATTAATTTAC 3'; FIME, 20-mer, 5' GCAGGCGGTTTGTACGGGG 3'; and FIMA, 20-mer, 5' GATGCGGTACGAACCTGTCC 3'. The pairing of the INV and FIME primers amplified the off-oriented invertible element DNA, while the pairing of INV and FIMA amplified the on-oriented DNA.

Plasmid constructions. Cesium chloride gradient-purified plasmid DNA from pSH2 and pHSS22 was digested with *SalI*, ligated, and transformed into *E. coli* DH5α MCR. Transformants (Km^r) were screened for the presence of the 11.2-kb *SalI* fragment containing *fimB-H* on pHSS22. Both orientations of *fimB-H* in pHSS22 were represented by recombinant plasmids pWRS1-17 and pWRS2-15. Strain RDP146/pRK212.1 was conjugated with DH5α MCR containing recombinant plasmids, and the transconjugants were used to mate the recombinant plasmids into either the

clinical *E. coli* isolate 149 Sm^r or *E. coli* HB101. Piliation of 149 and HB101 transconjugants was determined as noted above. Subclones of pWRS1-17 were constructed in pHSS22 by using appropriate restriction enzymes, and recombinant plasmids were conjugated into strain 149 as described above.

Plasmids pPR274 and pMMB91 were cut with *Sal*I, ligated with *Sal*I-digested pWRS2-15 plasmid DNA, and transformed into HB101 cells. Transformants were screened for insertion of the 11.2-kb *fim* gene cluster.

Strand-specific RNA probes were constructed by using the pGEM4Z plasmid (Promega), which contains a poly-linker sequence for cloning and T7 and SP6 promoter sites for transcription with T7 or SP6 RNA polymerase. Three strand-specific RNA probes were constructed: *fimA* (pWR S16-4Z11; 388-bp *Hpa*I-*Sma*I fragment from pWRS2-10 blunt-end ligated to *Sma*I-digested pGEM4Z); *fimB* (pWR S15-4Z14; 1,007-bp *Bam*HI-*Sal*I fragment from pWRS2-12 ligated to *Bam*HI-*Sal*I-digested pGEM4Z); and *fimE* (pWR S17-4Z7; 500-bp blunt-end *Dra*III-*Eco*RI fragment from pWRS4-1 ligated to *Sma*I-*Eco*RI-digested pGEM4Z).

Southern blotting and hybridization. Chromosomal DNA from broth- or agar-grown strain 149 cells was isolated from 50 ml of LB or 20 plates of LA by the method of Sambrook et al. (45) and digested with *Hin*FI. Transfer of the DNA from agarose gels to nylon filters (GeneScreen Plus; DuPont, Boston, Mass.) was as described by Sambrook et al. (45). ³²P-labeled probes were prepared by random primer labeling (16). Hybridizations were done at 65°C, and blots were washed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–sodium dodecyl sulfate (SDS) at 65°C before autoradiography.

RNA isolation and Northern (RNA) blot hybridization. Total RNA was isolated from broth-grown *E. coli* 149 and 2-7 cells by the hot-phenol treatment of von Gabain et al. (52). Six to eight overnight LA plates showing confluent growth of strain 149 or strain 2-7 were used to extract total RNA from agar-grown cells. These cells were treated like the broth-grown cells in subsequent manipulations by the method of von Gabain et al. (52). Twenty micrograms of RNA per lane was run on a 1% formaldehyde agarose gel (2). Nitrocellulose filters were baked in vacuo at 80°C for 2 h. Prehybridizations were done for 4 to 6 h at 55°C. Transcription of ³²P-labeled strand-specific RNA probes from pGEM4Z constructs was done by the method of Melton et al. (36). Strand-specific RNA probes were hybridized to RNA preparations at 55°C for 12 to 16 h in 50% formamide–5× SSC–0.2% SDS–5× Denhardt's solution–200 µg of salmon sperm DNA (Sigma) per ml. Two washes in 1× SSC–0.5% SDS for 20 min at room temperature were followed by three washes at 67°C in 0.1× SSC–0.5% SDS. In addition, the probed filters were washed with 500 ng of RNase A (Sigma) per ml in 0.1× SSC for 5 min at room temperature and then washed once with 1× SSC–0.5% SDS to reduce nonspecific binding of the probe (53). In some experiments, hybridization with the *fimE* probe was done at 44°C with a final wash temperature of 50°C. Blotted filters were exposed to X-ray film at –80°C for 2 h to 4 days, using intensifying screens. The concentration of RNA from broth- and agar-grown cells was standardized by visualization of ethidium bromide-stained rRNA in denaturing agarose gels and confirmed by using a probe for rRNA. Linearized plasmid DNA from pKK3535 containing the *rmb* operon was radiolabeled by random primer labeling (16) and used to probe total RNA from broth- or agar-grown cells. These blots were washed in 0.1× SSC–0.1% SDS at 65°C before autoradiography.

FIG. 1. Analysis of the invertible element in strain 149. (A) Southern hybridization analysis of chromosomal DNA from broth- and agar-grown strain 149 cells digested with *Hin*FI and probed with a 2.4-kb *Nru*I-*Eco*RI fragment from pSH2 (34). Shown is DNA from broth-grown (lane B) and agar-grown (lane A) cells. (B) Schematic representation of the invertible DNA element controlling phase variation of type 1 pili in the on and off orientations. The relative locations of the *fimE*, *fimA*, and *fimC* genes (hatched boxes) and the invertible element (stippled box) are shown. The arrowhead designates the orientation of the *fimA* promoter. Chromosomal *Hin*FI (H) sites were used to determine the orientation of the invertible element.

RNase protection assay. An RNase protection assay kit (RPA II; Ambion, Austin, Tex.) was used to detect *fimE* transcripts (20). A radiolabeled strand-specific RNA probe was transcribed as noted above and hybridized with 20 µg of total RNA from broth- or agar-grown cells from strain 149 or 2-7. The RNase protection assay was done by following the manufacturer's instructions. Protected fragments were isolated on a 5% polyacrylamide–8 M urea gel. The gels were wrapped in Saran wrap and exposed to X-ray film at –80°C for 2 days, using intensifying screens.

RESULTS

Orientation of the switch region in broth- and agar-grown cells of strain 149. *E. coli* 149 is a uropathogenic isolate which phase varies type 1 pilus production (Pil⁺/Pil[–]) in broth cultures, but which becomes completely nonpiliated (agar phase locked) during growth on various types of agar media (24). To determine whether the invertible element which mediates phase switching is involved in the loss of pilus expression on agar, we performed Southern hybridizations on DNA isolated from broth-grown (149-B) and agar-grown (149-A) cells. The static 149-B cultures had hemagglutination titers of ≥128, corresponding to a population in which >50% of the cells are piliated (unpublished data). Similar broth cultures were used as the initial inoculum for agar plates. The invertible element containing the *fimA* promoter has an asymmetric *Hin*FI site which can be used to determine its orientation (1, 14). Strain 149 chromosomal DNA digested with *Hin*FI was probed with a 2.4-kb *Nru*I-*Eco*RI fragment from pSH2 that spans the invertible element (see Fig. 3A). Autoradiographs revealed five bands in the 149-B lane and four bands in the 149-A lane (Fig. 1A). A map of the off and on orientations for the invertible element was generated based on a *Hin*FI digestion of the 2.4-kb probe (Fig. 1B). The 260- and 540-bp bands were present in both the 149-B and 149-A lanes independent of the orientation of the invertible element. The remaining three bands in the 149-B lane reflected the two different orientations of the invertible element; a 1,600-bp on orientation band as well as 350- and 1,250-bp off orientation fragments (Fig. 1A). A fourth band of ca. 30 bp is not seen. Only the off orientation bands, 350

FIG. 2. Determination of the invertible element orientation in strains 149 and 2-7 by PCR analysis. (A) PCR analysis of chromosomal DNA from broth (lanes B)- and agar (lanes A)-grown strain 149 cells, using INV and FIMA primers to amplify on-oriented DNA (450-bp product) and FIME and INV primers to amplify off-oriented DNA (750-bp product). (B) PCR analysis of chromosomal DNA from broth (lanes B)- and agar (lanes A)-grown strain 2-7 cells, using the INV and FIMA primers to amplify on-oriented DNA (450-bp product) and FIME and INV primers to amplify off-oriented DNA (750-bp product).

and 1,250 bp, were seen in the 149-A lane, corresponding to the Pil^- phenotype.

To confirm that the switch element was present only in the off orientation in Pil^- agar-grown strain 149 cells, we did a PCR analysis of the invertible element in broth- and agar-grown cultures. These studies made use of an oligonucleotide primer (INV) complementary to a DNA sequence in the invertible element. This primer was paired with one of two flanking primers, either FIMA, complementary to a region downstream of the invertible element, or FIME, complementary to an upstream region. The INV and FIMA primers produce a 450-bp product when the invertible element is in the on orientation, whereas the pairing of INV and FIME primers produces a 750-bp product when the invertible element is in the off orientation. The results (Fig. 2A) indicate that PCR products corresponding to both on and off orientations are generated from DNA isolated from broth-grown cultures. In contrast, only the 750-bp product corresponding to the off orientation of the promoter element was produced with DNA isolated from agar-grown cultures.

Unlike strain 149, *E. coli* 2-7 and 2-14 are clinical isolates that continue to produce and phase vary type 1 pili during growth on agar (25). Southern hybridization analysis of the

switch region of 2-14 revealed that DNA fragments corresponding to both the on and off orientations were present in agar-grown as well as broth-grown cells (data not shown). The phase switching of the invertible element on agar was confirmed by PCR analysis for strains 2-14 (data not shown) and 2-7 (Fig. 2B). Bands corresponding to both the on and off orientations were present in agar- as well as broth-grown cultures.

Effect of the J96 *fim* gene cluster in a multicopy plasmid on pilus expression in strain 149. *E. coli* J96 is a clinical isolate (23) which becomes Pil^- when cultured on agar. However, the cloned J96 *fim* gene cluster in pACYC184 (pSH2) behaves in an agar-variable manner in *E. coli* K-12 strains (42) (Table 2). To determine whether strain background affects the expression of pili on agar and to test the effect of various *fim* genes in *trans* on the regulation of phase switching, we subcloned the 11.2-kb *fim* gene cluster from pSH2 (42) into a mobilizable plasmid, pHSS22. Two subclones, pWRS1-17 and pWRS2-15 (Fig. 3B), were then conjugated into strain 149, and piliation after growth on agar was tested by MSHA assays and by fluorescence immunoassays (BCFIA). Pili produced from strain 149 chromosomal *fim* genes were assayed with a monoclonal antibody that does not react with J96 type 1 pili. Pili expressed from the plasmid-borne J96 *fim* gene cluster were assayed with a polyclonal antiserum that was diluted to give reactivity with J96 pili, but no reactivity with strain 149 pili.

Conjugal transfer of either pWRS1-17 or pWRS2-15 into strain 149 resulted in recombinant strains that now produced strain 149 pili when grown on agar (Table 2). Both recombinant strains produced J96 type 1 pili when grown in broth, but only 149/pWRS1-17 exhibited production of J96 type 1 pili when passaged on agar. Southern hybridization of chromosomal DNA from agar-grown 149/pWRS1-17 cells showed bands corresponding to both the on and off patterns found in DNA from broth-grown 149/pWRS1-17 cells and 149-B cells (data not shown). To show that the J96 *fim* gene cluster remained extrachromosomal in these studies, we passaged the recombinant strains in the absence of antibiotic selection. This procedure resulted in the loss of both kanamycin resistance and pilus expression on agar.

TABLE 2. Hemagglutination (HA) titers and antibody reactivity of strains HB101 and 149 containing recombinant plasmids

Strain	Plasmid (relevant genes)	HA titer ^a		Antibody reactivity (RFU) ^d			
		Broth ^b	Agar ^c	Anti-149 ^e		Anti-J96 ^f	
				Broth ^b	Agar ^c	Broth ^b	Agar ^c
HB101		0	0	400	300	300	250
HB101	pSH2 (<i>fimB-fimH</i>)	256	64	400	400	7,000	3,000
149	pHSS22	256	0	7,200	200	500	200
149	pWRS1-17 (<i>fimH-fimB</i>)	64	64	9,000	4,500	2,000	1,100
149	pWRS2-15 (<i>fimB-fimH</i>)	256	8	8,300	1,500	1,800	600
149	pWRS9-2 (<i>fimA-fimE</i>)	64	12	6,400	1,100	2,000	900
149	pWRS2-10 (<i>fimA-fimB</i>)	1,024	256	7,000	2,000	4,500	3,000
149	pWRS1-23 (<i>fimA</i>)	256	0	7,000	300	1,100	400
149	pWRS4-1 (<i>fimB</i>)	512	256	8,000	6,900	500	600
149	pWRS5-2 (<i>fimB-5'</i>)	512	0	7,600	200	400	300
149	pWRS6-1 (<i>fimB-3'</i>)	256	0	7,900	500	600	300

^a MSHA titer.

^b Cultures passaged five or more consecutive times in LB.

^c Cultures passaged five or more consecutive times on LA.

^d Antibody binding measured as relative fluorescence units.

^e Monoclonal antibody to strain 149 type 1 pili.

^f Polyclonal antibody to strain J96 type 1 pili.

FIG. 3. Maps of recombinant plasmids. (A) pSH2 is a construct containing the 11.2-kb J96 *fim* gene cluster insert (hatched bar) in pACYC184 (34). The gene organization of the *fim* cluster is indicated below pSH2. Arrowheads between *fimE* and *fimA* represent the 314-bp invertible element containing the *fimA* promoter. Restriction sites indicated are: S, *SalI*; C, *ClaI*; N, *NruI*; E, *EcoRI*; and B, *BamHI*. (B) Series of pWRS constructs in pHSS22 containing the J96 *fim* gene cluster in both orientations (1-17 and 2-15) or subclones of the *fim* genes in the same orientation as pWRS1-17.

Effect of individual *fim* genes in strain 149. To pinpoint which *fim* gene(s) was responsible for overcoming the suppressive effect of agar on 149 pilus expression, we constructed subclones of the J96 *fim* gene cluster in the same orientation as pWRS1-17. These were introduced into strain 149 and tested for piliation as described above. A subclone spanning *fimBEA* (Fig. 3B) conferred a piliated phenotype on the recombinant strain 149/pWRS2-10 on agar as well as in broth. The pili were recognized by both anti-149 and anti-J96 antibodies (Table 2). The presence of *fimA* alone (including the invertible element) in 149/pWRS1-23, however, did not result in piliation on agar. A subclone containing the *fimEA* genes, 149/pWRS9-2, produced a low level of MSHA and reactivity with both anti-149 and anti-J96 antibody after growth on agar.

The role of *fimB* in allowing expression of type 1 pili on agar was assessed with the *fimB* subclone pWRS4-1 (Fig. 3B). Recombinant strain 149/pWRS4-1 showed a high level of expression of 149 pili on agar (Table 2). To determine whether an intact *fimB* gene was necessary to overcome the inhibitory effect of agar growth, we used a *ClaI* site in the *fimB* gene to subclone the DNA 5' (pWRS5-2) and 3' (pWRS6-1) of the restriction site in pWRS4-1 (Fig. 2B). Neither construct in strain 149 exhibited a Pil⁺ phenotype on agar.

Expression of the *fim* gene cluster on single- or low-copy-number plasmids. Although wild-type J96 is an agar phase-locked strain, recombinant *E. coli* strains bearing the J96 *fim* gene cluster on plasmid pACYC184 or pHSS22 produce pili on agar (42) (Table 2). To determine whether copy number or other properties of these vectors were responsible for the absence of *fim* regulation on agar, we introduced the J96 *fim* gene cluster in both orientations into a single-copy plasmid, pPR274, yielding pWRS12-27 and pWRS12-46. These con-

TABLE 3. Hemagglutination (HA) titers and antibody reactivity of HB101 cells containing single- and low-copy-number plasmids

Plasmid	HA titer ^a		Antibody reactivity ^b	
	Broth ^c	Agar ^d	Broth ^c	Agar ^d
None	0	0	200	300
pSH2	256	32	20,000	2,400
pHSS22	0	0	100	100
pWRS1-17	64	64	6,000	2,500
pWRS2-15	256	16	10,000	1,600
pPR274	0	0	0	0
pWRS12-27	64	0	4,300	0
pWRS12-46	92	0	4,800	0
pMMB91	0	0	400	500
pWRS8-25	128	0	5,500	200

^a MSHA.

^b Measured as relative fluorescence units.

^c Cultures passaged five or more consecutive times in LB.

^d Cultures passaged five or more consecutive times on LA.

structs were analyzed in strain HB101 because pPR274 is not mobilizable. The *fim* gene cluster in one orientation was also introduced into the low-copy-number plasmid pMMB91, producing pWRS8-25. No expression of type 1 pili was detected in agar-grown cells in either the single-copy construct (HB101/pWRS12-27 and HB101/pWRS12-46) or the low-copy-number plasmid (HB101/pWRS8-25) (Table 3). Type 1 pili were expressed, however, in each recombinant strain during growth in broth. The pMMB91 derivative pWRS8-25 was also conjugally transferred into clinical strain 149; no type 1 pili were expressed during growth on agar (data not shown).

Transcription of *fimA*, *fimB*, and *fimE* under different growth conditions. The PCR analysis described above indicates that the invertible *fimA* promoter element becomes phase locked during growth of strain 149 on agar and argues that the Pil[−] phenotype on agar is not simply due to more rapid growth of nonpiliated versus piliated cells. In addition, our complementation studies show that the *fim* gene cluster, particularly *fimB*, acts in *trans* to alter the Pil[−] phenotype of strain 149 grown on agar. Given the differential production of type 1 pili in broth and agar cultures, we predicted that the expression of the *fim* genes involved in phase switching would change under the two growth conditions. Therefore, we did Northern hybridizations to determine whether the *fimB*, *fimE*, and *fimA* genes were differentially expressed in strain 149. Total RNA was isolated from broth- and agar-grown cells, and transcripts of *fimB*, *fimE*, and *fimA* were analyzed with strand-specific RNA probes produced from *fim* gene subclones in pGEM4Z. Hybridization analysis revealed the presence in broth-grown cells of a *fimA* transcript of 900 nucleotides and a *fimB* transcript of 800 nucleotides (Fig. 4). An antisense *fimE* transcript of approximately 650 nucleotides was also detected in broth-grown strain 149 RNA preparations when analyzed under lower-stringency conditions (Fig. 5). Transcripts of *fimA*, *fimB* (Fig. 4), and the antisense *fimE* transcript (Fig. 5) were not detected in agar-grown cultures of strain 149. The difference in transcript levels between broth- and agar-grown RNA was not attributable to differences in RNA concentrations; hybridization with an rRNA probe indicated that equal amounts of RNA were present for both growth conditions.

Northern hybridization analysis of *fimE* transcription suggested that a *fimE* transcript was present in agar-grown but not broth-grown cells of strain 149 (data not shown). The

FIG. 4. Northern hybridization analysis of strand-specific transcription of *fimA* and *fimB* as well as detection of rRNA from total RNA isolated from broth (lanes B)- and agar (lanes A)-grown strain 149 cells. The 16S and 23S rRNAs (*) and RNA standard markers are indicated. The lanes are marked to indicate sense (S) and antisense (AS) transcripts. Autoradiograph exposure times are indicated.

transcript was not consistently detected in agar-grown cells, and when it was observed, it appeared to be degraded. An RNase protection assay (20) was also used to examine *fimE* transcription. Again, the *fimE* transcript in agar-grown cells of strain 149 was not consistently observed (data not shown). No transcript was ever detected in RNA from broth-grown cells.

Agar phase-variable strains such as 2-7 and 2-14 continue to phase vary the production of type 1 pili when grown on agar. The invertible *fimA* promoter element continues to switch between Pil^+ and Pil^- orientations (Fig. 2), suggesting that the expression of *fimB* and *fimE* should be different in these strains compared with the agar phase-locked strain 149. To investigate this question, we probed total RNA from broth- and agar-grown strain 2-7 cells with the same strand-specific RNA probes used to analyze RNA from strain 149. Northern hybridization analysis revealed the presence of a *fimA* transcript of 900 nucleotides and a *fimB* transcript of

FIG. 5. Northern hybridization analysis of antisense transcription of *fimE* from total RNA isolated from broth (lanes B)- and agar (lanes A)-grown strain 149 and strain 2-7 cells. The 16S and 23S rRNA (*) and RNA standard markers are indicated. The lanes are marked to indicate antisense (AS) transcripts. Autoradiograph exposure times are indicated.

FIG. 6. Northern hybridization analysis of strand-specific transcription of *fimA* and *fimB* as well as detection of rRNA from total RNA isolated from broth (lanes B)- and agar (lanes A)-grown strain 2-7 cells. The 16S and 23S rRNA (*) and RNA standard markers are indicated. The lanes are marked to indicate sense (S) and antisense (AS) transcripts. Autoradiograph exposure times are indicated.

800 nucleotides in both broth- and agar-grown cells (Fig. 6). The presence of 650-nucleotide antisense *fimE* transcripts in broth- and agar-grown cells was detected under conditions of reduced stringency in the Northern blot hybridizations (Fig. 5). In addition, RNase protection assays consistently revealed the presence of 145-nucleotide *fimE* transcripts in both broth- and agar-grown cells (data not shown).

DISCUSSION

It has been known for many years that type 1-piliated bacteria tend to become nonpiliated during growth on agar (11), but the relationship between this phenomenon and random phase switching has not been explored. We previously described three groups of type 1-piliated *E. coli* that were isolated from patients with urinary tract infections and that behaved differently in their ability to produce pili on agar (25, 26). The results obtained in this study indicate that in the agar-negative strain 149, the *fimA* promoter element is phase locked in an off orientation during growth on agar, whereas in the agar variable strains 2-7 and 2-14, the invertible element continues to undergo switching. Furthermore, the difference in production of type 1 pili in broth and agar cultures of agar phase-locked strain 149 appears to be the result of differential expression of *fimB* and *fimE*.

Broth-grown cells of strain 149 displayed both orientations of the invertible switch region in Southern and PCR analyses, an indication that phase variation was occurring (Fig. 1). The Southern hybridization fragment patterns are similar to those described by Eisenstein et al. (14). From the PCR analysis of broth-grown cells, we noted that the DNA product corresponding to the on orientation of the invertible element was more intense than the band corresponding to the off orientation. This presumably reflects the selective outgrowth of piliated cells over nonpiliated cells that has been described in broth cultures (41). In our previous studies of strain 149 and other agar-negative strains, the absence of pili after growth on agar was documented by electron microscopy and by the absence of MSHA activity and reactivity of the cells with antipilus antibody (25). The results of the experiments reported here confirm at the DNA level that the cells of strain 149 are indeed locked in the Pil^- phase on agar. Southern hybridization and PCR analyses (Fig. 1 and 2) demonstrated that DNA corresponding only to the off orientation of the *fimA* promoter could be detected in

agar-grown cultures. The sensitivity of the PCR assay in detecting a product corresponding to the on orientation was determined based on the amount of DNA added in the PCR (300 µg), the number of amplifications (28 cycles), the size of the on product (450 bp), and the size of the genome (4×10^6 bp). Assuming that 10 ng of DNA could be detected on the gels, we calculate that the number of on cells in the agar-grown population of strain 149 is less than 1 in 8×10^7 cells.

To assess the effect of providing the *fim* genes in *trans* on agar-grown cells, we introduced the J96 *fim* gene cluster on pHSS22 into strain 149. The J96 *fim* gene cluster on pHSS22 affected pilus expression differentially in strain 149, depending on the orientation of the cluster on the plasmid. Type 1 pili encoded by the chromosomal *fim* gene cluster (strain 149 pili), as well as pili encoded by the recombinant plasmid (strain J96 pili) were produced in agar-grown strain 149/pWRS1-17. In contrast, only the chromosome-encoded *fim* genes were expressed in agar-grown strain 149/pWRS2-15 (Table 2). The absence of J96 pilus expression from pWRS2-15 during growth on agar is puzzling, especially since the presence of this plasmid overcomes the suppressive effect of agar on pilus expression from chromosomal genes. At present, we are unable to explain the difference between pWRS1-17 and pWRS2-15 in their ability to express J96 pili on agar.

To determine which *fim* genes are involved in producing the phase-locked promoter element seen in agar-grown cells, subclones of various *fim* genes on pHSS22 were transferred into strain 149. These experiments revealed that the presence of an intact *fimB* gene correlated with the expression of 149 pili at a high level on agar (Table 2). Our results documenting the role of *fimB* in expression of the chromosomal *fim* gene cluster in a clinical strain are similar to those of Pallesen et al. (43), who found that *fimA-lacZ* fusions on low-copy-number plasmids in K-12 strains of *E. coli* could be complemented by *fimB* on a multicopy plasmid, resulting in Lac⁺ colonies. Those investigators also indicated that the *fimE* gene product may be able to mediate an on configuration of the switch, although very inefficiently. That suggestion is supported by our results, which showed that a *fimAE* construct (pWRS9-2) allowed a low level of expression of 149 type 1 pili on agar (Table 2). Our results are also consistent with the observations of McClain et al. (35), who found that *fimB* promoted recombination of the invertible element in both orientations, while *fimE* results in the preferential rearrangement of the element to the off orientation.

Pili that were recognized by anti-J96 pilus antiserum were produced in strain 149 recombinants containing *fimBEA* or *fimEA* from the J96 *fim* gene cluster (Table 2). Thus, the chromosomal 149 *fim* assembly genes (*fimC*, *fimD*) can apparently complement the assembly genes that are missing in these J96 constructs. Complementation of heterologous *fimA* has been previously documented for type 1 pili (19), and serologically identical pili containing functionally distinct adhesins have been described in the Pap pilus system (33). We do not know whether J96 pilin monomers are assembled into distinct J96 pili in these recombinants, or whether both 149 and J96 pilin monomers are assembled into a single pilus fiber.

E. coli J96 is a clinical isolate that produces several different types of pili (23). The expression of type 1 pili by J96 indicates that this organism behaves as an agar phase-locked strain, becoming nonpiliated for type 1 pili after growth on agar (data not shown). However, when the J96 *fim* gene cluster is cloned into pACYC184 or pHSS22 and then

transformed into nonpiliated HB101, the recombinant strain continues to produce pili on agar (42) (Table 2). The results of our experiments (Table 2) indicate that this behavior may result from the multicopy nature of the plasmid vectors. These observations are consistent with the idea that a *trans*-acting regulatory factor which influences *fim* gene expression is titrated out when the *fim* genes are present in high copy number. Alternatively, other characteristics of these vectors such as transcription from a plasmid promoter or supercoiling properties may be responsible for the expression in these constructs.

Experiments utilizing strain 149 derivatives containing subclones of the *fim* gene cluster suggested that agar regulation involves *fimB* and/or *fimE*. This was confirmed by examining the transcription of these genes in broth and agar cultures. RNA analyses demonstrated that in broth-grown cells of strain 149, transcripts of *fimB* and *fimA* are present along with an antisense transcript recognized by the *fimE* probe. The latter presumably originates from the *fimA* promoter present in the off orientation. A *fimE* transcript was detected when the cells were cultured on agar, but this result was not always reproducible, and the transcript appeared to be degraded. It is puzzling that antisense but not sense transcripts of *fimE* could be detected in broth-grown cells of strain 149, whereas only sense transcripts of *fimE* apparently are present in agar-cultured cells. The basis of this difference is not clear, but we assume it reflects regulatory events taking place during growth of this strain on agar. It is also unclear why the *fimE* transcript in agar-grown cultures was inconsistently observed. This may be due to degradation of these transcripts, which was seen in the Northern analysis of *fimE*. In addition, reduced homology between the *fimE* probe derived from strain J96 and strain 149 RNA may contribute to our inability to consistently detect the *fimE* transcript.

The results of these experiments do not allow us to conclude whether the phase-locked state of the *fimA* promoter in agar-grown strain 149 cells is due to the absence of *fimB* transcription, the transcription of *fimE*, or both. What is clear is that the *fim* genes which mediate phase switching, as well as the *fimA* gene itself, are differentially transcribed under different growth conditions in strain 149.

Agar phase-locked strains are phenotypically distinct from agar phase-variable strains as determined by MSHA and electron microscopy of agar-grown cells (25). PCR analysis (Fig. 2B) confirmed that in contrast to what is seen with strain 149, the invertible element continues to switch in strains 2-7 and 2-14 during passage on agar media. The results of transcriptional analysis of the *fim* genes in cells of strain 2-7 are consistent with the phenotypic observations and PCR studies indicating continued phase switching of the *fimA* promoter in agar cultures. Transcripts of *fimA* and *fimB*, as well as sense and antisense transcripts of *fimE*, were present in agar- and broth-grown cells of strain 2-7. A similar pattern of *fimA* and *fimB* expression is seen in broth-grown cells of strain 149. Whatever regulatory control is exerted on agar-grown strain 149 cells appears to be absent or modified in the phase-variable strains.

The agar phase-variable strains of *E. coli* we have characterized (25) behave like strain CSH50 (13), which expresses type 1-piliated colonies when passaged on agar. Blomfield et al. (3) recently demonstrated that strain CSH50 contains a copy of a 765-bp insertion sequence IS1 (22) in the *fimE* gene and localized the insertion sequence to a 3' region of *fimE* (3). Furthermore, construction of *fimE::IS1* mutations in strain MG1655 (in which piliation is largely sup-

pressed during growth on agar) resulted in *fimE* mutants that produced phase-variant colony types on agar. The two agar phase-variable strains we examined in this study (2-7 and 2-14) do not appear to have an *IS1* element in the 3' region of *fimE* as described for strain CSH50. The PCR products generated with the FIME primer, which hybridizes in the 5' region of *fimE*, are the same size in strains 2-7 and 2-14 and agar phase-locked strain 149.

The results described here demonstrate that in certain clinical isolates of *E. coli*, the expression of type 1 pili is affected by growth conditions. Regulation by growth on agar is seen as an effect on the *fimA* promoter element, which undergoes reversible switching in broth but becomes locked in the off orientation on agar. Differential expression of *fimB* and *fimE* clearly seems to be involved in regulation, but the signals that affect the transcription of these genes are not known. The immediate environment of bacteria growing on agar is obviously complex, with cells in contact with a solid surface as well as with neighboring cells. In addition, differences in viscosity, osmolarity, oxygenation, and availability of nutrients may exist in a bacterial colony. One (or more) of these conditions may trigger regulatory signals that alter the expression of the *fim* genes which mediate phase switching. The consequences of phase variation of pili *in vivo* are also uncertain. One could speculate that while pili promote adherence of bacteria to epithelial cells, phase variation allows some cells to desorb and spread to other sites on mucous membrane surfaces. In addition, type 1-piliated *E. coli* are known to attach to phagocytic cells in a mannose-sensitive reaction, resulting in lectinophagocytosis (39). *In vivo*, the presence of pili might prove to be advantageous at some sites (by promoting adherence), but disadvantageous under other circumstances (in the presence of phagocytes). It seems reasonable to assume that local environmental conditions (pH, anaerobiosis, osmolarity, etc.) existing at different sites *in vivo* could signal a change in the expression of *fimB* or *fimE*, thereby affecting the inversion of the *fimA* promoter element and the synthesis of pili. Thus, the ability to alter pilus expression in response to environmental signals may enhance the ability of a bacterial population to survive in the host.

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