Expression of Multiple Flagellin-Encoding Genes of *Proteus mirabilis*†

ROBERT BELAS*

Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202

Received 15 July 1994/Accepted 27 September 1994

The overproduction of flagella is a distinguishing characteristic of *Proteus mirabilis* swarmer cell differentiation. The synthesis of flagellin, the principal protein composing the flagellar filament, is coordinately regulated as part of a larger regulon of genes whose expression is a prerequisite in urinary pathogenesis. In this report, the regulation of expression of the *flaA* locus, comprising *flaA* and *flaB*, two tandemly linked and nearly identical copies of flagellin-encoding genes, is examined. Transcriptional expression studies reveal that *flaA*, but not *flaB*, is expressed by wild-type cells, and *flaA* transcription increases eightfold during differentiation. The *flaA* transcriptional start site for both swimmer and swarmer cells was determined to be located at a guanine, 8 bases downstream of the *flaA* σ^{28} promoter. *FlaA*[−] mutants are nonmotile and undifferentiated and do not synthesize flagellin, while *FlaB*[−] mutants are wild type, thus verifying that *FlaA* is the sole flagellin produced by wild-type cells and that *flaB* is silent. *FlaA*[−] mutants frequently revert to a Mot⁺ phenotype that is antigenically distinct from that of wild-type cells. Southern blot analysis of the *flaA* Mot⁺ revertants reveals a deletion of between 2 and 7 kb in the *flaA* locus. Biochemical analyses of revertant flagellin indicate major changes in protein size and composition but conservation of the first 28 N-terminal residues. The result of this process is to produce an antigenically distinct flagellum that may be significant in ensuring the survival of *P. mirabilis* during pathogenesis.

Proteus mirabilis is a dimorphic bacterium that is capable of undergoing dramatic morphological and physiological changes in response to growth on surfaces or in viscous environments (3, 12, 14). These changes, referred to as swarmer cell differentiation, are ultimately required to produce the motile behavior that is characterized by flagellum-assisted movement over a surface and known as swarming (27). The differentiation process leading to the development of a swarmer cell involves between 25 and 50 genes that are coordinately expressed when the appropriate environmental signals are sensed by the bacteria (14). Inhibition of the rotation of the flagella (3, 11) and the concentration of glutamine in the surrounding milieu (4) have been identified as important cues required to initiate the surface-induced transcription of the swarmer cell regulon. Moreover, the expression of these genes is also required for invasion of epithelial cells by *P. mirabilis* (2, 5). Thus, since *P. mirabilis* is frequently associated with urinary tract infections, particularly in patients with chronic urinary catheterization (41), knowledge of the regulation of swarmer cell gene expression is crucial to understanding the pathogenesis of this organism.

As part of the differentiation events leading from the swimmer to swarmer cell, the rate of synthesis of certain proteins undergoes a remarkable increase. The most evident of these overproduced proteins is flagellin (*FlaA*, the subunit of the flagellar filament and the product of the *flaA* gene). Although swimmer cells have only a few flagella, the elongated swarmer cells are profusely covered by thousands of newly synthesized flagella (28). In many ways, the increase in flagellin expression is the hallmark of the differentiated swarmer cell, as has been shown not just for *P. mirabilis* but also for *Serratia marcescens* (1, 42) and *Vibrio parahaemolyticus* (16, 35, 37).

V. parahaemolyticus swarmer cell differentiation is manifested through the interaction of two distinct types of flagella: a constitutively synthesized polar flagellum and lateral flagella, produced in many copies only during the differentiation events. The flagellins of the polar flagellum and lateral flagella are encoded by different and distinct genes (8, 35). The polar flagellum acts as a sensor of the environment. Conditions inhibiting flagellar rotation are sensed by the polar flagellum that then relays a secondary signal inducing the transcription of the swarmer cell differentiation regulon. A second signal, iron availability, is also required for differentiation to begin (36). Thus, *V. parahaemolyticus* responds to viscous environments and surfaces through a signal transduction process involving both the polar flagellum and iron limitation. This induces the transcription of *lafA* (encoding lateral flagellin), thus enabling the production of numerous lateral flagella filaments.

Unlike *V. parahaemolyticus*, biochemical studies of flagella isolated from *P. mirabilis* swimmer and swarmer cells (10, 14), along with genetic data from Tn5 mutageneses (13, 14), suggest that *P. mirabilis* synthesizes only a single flagellin species. If this is true, the flagellin-encoding gene, *flaA*, must be coordinately regulated along with all the other genes of the swarmer cell regulon. Such a hypothesis would predict that *flaA* should be minimally transcribed in the swimmer cell phase and induced to very high levels during swarmer cell differentiation. Thus, an investigation of the genetic control of *P. mirabilis* *flaA* during swarmer cell differentiation may reveal features common to all of the genes associated with this pathway.

Recently, Belas and Flaherty reported on the cloning of a region of *P. mirabilis* chromosomal DNA capable of complementing *Escherichia coli* *FliC*[−] mutants (15). Nucleotide and deduced amino acid sequence analyses revealed that this region contains three open reading frames that were identified on the basis of their homology to other known flagellar genes (Fig. 1). The region included the 5' end of *flaD*, a putative homolog of *fliD* (an essential flagellar gene responsible for filament assembly), and two nearly identical copies of flagellin-encoding genes called *flaA* and *flaB*. (A third copy of a

* Corresponding author. Mailing address: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 600 East Lombard St., Baltimore, MD 21202. Phone: (410) 783-4825. Fax: (410) 783-4806. Electronic mail address: Belas@mbimail.umd.edu.

† Contribution no. 1011 from the Center of Marine Biotechnology.

FIG. 1. The *P. mirabilis* *flaA* locus. (A) Schematic representation of the region including *flaD*, *flaA*, and *flaB*. Recognition sites for the enzymes *Hind*III, *Eco*RV, and *Pst*I are indicated, as are the putative promoter regions for these genes (*flaD*_p, *flaA*_p, and *flaB*_p). The direction of transcription of each gene is shown by an arrow. The bar represents 1 kb. (B) Features of the *flaD*_p, *flaA*_p, and *flaB*_p regulatory regions. The nucleotide sequence immediately surrounding the σ^{28} promoter -10 and -35 regions continuing to the start codon for each gene is shown. Numbering of nucleotides coincides with the previously reported GenBank sequence L07270 (15). The -10 and -35 regions of the σ^{28} promoters are highlighted, as are the ribosome binding site and start codon.

potential flagellin-encoding gene, referred to as *flaC*, was also found in this study by DNA-DNA homology.) All three genes possess an upstream regulatory sequence with strong homology to the well-characterized σ^{28} promoter, specific to flagellar genes (7, 26, 40). Surprisingly, while *flaA* encodes a protein that complements *E. coli* FliC⁻ mutants, *flaB* fails to complement any *E. coli* defects (15). Moreover, *flaB* encodes a protein that is larger than predicted from its deduced amino acid sequence when the gene was used in *E. coli* minicell protein programming experiments. These data suggest that although *flaA* is functional in *E. coli*, *flaB* is not. In light of these observations made in an *E. coli* background, a more detailed study of the regulation of *flaA* and *flaB*, as well as *flaD*, in *P. mirabilis* was sought. Thus, the aim of this study was to delineate the expression of each flagellin-encoding gene and determine how the transcription of *flaA* and *flaB* is influenced during swarmer cell differentiation. Analysis of the data from these experiments reveals that *P. mirabilis* flagellin can undergo antigenic variation under certain circumstances.

The *flaA*, *flaB*, *flaC*, and *flaD* genes were originally named *fliC1*, *fliC2*, *fliC3*, and *fliD*, respectively (15). The change in the designations used in this report agrees with the conventional genetic nomenclature.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, and media. The strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *P. mirabilis* BB2000 is wild type for swimming and swarming behaviors. *E. coli* and *P. mirabilis* strains were grown as previously described (13, 14). Swarming motility of *P. mirabilis* was observed on L agar, whereas LSW⁻ agar (10 g of tryptone, 5 g of yeast extract, 0.4 g of NaCl, 5 ml of glycerol, 20 g of Bacto Agar, 1,000 ml of distilled H₂O) was used to phenotypically inhibit swarming (13). Ampicillin (100 mg/liter), chloramphenicol (40 mg/liter for *E. coli* strains and 80

mg/liter for *P. mirabilis*), rifampin (100 mg/liter), spectinomycin (100 mg/liter), and streptomycin (100 mg/liter) were used for antibiotic selection. Swimming motility was determined by using liquid motility (Mot) broth (10 g of tryptone, 5 g of NaCl, 1,000 ml of distilled H₂O) and semisolid Mot agar (Mot broth plus 3.5 g of agar per liter) as described by Belas et al. (13).

DNA manipulation. *E. coli* cells were made competent by using a modification of the CaCl₂ procedure (34) as described by Ausubel et al. (9). Standard methods were used for the manipulation of DNA (9, 45, 48) unless specified otherwise. Plasmid DNA was purified by the alkaline lysis method (17). Supercoiled DNA was purified by CsCl density gradient centrifugation (45). Restriction and DNA-modifying enzymes were used as specified by the enzyme suppliers. Oligonucleotide primers were synthesized by an on-site oligonucleotide synthesizer (Oligol000; Beckman Instruments) or obtained commercially (DNA Express; Macromolecular Resources, Colorado State University).

Sequence analysis of the *flaA* locus. The nucleotide and deduced amino acid sequences of *flaD*, *flaA*, and *flaB* have been previously described (15) and are deposited in GenBank (accession number L07270). Nucleotide sequencing of this DNA was done as described by Belas and Flaherty (15).

Construction of *lacZ* transcriptional fusion plasmids. Transcriptional control of *flaA*, *flaB*, and *flaD* (as an internal flagellar gene control) was assessed in *P. mirabilis* by using a derivative of a low-copy-number plasmid, pCED6 (21), carrying a promoterless *lacZ* gene. To permit plasmid transfer from *E. coli* to *P. mirabilis*, plasmid pCED6 was modified to carry the RP4 *mob* gene. Plasmid pUT/mini-Tn5-CM (20) was digested with *Bam*HI, which cuts at either end of the *mob* gene. The *Bam*HI fragment harboring the intact *mob* gene was then ligated to pCED6, producing pCED-Mob. This plasmid, when placed into a suitable donor strain such as *E. coli* S17-1 (Δ pir), can be transferred to recipient bacteria through biparental conjugal mating. To construct *flaA*::*lacZ* and *flaD*::*lacZ* transcriptional fusions, the 1.2-kb *Hind*III fragment from pUCf1C1 was ligated in both orientations to the *Hind*III site on pCED-Mob that is immediately 5' to the promoterless *lacZ* gene. This construction resulted in two plasmids: pflaD::*lacZ* and pflaA::*lacZ*. Transcriptional fusions to *flaB* were constructed by digesting pflaD::*lacZ* with *Nru*I plus *Eco*RV and isolating the 447-bp fragment containing the *flaB* promoter region and 5' part of the gene (nucleotides [nt] 2287 to 2734 on the GenBank sequence). Mung bean exonuclease treatment to blunt the ends of this fragment was followed by ligation of *Hind*III linkers to both ends. The fragment was then digested with *Hind*III and ligated to pCED-Mob, and ampicillin-resistant (Ap^r) transformants were obtained. Several transformants were chosen, and the nucleotide sequence of each was analyzed to determine the orientation of the insert. Plasmid pflaB::*lacZ*, having the correct orientation of *flaB* to *lacZ*, was obtained and used for subsequent work. All three plasmids and pCED-Mob (as a negative control for plasmid-borne background enzyme activity) were conjugally transferred to *P. mirabilis* BB2000 by using the filter mating technique described previously (13).

β -Galactosidase assay. Both qualitative and quantitative measurements of β -galactosidase activity were performed on *P. mirabilis* strains harboring plasmid-borne *lacZ* transcriptional fusions. For qualitative assays, bacteria were incubated in L broth with appropriate antibiotics at 37°C for 4 to 6 h, and a loopful of culture was streaked onto either L agar or LSW⁻ agar containing 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-Gal) plus antibiotic per ml. Production of the chromogen 5-bromo-4-chloro-3-indole was assessed after incu-

TABLE 1. Strains, plasmids, and oligonucleotides used

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) ^a or sequence	Derivation or description	Source and/or reference
<i>E. coli</i> K-12			
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1</i> <i>supE44</i> λ^- <i>gyrA96 relA1</i>		Laboratory stock (45)
S17-1 (λ pir)	<i>thi pro recA hsd</i> (r ⁻ m ⁺) RP4-2Tc::Mu-Km::Tn7 Sm ^r λ pir	294	20
<i>P. mirabilis</i>			
BB2000	Wild-type, R ^f	Spontaneous from PRM1	13
BB2301	<i>flaB</i> :: <i>cam</i> :: <i>'flaB</i> Cm ^r R ^f	<i>cam</i> gene from pUT/mini-Tn5-CM inserted at the <i>EcoRV</i> site in BB2000 <i>flaB</i> gene	This study
DF1000	<i>flaD</i> :: <i>cam</i> :: <i>'flaD</i> FlaA ⁻ FlaD ⁻ Mot ⁻ Cm ^r R ^f	<i>cam</i> gene from pUT/mini-Tn5-CM inserted at the <i>EcoRV</i> site in BB2000 <i>flaD</i> gene	This study
DF1001	<i>flaA</i> :: <i>cam</i> :: <i>'flaA</i> FlaA ⁻ Cm ^r R ^f	<i>cam</i> gene from pUT/mini-Tn5-CM inserted at the <i>EcoRV</i> site in BB2000 <i>flaA</i> gene	This study
DF1002	<i>flaA</i> Mot ⁺ revertant, Cm ^s R ^f	Spontaneous from DF1001	This study
DF1003	<i>flaA</i> Mot ⁺ revertant, Cm ^s R ^f	Spontaneous from DF1001	This study
Plasmids			
Vectors			
pBluescript	Ap ^r	pBluescript SK(-) and KS(-)	Stratagene
pCED6	Ap ^r	Low-copy-number, <i>lacZ</i> transcriptional fusion	21
pCED-Mob	Ap ^r	pCED6 with RP4 <i>mob</i>	This study
pGP704	Ap ^r		39
pUC18/19	Ap ^r		45
pUT/mini-Tn5-Cm	Ap ^r Cm ^r	pGP704 Tn5 vector	20
<i>flaA</i>			
pBSSKfliC1	<i>flaA</i> ⁺ Ap ^r	Intact <i>flaA</i> region, nt 834–2287	15
pflaA::lacZ	<i>flaA</i> :: <i>lacZ</i> Ap ^r	<i>flaA</i> transcriptional fusion to <i>lacZ</i>	This study
pflDC1C2	<i>'flaD flA</i> ⁺ <i>flaB</i> ⁺ Ap ^r	pBluescript SK(-) with a 3.6-kb <i>HindIII-PstI</i> fragment, nt 1–3567	15
pDFFL1	<i>flaD</i> :: <i>cam</i> :: <i>'flaD</i> Ap ^r	1.25-kb <i>HindIII</i> fragment from pUCfliC1 subcloned on pGP704 with <i>cam</i> gene inserted at the <i>EcoRV</i> site in <i>flaD</i> (nt 270)	This study
pGPfliC1	<i>flaA</i> ⁺ Ap ^r	<i>EcoRI-KpnI</i> fragment from pBSSKFlaA containing intact <i>flaA</i> nt 834–2287 on pGP704	This study
pGPfliC1-CM	<i>flaA</i> :: <i>cam</i> :: <i>'flaA</i> , Ap ^r , Cm ^r	<i>flaA</i> (nt 834–2287) from pGPfliC1 on pGP704 with <i>cam</i> inserted at the <i>EcoRV</i> site (nt 1325) in <i>flaA</i>	This study
pUCfliC1	<i>'flaD-flaA</i> ⁺ Ap ^r	1.25-kb <i>HindIII</i> subclone carrying the 5' end of <i>flaA</i> and 5' end of <i>flaD</i> on pUC18	15
<i>flaB</i>			
pBSSKfliC2	<i>flaB</i> ⁺ Ap ^r	<i>NruI-PstI</i> subclone of pflDC1C2 containing intact <i>flaB</i> gene on pBluescriptII SK (nt 2287–3567)	15
pflaB::lacZ	<i>flaB</i> :: <i>lacZ</i> Ap ^r	<i>flaB</i> transcriptional fusion to <i>lacZ</i>	This study
pGPfliC2	<i>flaB</i> ⁺ Ap ^r	<i>flaB</i> (nt 2287–3566) on pGP704	This study
<i>flaD</i>			
pflaD::lacZ	<i>flaD</i> :: <i>lacZ</i> Ap ^r	<i>flaD</i> transcriptional fusion to <i>lacZ</i>	This study
Oligodeoxyribonucleotides			
PfliC1	5'-CAAATCTCAGGGAAGCTTTAGGAAG-3'	<i>flaA</i> -specific DNA probe, nt 1058–1081	This study
PfliC2	5'-ATACTAATTATCTGTCTTTAGTCA-3'	<i>flaB</i> -specific DNA probe, nt 2427–2450	This study
PEfliC1	5'-CTTCCTAAAGTTCCCTGAGATTTG-3'	<i>flaA</i> -specific DNA probe, nt 1081–1058	This study
PEfliC2	5'-TGACTAAAGACAGATAAATTAGTAT-3'	<i>flaB</i> -specific DNA probe, nt 2450–2427	This study
PEfliC2-2	5'-AATAACTTGTGCCATACAATATTC-3'	<i>flaB</i> -specific DNA probe, nt 2425–2402	This study
PEfliC2-3	5'-TGTGAACGATTTAGATTATTTTGA-3'	<i>flaB</i> -specific DNA probe, nt 2475–2452	This study

^a R^f, rifampin; Sm, streptomycin; Sp, spectinomycin; Mob⁺, conjugally mobilizable; Mot⁺, motile (both swimming and swarming).

bation of plates at 37°C for 14 to 16 h. Comparison of β -galactosidase activity in swimmer and swarmer cells was obtained by using a quantitative assay for enzyme activity. Bacteria were incubated overnight at 37°C in L broth plus suitable antibiotics. To measure enzyme activity in swimmer cells, 100 μ l of the overnight culture was inoculated into 100 ml of fresh L broth, and the culture was incubated at 37°C for 4 h. The production of β -galactosidase in swarmer cells was deter-

mined by spreading 100 μ l of an overnight culture of swimmer cells on the surface of a fresh L-agar plate. Following incubation for 4 h at 37°C, swarmer cells were scraped off the agar surface with an L-shaped glass rod and suspended in 100 ml of ice-cold phosphate-buffered saline (PBS; 20 mM sodium phosphate, 100 mM NaCl [pH 7.5]). Swimmer and swarmer cell cultures were harvested by centrifugation and resuspended in 25 ml of ice-cold PBS. β -Galactosidase was then measured by

lysing 100 μ l of washed, concentrated cells with 50 μ l of 0.1% sodium dodecyl sulfate (SDS) and adding 400 μ l of modified (38) Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄) plus 1 mg of *o*-nitrophenyl- β -D-galactoside (ONPG) per ml. This mixture was incubated at 37°C until a pale yellow color had developed. The time of incubation was noted, and the reaction was stopped with 1 M Na₂CO₃. The amount of *o*-nitrophenol produced was assayed spectrophotometrically at 420 nm. The specific activity of β -galactosidase in the sample was calculated in units of enzyme activity and represents the amount of enzyme required to generate 1 nmol of *o*-nitrophenol from ONPG per min at pH 7.0 and 37°C per mg of total protein (48).

Northern (RNA) blot analysis. Transcription of *flaA* and *flaB* in swimmer and swarmer cells was assessed by Northern blot analysis of total *P. mirabilis* BB2000 RNA. Bacteria were incubated overnight at 37°C in L broth, and 100 μ l of the overnight culture was either inoculated into 100 ml of fresh L broth (for swimmer cells) or, for swarmer cells, spread on the surface of a fresh L-agar plate. Following an incubation of 4 h at 37°C, swarmer cells were scraped off the agar surface with an L-shaped glass rod and suspended in 100 ml of ice-cold PBS. Swimmer and swarmer cell cultures were then harvested by centrifugation. Total cellular RNA was purified from swimmer and swarmer cells by the hot phenol technique (53). Equal amounts (20 μ g) of total RNA were applied to wells on a 1.2% agarose-formaldehyde gel, and individual RNA species were separated by the procedure of Ausubel et al. (9). Duplicate samples and RNA size standards were mixed with ethidium bromide and applied to the same gel. After electrophoresis, the portion of the gel containing ethidium bromide-stained samples and RNA standards was illuminated with UV light and photographed for later reference. The unstained RNA molecules were transferred to nitrocellulose (Schleicher & Schuell).

Nitrocellulose filters with immobilized total RNA from swimmer and swarmer cells were prehybridized as described by Ausubel et al. (9). An oligonucleotide called PflC1 (5'-CAA ATCTCAGGGAAGTTTAGGAAG-3', corresponding to nt 1058 to 1081) was synthesized as a probe specific for *flaA*, while PflC2 (5'-ATACTAATTATCTGTCTTTAGTCA-3', corresponding to nt 2427 to 2450) was produced as a *flaB*-specific probe. Each oligonucleotide was end labeled with [γ -³²P]ATP, using T4 polynucleotide kinase according to established procedures (45). Hybridization and washing of the Northern blots were done under high-stringency conditions.

RNA slot blots were also performed to allow more accurate measurement of relative amounts of *flaA* and *flaB* mRNA found in swimmer and swarmer cells. Total RNA from 4-h swimmer or swarmer cells was bound to nitrocellulose. A twofold dilution series starting with an initial concentration of 25 μ g of total RNA was applied to consecutive slots in the manifold, providing a final dilution point of 1:2,048 (0.01 μ g). The probes as used in the Northern blot analysis (above) were also used for RNA slot blots.

Primer extension. The 5' ends of the *flaA* and *flaB* transcripts were mapped by primer extension (9). The oligonucleotide primer PflC1 (5'-CTTCCTAAAGTTCCCTGAGAT TTG-3', corresponding to nt 1081 to 1058) was used to map *flaA*, while PflC2 (5'-TGACTAAAGACAGATAATTAGT AT3', nt 2450 to 2427), PflC2-2 (5'-AATAACTTGTGCCATA CAATATTC-3', nt 2425 to 2402), and PflC2-3 (5'-TGTGAAC GATTTAGATTATTTTGA-3', nt 2475 to 2452) were used for *flaB*. RNA (25 μ g) prepared from 4-h swimmer or swarmer cell cultures of strain BB2000 was annealed to 200 ng of each primer that had been end labeled with [γ -³²P]ATP by using T4 polynu-

cleotide kinase (45). After annealing, reverse transcription was initiated by using avian myeloblastosis virus reverse transcriptase (50 U; Promega) and actinomycin D for 1 h at 42°C. After treatment with RNase, phenol extraction, and ethanol precipitation, the DNA pellets were resuspended in 10 μ l of Sequenase stop/loading dye (U.S. Biochemical) and stored at -20°C until use. Samples were heated to 85°C just prior to loading, and 3 μ l was loaded onto a polyacrylamide DNA sequencing gel alongside a standard sequencing reaction mixture of pflDC1C2 and the respective primer.

Construction of FlaA⁻, FlaB⁻, and FlaD⁻ mutants of *P. mirabilis*. Mutations in *flaD*, *flaA*, and *flaB* were constructed by using gene replacement techniques employing the suicide plasmid pGP704 (39) and wild-type strain BB2000. In brief, the cloned flagellar gene on pGP704 was modified in vitro by recombinant DNA techniques to incorporate the insertion of the chloramphenicol resistance (*cam*) gene. The recombinant plasmid was then conjugally transferred to BB2000, where failure of plasmid replication and allelic exchange between the mutant flagellar gene and the wild-type chromosomal copy resulted in gene replacement and consequent mutagenesis.

A FlaD⁻ mutant was constructed by subcloning the 1.2-kb *Hind*III fragment (nt 1 to 1189) from pUCfliC1 to pGP704. This was accomplished by enzymatic digestion of pUCfliC1 with *Hind*III and electroeluting the 1.2-kb fragment after agarose gel electrophoresis. The 1.2-kb DNA fragment was digested with mung bean exonuclease, and the resulting DNA fragment was blunt-end ligated to the *Eco*RV site in pGP704. The ligation mixture was used to transform competent *E. coli* S17-1 (λ pir) with Ap^r selection for pGP704. Resulting Ap^r colonies were isolated, plasmid DNA was extracted, and restriction enzyme sites were mapped to confirm the insertion of the *P. mirabilis* 1.2-kb DNA. A *cam* gene was obtained from pUT/mini-Tn5-CM by *Hind*III digestion and electroelution of the *cam*-containing 3.4-kb fragment from an agarose gel. After mung bean treatment, the blunt-end *cam* gene fragment was ligated to the *Eco*RV site (nt 270) in *flaD*, and the ligation mixture was used to transform *E. coli* S17-1 (λ pir), with selection for both Ap^r and chloramphenicol resistance (Cm^r). The construction was confirmed through restriction site mapping of the recombinant plasmid DNA, and a positive clone, referred to as pDFFL1, was chosen for further studies. Plasmid pDFFL1 was conjugally transferred into *P. mirabilis* by filter mating (below), resulting in DF1000 (*flaD*::*cam*::*flaD*) as described below.

FlaA⁻ mutants were constructed by using pBSSKfliC1, which contains the intact *flaA*, nt 834 to 2287. This plasmid was cleaved with *Eco*RI and *Kpn*I, and the *flaA*-containing DNA fragment was isolated by electroelution and ligated to pGP704. The ligation mix was transformed into competent *E. coli* S17-1 (λ pir) with selection for Ap^r. Several Ap^r colonies were screened through restriction site analysis, and one colony with the correct *flaA* DNA insert was chosen. This plasmid, pGPfliC1, was next digested with *Eco*RV, which internally cleaves the *flaA* gene (at nt 1325). The *cam* gene from pUT/mini-Tn5-CM was isolated by using the strategy described above, digested with mung bean nuclease to produce a blunt-ended fragment, and ligated with *Eco*RV-cut pGPfliC1 DNA. Following transformation of S17-1 (λ pir) and selection for Ap^r plus Cm^r, plasmid pGPfliC1-CM, containing the *cam* gene inserted at the *Eco*RV site in *flaA*, was obtained. This plasmid was used to produce a FlaA⁻ mutant in *P. mirabilis*, called DF1001 (*flaA*::*cam*::*flaA*).

Similarly, mutations in *flaB* were produced by using pBSSKfliC2, which is an *Nru*I-*Pst*I subclone from pflDC1C2 containing an intact *flaB* (nt 2287 to 3566). A *Sma*I-*Sal*I

digestion of pBSSKfiC2 was used to subclone the *flaB* gene to the *EcoRV* site on pGP704, resulting in pGPfiC2. The same cloning and insertion strategy used to produce the FlaA⁻ mutants was used to produce mutations in *flaB*; in this case, however, an *EcoRV* site (nt 2734) in *flaB* was used. The resulting strain was called BB2301 (*flaB*::*cam*::*flaB*).

Gene replacement mutagenesis. Each of the *flaD*, *flaA*, and *flaB* suicide plasmid constructs harbored in *E. coli* S17-1 (λ pir) was conjugally mated to *P. mirabilis* BB2000 by using the plate mating technique. Following mating, *P. mirabilis* exconjugants were selected by plating on LSW⁻ agar containing chloramphenicol. Antibiotic-resistant colonies from each mating were then screened for ampicillin susceptibility (Ap^s; i.e., loss of the plasmid), and a single representative Ap^s colony was chosen for each. Genomic DNA from each gene replacement strain and from BB2000 was prepared as described above. Chromosomal DNA was digested with *PvuII*, which cleaves asymmetrically in the *cam* gene, thereby providing a diagnostic test for replacement of the wild-type copy of either *flaD*, *flaA*, or *flaB* with the mutant construction. The FlaD⁻, FlaA⁻, and FlaB⁻ strains were then assayed for swimming motility, swarming motility and behavior, and swarmer cell differentiation.

Motility and differentiation analyses. The analysis of swarming motility and behavior was performed as previously described (14). In all swarming assays, L-agar plates were used after thorough drying at 42°C (45 to 60 min) to provide uniform conditions for evaluating swarming motility. A colony of the cells to be tested was incubated for 14 to 16 h in 2 ml of L broth at 37°C, with the appropriate antibiotics added to the medium prior to inoculation as required. Five microliters of the overnight culture was applied to the center of a dry L-agar plate, and the inoculum was given sufficient time to be absorbed into the agar (usually less than 2 min). The bacteria were incubated in the inverted plate at 37°C and examined at 4, 8, and 16 h postinoculation. *P. mirabilis* BB2000 (wild-type swarming) served as a control. Swarmer cell differentiation, i.e., the overproduction of flagella, cellular elongation, and polyploidy, was also examined microscopically as described by Belas et al. (14).

Swimming motility was assessed by both Mot agar analysis and light-microscopic examination of the motility of cells grown in Mot broth. A loopful of an overnight inoculum was used as an inoculum and gently stabbed into the center of a Mot agar plate. The plate was placed at 37°C, and swimming, i.e., outward movement of the cells through the agar matrix, was recorded at 4, 8, and 16 h after inoculation. The results of this test were confirmed by further analyses by light microscopy of individual cells grown in Mot broth for 4 to 6 h at 37°C to a cell density of 10⁷ to 10⁸ cells per ml. *P. mirabilis* BB2000 (wild-type swimming) served as a control.

Southern blot analysis of *flaA* Mot⁺ revertants. Chromosomal DNA from *P. mirabilis* BB2000, DF1001, DF1002, and DF1003 was isolated by the spooling method (48) and digested with either *HindIII* or *EcoRV*, and the resulting DNA fragments were separated on a 1.0% agarose gel in 1× Tris-borate-EDTA buffer (45). Following ethidium bromide staining and photography, the DNA fragments were transferred to a Nytran membrane (Schleicher & Schuell) as recommended by the manufacturer. Plasmid pfiDC1C2 (Table 1) was used as a probe and labeled by nick translation (45) using [α -³²P]dCTP (New England Nuclear). Stringent conditions were maintained throughout hybridization and washing. Following thorough washing of the membrane to remove unbound probe DNA, the membrane was autoradiographed, and the resulting film was examined.

Isolation of flagella and preparation of flagellin. Flagella

from wild-type cells and FlaA⁻ motile revertants were isolated by mechanical shearing, and the flagellin was purified as described by Belas et al. (14).

Production and use of anti-FlaA antiserum. Polyvalent rabbit antiserum to wild-type flagellin (FlaA) was prepared as previously described (14). To test for bacterial tethering, the antiserum was added to molten Mot agar at concentrations of 1:100, 1:500, and 1:1,000. The antiserum was mixed thoroughly with the Mot agar, which was then poured into petri dishes and allowed to gel. BB2000, DF1001, and FlaA⁻ motile revertants DF1002 and DF1003 were inoculated into Mot agar with and without antiserum and incubated for 6 to 8 h at 37°C to allow adequate swimming migration.

Protein analyses. Protein concentration was determined by using the micro-bicinchoninic acid protein assay reagent as recommended by the manufacturer (Pierce Chemical Co.). Polyacrylamide gel electrophoresis (PAGE) was done by using the system established by Laemmli (32). N-terminal amino acid sequence analysis was done as previously described (15) and performed as a gift by Beckman Instruments, Inc. Flagellin was purified by transferring the protein from a polyacrylamide gel to a nitrocellulose membrane and using the electroelution method of Harrington (25). Amino acid composition was determined by using acid hydrolysis and standard procedures (43). Enzymatic digestion of FlaA and revertant flagellins was done by the procedure of Alberti and Harshey (1). The resulting peptides were separated by PAGE on a 15% polyacrylamide gel and visualized by Coomassie blue staining (22). Western blot (immunoblot) analyses of partially digested flagellins were performed with an anti-FlaA rabbit polyvalent antiserum as described previously (14).

Materials and reagents. All reagents were of the highest purity available. Components of bacteriological media were purchased from Difco. Restriction endonucleases and DNA-modifying enzymes were obtained from either New England Biolabs, Boehringer Mannheim Biochemicals, or Promega and used according to the supplier's recommendations.

RESULTS

Plasmid-borne *lacZ* transcriptional fusion studies. To investigate the regulation of *flaA* and *flaB* during swarmer cell differentiation, transcriptional fusions that coupled these genes to a promoterless *lacZ* gene carried on a low-copy-number plasmid, pCED6, were constructed (21). As described in Materials and Methods, pCED6 was modified to allow for conjugal transfer of the plasmid from *E. coli* donors into BB2000. The plasmid by itself and harboring a *flaD*::*lacZ* transcriptional fusion served as controls for these experiments in which the levels of the enzyme were measured and compared between uninduced swimmer cells grown in liquid medium versus induced swarmer cells harvested from the same medium solidified by the addition of 1.5% agar.

The results of these experiments are shown in Table 2. When β -galactosidase levels are compared between swarmer and swimmer cells, there is approximately a 7.5-fold increase in the expression of *flaA*. The data obtained from *flaA*::*lacZ* fusions suggest that *flaA* is coordinately regulated as part of swarmer cell differentiation. On the other hand, *flaB* does not appear to have a regulatory scheme similar to that of *flaA*; moreover, the level of *flaB* expression is very similar to background levels (pCED6-Mob). This may indicate that *flaB* is not expressed in either wild-type swimmer or swarmer cells. Finally, the data show that the expression of *flaD* is much lower than that of *flaA*. The ratios of expression of *flaA* to *flaD* expression are ca. 20:1 in swimmer cells and 100:1 in swarmer cells. These results

TABLE 2. Measurement of *flaA* and *flaB* expression through plasmid-borne *lacZ* transcriptional fusions

Plasmid	β -Galactosidase activity ^a (nmol/min/mg of protein)		
	SWM	SWR	Ratio ^b
pCED6-Mob	2.12	1.54	0.73
pflaA::lacZ	71.1	536	7.54
pflaB::lacZ	2.11	2.00	0.95
pflaD::lacZ	3.57	5.14	1.44

^a See Materials and Methods for experimental details. SWM, swimmer cell; SWR, swarmer cell.

^b β -Galactosidase produced by swarmer cells compared with that produced by swimmer cells.

are in keeping with the stoichiometry of FlaD to FlaA, whereby only a few molecules of FlaD are required for several thousand FlaA molecules composing the flagellar filament (19, 29). The reason for the increase in the FlaA-to-FlaD ratio between swimmer and swarmer cells is unknown and is the subject of ongoing research.

Northern blot analyses of *flaA* and *flaB* transcription. Since the preceding experiments were done with a plasmid-borne *lacZ* transcriptional fusion, there was concern that a plasmid copy number effect might be responsible for reducing the relative change in transcription between swimmer and swarmer cells. Therefore, direct measurement of swimmer and swarmer cell *flaA* and *flaB* mRNA was undertaken, using Northern blots and highly specific oligonucleotide probes to each flagellin gene. The Northern blot data obtained from such experiments are presented in Fig. 2. When a *flaA*-specific oligonucleotide probe was used, a single mRNA species of ca. 1.2 kb was labeled. The size of this mRNA is appropriate for the size of the *flaA* coding region and confirms earlier reports (15) which suggest that *flaA* is transcribed as a monocistronic message. The size of the message remains constant when swimmer and swarmer cells are compared, indicating that the same DNA is transcribed in both uninduced and induced conditions. The amount of the message increased during differentiation, and densitometric analyses suggest that this change (8- to 10-fold increase) was similar to that seen when plasmid-borne *lacZ* transcriptional fusions were used (see above). Therefore, *flaA* appears to be regulated coordinately with swarmer cell differentiation, and the amount of *flaA* transcription increases about eightfold during that event. However, the eightfold increase in *flaA* expression is much less than expected and, by itself, may not fully explain the increase in flagellin observed on swarmer cells (see Discussion).

Unlike the case for *flaA*, *flaB*-specific oligonucleotide probe PflC2 failed to hybridize to *flaB* mRNA, even though several different oligonucleotide sequences were used in these experiments (data not shown). These data corroborate the results of the plasmid-borne *flaB*::*lacZ* transcriptional fusion experiments and suggest that *flaB* is not normally transcribed by either swimmer or swarmer cells.

Identification of the promoter directing *flaA* transcription. The data from Northern blots of *flaA* mRNA suggest that this gene is transcribed as a monocistronic message. Previous work examining the nucleotide sequence of this locus (15) found a possible promoter region upstream of the *flaA* start codon with homology to the flagellar gene-specific σ^{28} promoter (7, 26). To confirm the actual site of RNA transcription, primer extension experiments using mRNA from swimmer and swarmer cells, and *flaA*- as well as *flaB*-specific oligonucleotide primers, were undertaken.

FIG. 2. Northern blot analyses of *flaA* mRNA. Shown are hybridizations of transcripts from *P. mirabilis* BB2000 swimmer cells (SWM) and swarmer cells (SWR). The *flaA*-specific probe used for the analysis is an oligonucleotide (PflC1) corresponding to nt 1058 to 1081 from the *flaA* gene. (A) Twenty micrograms of the isolated RNA sample was loaded on each lane. RNA size standards (Gibco-BRL Lifescience Technology) used to measure the relative size of the *flaA* transcript are indicated at the left. (B) Northern slot blots of the same samples of mRNA from swimmer and swarmer cells, labeled with the probe used for panel A. A twofold dilution (dil) series was used to dilute the mRNA from 20 μ g (1:1) to 10 ng (1:2,048). Analysis of the dilute samples of equal density indicates that transcription of *flaA* increases approximately eightfold in swarmer cells.

Figure 3 shows the results of primer extension experiments using a *flaA*-specific primer and mRNA taken from swimmer and swarmer cells. As indicated by these experiments, the start of *flaA* transcription begins at the G at position 932 (relative to the published sequence [15] and that shown in Fig. 1B). This places the site of transcription initiation at ca. 8 bases 3' to the σ^{28} promoter -10 sequence, which is in good agreement with the observations for similar flagellar genes from *E. coli* and *Salmonella typhimurium* (26). There appear to be no other start points of transcription, and as indicated by Northern blot analyses of *flaA* transcripts, transcription is initiated at the same site in both swimmer and swarmer cells. Primer extension experiments also confirm the results of earlier *flaA* transcription studies, indicating a relative 8- to 10-fold increase in transcription of *flaA* in the swarmer cell.

Once again, as was seen with either *lacZ* transcriptional fusions or Northern blot procedures, no *flaB*-specific transcript could be found with use of three different oligonucleotide primers (PflC2, PflC2-2, and PflC2-3; Materials and Methods).

Phenotypes of FlaD⁻, FlaA⁻, and FlaB⁻ mutants. Mutations in either *flaD*, *flaA*, or *flaB* were constructed to define the function of each of these genes in swimming and swarming behavior and in swarmer cell differentiation. This was accomplished through a process of allelic exchange involving a conjugative suicide plasmid bearing a copy of the mutated gene

FIG. 3. Location of the *flaA* transcriptional start site by primer extension. The primer used, PEfiC1 (5'-CTTCCTAAAGTTCCCT GAGATTTG-3', corresponding to nt 1081 to 1058 on the *flaA* map), was hybridized to equal amounts of mRNA from swimmer cells (lane 1) and swarmer cells (lane 2) and extended with avian myeloblastosis virus reverse transcriptase. Sequencing reactions, carried out on a single-stranded DNA template by using the same primer, were analyzed on a 6% acrylamide-7 M urea gel and autoradiographed. The sequence flanking the start site is shown, and the apparent transcriptional start site is marked by an arrowhead.

and BB2000. Loss of plasmid sequences and the exchange of the wild-type copy of the gene for the mutated copy were determined as described in Materials and Methods. The resulting phenotypes of the FlaD⁻, FlaA⁻, and FlaB⁻ mutants are listed in Table 3.

Mutations in *P. mirabilis* *flaD* (the homolog to *E. coli* *fliD*) are defective in swimming motility and fail to produce flagella, as is also true for FliD⁻ *E. coli*, *S. typhimurium*, and *Bacillus subtilis* (19, 29). Production of flagellin protein in FlaD⁻ mutants does occur, since concentrated supernatants from DF1000 (*flaD*::*cam*::*flaD*) produce a positive result when analyzed by Western blotting using a polyvalent antiserum against *P. mirabilis* flagellin (11). These mutants also fail to produce a differentiated swarmer cell and do not exhibit swarming motility and behavior, suggesting that the flagellar filament is integral in the sensory transduction pathway leading

TABLE 3. Motility and differentiation phenotypes of FlaD⁻, FlaA⁻, and FlaB⁻ mutants

Relevant genotype	Motility (mm)		Swarmer cell differentiation		
	Swimming ^a	Swarming ^b	Elongation ^c	Flagella ^d	Polyoidy ^e
Wild type	65	75	+	+	+
<i>flaD</i> :: <i>cam</i> :: <i>flaD</i>	0	0	-	-	-
<i>flaA</i> :: <i>cam</i> :: <i>flaA</i>	2.5 ^f	0	-	-	-
<i>flaA</i> Mot ⁺ revertant	60	73	+	+	+
<i>flaB</i> :: <i>cam</i> :: <i>flaB</i>	62	70	+	+	+

^a Swimming motility through semisolid Mot agar recorded after 24 h of incubation at 37°C.

^b Swarming motility over L agar recorded after 24 h of incubation at 37°C.

^c Swarmer cell elongation compared with wild type (+, wild type; -, mutant phenotype).

^d Overproduction of flagella compared with wild type (+, wild type; -, mutant phenotype).

^e Swarmer cell polyoidy compared with wild type (+, wild type; -, mutant phenotype).

^f Nonmotile except at zones of Mot⁺ revertant flares.

to induction of the genes associated with the swarmer cell differentiation regulon.

When gene replacement mutagenesis is done by using *flaA*, the resulting FlaA⁻ mutants are defective in swimming and swarming motility. FlaA⁻ mutants are also defective in normal swarmer cell differentiation (cellular elongation, overproduction of flagella, and polyploidy) and, unlike FlaD⁻ mutants, fail to produce any detectable amounts of flagellin when tested by Western blot techniques. These results suggest a major role for *flaA* in the synthesis of the flagellin of both swimmer and swarmer cells, support the data obtained from the transcription assays described above, and point to the integral role of the flagellar filament in this signal transduction pathway.

In contrast to *flaA*, mutations in *flaB* were wild type in all aspects of swimming and swarming motility and behavior and in the three aspects of swarmer cell differentiation measured in this study. These data provide substantial evidence that in wild-type cells, FlaA is the only flagellin expressed. The additional copy of the flagellin-encoding genes described here (*flaB*), and a third flagellin-encoding gene (*flaC*) discovered by DNA-DNA hybridization (15), appear to be silent in the wild-type cell.

FlaA⁻ mutants were found to be unstable, frequently producing flares of motile cells when incubated in semisolid Mot agar for extended periods (more than 24 h at 37°C). This instability occurred under both selective (plus chloramphenicol) and nonselective (no chloramphenicol) conditions and appeared to be specific to the *flaA* region, because *flaD*::*cam*::*flaD* mutations were, in contrast, very stable and were not seen to revert to a Mot⁺ phenotype. The *flaA* Mot⁺ revertants swam through Mot agar and swarmed over L agar at rates nearly indistinguishable from those of wild-type cells. When cells taken from the swarming periphery were examined microscopically, the full complement of swarmer cell characteristics was seen (Table 3). For all practical purposes, *flaA* Mot⁺ revertants appear to have the wild-type phenotype.

***flaA* Mot⁺ revertants possess an antigenically distinct flagellum.** Twelve *flaA* Mot⁺ revertants were selected from separate flares originating from a group of eight distinct *flaA* mutants. Each of the 12 *flaA* Mot⁺ revertants was analyzed for swimming and swarming characteristics as described above, but as a means to test the antigenicity of the flagellar filament, motility studies were conducted in the presence or absence of rabbit anti-flagellin antiserum. Previous experiments (14) have demonstrated that this antiserum developed against whole flagellar filaments is highly specific to BB2000 flagellin and most likely directed to FlaA, since this is the only flagellin species found to be expressed by wild-type cells under the conditions of these studies.

An example of the results of the analyses of *flaA* Mot⁺ revertant DF1003 motility through or over anti-FlaA-amended medium is shown in Fig. 4. As indicated above, although FlaA⁻ mutants are nonmotile, *flaA* Mot⁺ revertants are as motile as wild-type cells (Fig. 4A). In contrast, when anti-FlaA antiserum is added to the same medium (Fig. 4B), the motility of wild-type cells is severely impeded. This is most likely the result of antibody molecules tethering groups of flagella together, thereby reducing flagellar rotation and cell movement. Unlike wild-type cells, however, *flaA* Mot⁺ revertants move through the antibody-amended medium with relative ease and travel a distance comparable to that obtained in medium without antiserum. In both media, FlaA⁻ mutants remain at the initial point of inoculation (although small flares may be observed emanating from the FlaA⁻ colony in Fig. 4). Similar patterns of diminished swarming over anti-FlaA-amended L agar were also observed for wild-type cells, while the *flaA*

FIG. 4. Antigenic variation of *P. mirabilis* flagellin. Swimming motility of the wild type (wt), a *flaA* Mot⁺ revertant (DF1003), and the parent *flaA* strain (DF1001) is shown after 24 h of incubation at 37°C in semisolid Mot agar (A) or Mot agar amended with a 1:500 dilution of anti-FlaA antiserum (B). The swimming zone of the wild type is severely affected by the addition of the anti-FlaA antiserum, while the motility of the *flaA* Mot⁺ revertant is unaffected.

Mot⁺ revertants swarmed over this agar apparently unhampered by the presence of the antibody (11). These data indicate that the *flaA* Mot⁺ revertants produce an antigenically distinct flagellar filament.

Southern blot analysis. Southern blot DNA-DNA hybridization was used to determine the genetic cause for the change in flagellin antigenicity. The chromosomal DNA from each of 12 *flaA* Mot⁺ revertants was isolated and digested with either *EcoRV* or *HindIII*. These enzymes provide a unique DNA fragment pattern for the *flaD-flaA-flaB* region (Fig. 1A) and are therefore useful for diagnosing changes that might occur therein. Moreover, the *cam* gene inserted into *flaA* carries an *EcoRV* site, thus providing a convenient way of identifying its presence. Plasmid pfiDC1C2, covering the entire 3.6-kb region (*flaD flaA flaB* but not *flaC*), was radioactively labeled and used as a probe. Figure 5 shows the results from such experiments with 2 of the 12 *flaA* Mot⁺ revertants.

As the DNA fragment pattern on Fig. 5 indicates, the insertion of the *cam* gene in *flaA* results in an increased size of the hybridizing DNA compared with wild-type DNA. However, for each of the *flaA* Mot⁺ revertants analyzed, the reversion to a motile phenotype produces a hybridizing DNA fragment of smaller size, possibly due to a deletion or rearrangement in the *flaA* locus. For the 12 mutants thus far examined, the deletion size varied from a low of 2 kb (DF1002) to as much as 7 kb (DF1003). Apparently the 7-kb deletion removes the DNA 3' from the *EcoRV* site in *flaA*, all of the *flaB* gene, and downstream into other uncharacterized

FIG. 5. Southern hybridization of chromosomal DNA isolated from wild-type, *flaA'*::*cam*::*flaA* parental, and two *flaA* Mot⁺ revertant strains. Chromosomal DNA was digested with either *EcoRV* or *HindIII*, as indicated, and the resulting DNA fragments were separated on a 1.0% agarose gel prior to Southern blotting. The probe used to label the *flaA* region was pfiDC1C2, covering a portion of *flaD* and all of *flaA* and *flaB*. The physical map of this region predicts fragments of 1.1 and 1.4 kb (plus an additional fragment extending beyond the *flaA* locus) when cut with *EcoRV*. When cut with *HindIII*, a fragment of 1.2 kb and a larger fragment composed of flanking DNA are predicted. Lanes: 1, wild-type BB2000; lane 2, *flaA'*::*cam*::*flaA* parent DF1001; 3, *flaA* Mot⁺ revertant DF1002; 4, *flaA* Mot⁺ revertant DF1003. Blunt-end insertion of the *cam* gene disrupts the *flaA* gene, removing the original *flaA* *EcoRV* site at nt 1325, and adds an additional 3.75 kb (with a new *EcoRV*) to this locus. This disruption is seen as a change in fragments when DF1001 (*flaA'*::*cam*::*flaA*; lane 2) chromosomal DNA is cut with *EcoRV*. The *flaA* Mot⁺ mutants have deletions of ca. 2.0 and 7 kb, respectively, as can be seen in the *HindIII* digests.

genomic sequences (presumably another flagellin gene, e.g., *flaC*).

Analysis of revertant flagellin. To confirm that the *flaA* Mot⁺ revertants were indeed producing novel flagellin proteins, flagella were isolated from both wild-type and *flaA* Mot⁺ revertant (DF1003) swarmer cells, and the relative molecular weight of the flagellin was determined. Preparation of flagella by this method results in greater than 99% pure flagellin from wild-type swarmer cells (13, 14). Aliquots of each preparation were measured for protein concentration and denatured with sodium dodecyl sulfate (SDS), and equal amounts were applied to a denaturing 10 to 20% linear gradient polyacrylamide gel to analyze the relative size of the revertant flagellin. A photograph of a representative gel is shown in Fig. 6 (lanes 1 and 2).

The molecular size of FlaA was calculated to be ca. 39.4 kDa from these analyses, in good agreement with previous estimates of FlaA size (14, 15). The flagellin from the *flaA* Mot⁺ revertant DF1003, hereafter referred to as FlaR, has a larger molecular size, estimated to be ca. 40.3 kDa. The size difference between FlaR and FlaA provides further evidence supporting the conclusion that the reversion process produces a

FIG. 6. SDS-polyacrylamide gel analysis of FlaA and FlaR. Intact flagella from either the wild type (BB2000) or *flaA* Mot⁺ mutant DF1003 were prepared as described in Materials and Methods. Samples were mixed with 2× loading buffer (32) and boiled for 5 min prior to electrophoresis. Equivalent amounts (1 µg) of samples were loaded into all lanes. Samples were either stained with Coomassie blue (lanes 1 and 2) or immunoblotted with an anti-FlaA polyvalent antiserum and reacted with goat anti-rabbit alkaline phosphatase (lanes 3 to 6). Lanes: 1 and 3, FlaA; 2 and 4, FlaR; 5, FlaA partially digested with V8 protease; 6, FlaR partially digested with staphylococcal V8 protease. Unique V8 digestion products of FlaA and FlaR are indicated by the dots to the right of the corresponding peptides.

new flagellin. FlaR preparations also contained two additional, unidentified proteins of 54 and 38.3 kDa. The 54-kDa protein is also seen as a minor band in FlaA preparations (11), but the 38.3-kDa protein has been observed only in FlaR preparations. The identity of each of these proteins is currently unknown. Possible explanations for their existence are discussed below (Discussion).

To further define the differences between FlaA and FlaR, limited *Staphylococcus* V8 protease digestion of each flagellin was done, and the resulting peptides were transferred to polyvinylidene difluoride membranes and immunoblotted with anti-FlaA antiserum (Fig. 6, lanes 3 to 6). The FlaA antiserum has limited cross-reactivity to FlaR, suggesting that these two flagellins have common epitopes. A comparison of the undigested and digested samples of FlaA and FlaR reveals at least two distinct differences when these proteins are treated with limited V8 proteolysis (indicated by a dot next to the unique band in Fig. 6, lanes 5 and 6). Protease V8 treatment of FlaA produces a unique band of ca. 27 kDa, while the same treatment of FlaR produces a unique band of 35.2 kDa. The finding that each of these unique bands reacts with the FlaA polyvalent antiserum suggests that these two polypeptides have common antigenic components.

One hypothesis to explain *flaA* Mot⁺ reversion is that a change in DNA sequence due to a deletion (or rearrangement) produces a hybrid protein between the N-terminal end of FlaA and the C-terminal end of one of the other silent flagellin genes. To test this hypothesis, the N-terminal sequence of FlaR was determined. In keeping with the hypothesis, the first 28 residues of FlaR and FlaA are identical (11). However, the hypothesis also predicts that there should be amino acid differences between the two proteins. Therefore, amino acid composition analyses were done on FlaA and FlaR, and the results are shown in Table 4. Amino acid composition analyses indicate that there are major differences between FlaA and FlaR. The mole percent values for threonine, proline, valine, and methionine from FlaR are substantially different from those of FlaA, and other residues show smaller, yet still significant, differences. Thus, from these results and the data

TABLE 4. Amino acid composition analysis of FlaA and FlaR flagellin^a

Residue	Amt (pmol)		Mol%		
	FlaA	FlaR	FlaA	FlaR	Difference
Asp + Asn	1,838.13	2,004.14	17.61	15.22	-2.39
Glu + Gln	1,134.14	1,447.93	10.87	11.00	0.13
Ser	858.88	1,434.11	8.23	10.89	2.66
Gly	765.81	1,149.10	7.34	8.73	1.39
Arg	362.46	71.05	3.47	0.54	-2.93
Thr	1,008.11	313.62	9.66	2.38	-7.28
Ala	1,051.60	952.24	10.08	7.23	-2.85
Pro	81.27	1,312.88	0.78	9.97	9.19
Tyr	109.31	167.08	1.05	1.27	0.22
Val	665.45	1,397.61	6.38	10.61	4.23
Met	58.43	643.40	0.56	4.89	4.33
Cys-2	10.41	0.00	0.10	0.00	-0.10
Ile	600.19	546.49	5.75	4.15	-1.60
Leu	891.89	829.08	8.55	6.30	-2.25
Phe	253.18	241.67	2.43	1.84	-0.59
Lys	747.92	658.21	7.17	5.00	-2.17

^a FlaA isolated from wild-type BB2000 and FlaR isolated from *flaA* Mot⁺ revertant DF1003.

presented above, it appears that the *flaA* Mot⁺ revertants produce an antigenically distinct flagellin as a result of a deletion (or rearrangement) of DNA at or very near *flaA*. The result of the deletion may be to fuse the N-terminal portion of FlaA to the C-terminal end of a previously silent copy of the flagellin gene, either *flaB* or *flaC*. A hypothetical model describing this is shown in Fig. 7 and discussed below.

DISCUSSION

The discovery of multiple flagellin genes of *P. mirabilis* (15) has raised provocative questions concerning their regulation and function in association with swarmer cell differentiation. These issues gain further significance because the overproduction of flagella during swarmer cell differentiation is considered to be an important prerequisite for the colonization, invasion, and ultimate pathogenicity of this organism (2, 5). Many bacterial species have been observed to possess multiple copies of flagellin genes. For example, *S. typhimurium* *fliC* and *fliB* (47, 52, 54) and *Campylobacter coli* *flaA* and *flaB* (6, 23, 24) each encode two separate flagellin species that play a role in antigenic variation of the flagellum. Since flagella and specifically flagellin (H antigen) are extremely antigenic, changes in flagellin antigenicity may provide the bacteria with an effective means of escaping the immune response of the host (18). These investigations were undertaken to explore the role of multiple flagellin genes and understand the regulation of each gene during swarmer cell differentiation. On the basis of transcription regulation analyses, mutant phenotypes, and protein biochemistry, I have described a means by which *P. mirabilis* may alter the antigenicity of its flagella.

Regulation of *flaA* and *flaB*. Three methods were used to determine the transcriptional regulation of *flaA* and *flaB*: plasmid-borne *lacZ* transcriptional fusions, Northern blot DNA-RNA hybridization, and primer extension experiments. The results from each confirm that *flaA* is the sole flagellin gene transcribed by wild-type cells. Furthermore, *flaA* is transcribed as a monocistronic message and regulated coordinately with swarmer cell differentiation such that *flaA* transcription increases about eightfold during induction. The transcriptional analyses support the data from previous work using Tn5 mutants demonstrating that swimmer and swarmer cell flagel-

FIG. 7. Schematic model of *P. mirabilis* flagellin antigenic variation. (A) The *flaA* locus of *P. mirabilis* in which three copies of possible flagellin-encoding genes are found. *flaA* and *flaB* are physically adjacent to one another on the chromosome, while *flaC* is located at an undetermined distance downstream from *flaB*. On the basis of the evidence presented, only *flaA* is actively expressed in wild-type cells, while *flaB* and *flaC* are silent copies of flagellin-encoding genes. The transcription of *flaA* results in the synthesis of flagella composed solely of FlaA. (B) Introduction of a *cam* gene into *flaA* disrupts the *flaA* reading frame and prevents the expression of *flaA*, resulting in a Fla⁻ cell. (C) Spontaneous mutations within the *flaA* gene remove the *cam* gene insertion, the 3' end of *flaA*, and sequence in the *flaB*-*flaC* region. Some deletions result in the genetic fusion of the 5' region of *flaA* with the 3' region of either *flaB* or *flaC* and, occasionally, result in a functional hybrid flagellum composed of the N-terminal end of FlaA and the C-terminal end of either of FlaB or FlaC. Since the deletion process appears to be random, and the result is predicated solely on the basis of a functional flagellar filament, many antigenically different combinations of flagellin molecules may be produced by such a mechanism.

lin syntheses were interconnected, suggesting that only one flagellin gene was involved in the synthesis of both organelles (14).

The eightfold increase in *flaA* expression during induction of swarmer cell differentiation is less than expected from observations of the increase in flagellar filaments when swimmer cells differentiate into swarmer cells. On the basis of the increase in flagellin mass, an increase in *flaA* activity of 100- to 1,000-fold may be anticipated. Several possibilities exist to explain this disparity. First, *flaA* transcription was measured 4 to 6 h after induction of differentiation. Although cells begin to swarm actively at this time, 6 h may be an early stage in swarmer cell differentiation, when *flaA* transcription is less than maximal. Also, the sampling procedure used to obtain swarmer cells from agar media removes all cells, including highly differentiated swarmer cells along the swarming periphery as well as undifferentiated or poorly differentiated cells in the center of the colony. This heterogeneity of the population sampled may result in an artificially lower measurement of *flaA* transcription during differentiation. Experiments using a merodiploid *flaA::lacZ* transcriptional fusion in BB2000 and measuring β -galactosidase activity on L agar containing X-Gal, however, fail to show significant differences in *flaA* activity when the activity of the enzyme at the periphery is compared

with that at the colony center (11). Thus, it appears unlikely that *flaA* activity differs dramatically in cells at the swarming edge from the activity of cells at the colony center during the early stages (4 to 6 h) of swarming motility. It is also possible that other mechanisms, such as transcript stabilization, translational control, or posttranslational control, are involved in controlling the amount of FlaA synthesized during swarmer cell differentiation. Such alternative mechanisms are currently being explored in this laboratory.

The *flaA* transcript is a monocistronic message of 1.2 kb whose size remains constant during swarmer cell differentiation, suggesting that the same *flaA* promoter site is used by both the swimmer and swarmer cells. Primer extension experiments using *flaA*-specific primers and mRNA from swimmer and swarmer cells confirm this and place the *flaA* transcriptional start site at the G at position 932 relative to the published *flaD*-*flaA*-*flaB* sequence (15). The *flaA* σ^{28} promoter location, homology to the σ^{28} promoter consensus sequence, and placement of the transcription start site are in agreement with data for other σ^{28} promoters (26). Such conservation to the *E. coli* σ^{28} consensus suggests that the *P. mirabilis* *flaA* gene has regulatory control mechanisms in common with *E. coli*. This is not surprising since *flaA* regulation in the swimmer cell is likely to be identical to *E. coli* *flhC* regulation. The difference between the two genes may be manifested during swarmer cell differentiation, when regulatory control is probably exerted upon a gene associated with the upper levels of the flagellar control hierarchy (33), such as *flhA* or *flhDC*, rather than at the level of *flaA*.

Evidence obtained from this study and from previous work with *E. coli* (15) has shown that the *flaA* σ^{28} promoter, which is a direct match to the σ^{28} *E. coli* consensus promoter, is fully functional. In contrast, the putative *flaB* σ^{28} promoter, with two mismatches to the consensus, does not function in wild-type *P. mirabilis*. This interpretation is strengthened by previous *E. coli* minicell protein programming experiments showing that *flaB* fails to produce the predicted protein product in an *E. coli* background and does not complement *E. coli* FliC⁻ mutants (15). One possible explanation for this is that the two mismatches (underlined) in the putative *flaB* σ^{28} promoter sequence, TAAA(N-15)GACGATAG (Fig. 1B), apparently are sufficient to prevent recognition by *P. mirabilis* (or *E. coli*) σ^{28} RNA polymerase. [By comparison, *flaD* with one mismatch, TAAA(N-15)ICCGATAA, in its σ^{28} promoter is transcribed and functional in *P. mirabilis*.] Alternatively, a repressor of *flaB* may act to prevent expression of this gene, although no evidence in support of this possibility is currently available.

Mutant analyses. The function of the gene product of *flaD*, *flaA*, and *flaB* was established by constructing mutations in *P. mirabilis*. Mutations in *flaA* completely abolished all motility as well as swarmer cell differentiation (Table 3), while *flaB* mutations had no demonstrable change in wild-type phenotype. These observations emphasize the central role of FlaA flagellin in swarmer cell differentiation and behavior. Presumably, the cell responds to environmental conditions that prevent the normal rotation of the FlaA flagella (3, 12, 14). Loss of FlaA filaments resulted inappropriately in an undifferentiated swimmer cell under conditions that should elicit swarmer cell differentiation. This suggests that *flaA* is required for the induction of swarmer cell differentiation, because mutants defective in FlaA do not differentiate. This is contrary to what is observed during swarmer cell differentiation of *V. parahaemolyticus* (35, 46). In this case, defects causing loss of the polar flagellum (the sensing flagellum) result in a constitutively synthesized swarmer cell. The difference between these two mutants implies that the regulatory mechanisms operating to

control swarmer cell differentiation of *P. mirabilis* are probably different from those of *V. parahaemolyticus*, even though the initial signals (inhibition of flagellar rotation) triggering differentiation are the same for both species.

As indicated, FlaA⁻ mutants do not synthesize any flagellin species. This observation may be used as an indication that the third copy of *P. mirabilis* flagellin-encoding genes, *flaC*, is not actively transcribed. *flaC* was originally found through DNA-DNA hybridization of chromosomal DNA to a generalized flagellin DNA probe that had homology to both *flaA* and *flaB* (15). Cosmids bearing *flaC* recently have been identified and genetically (but not physically) link the third flagellin-encoding gene to a region on the chromosome downstream of *flaB* (11). Although the nucleotide sequence of *flaC* has not been determined and the transcription of this gene has not been assessed, the evidence obtained from FlaA⁻ mutants argues that *flaC* is not transcribed in a *flaA* mutation or wild-type cell, or if it is transcribed, the protein is not a functional flagellin. Therefore, both *flaB* and *flaC* are apparently silent copies of flagellin-encoding genes.

Mutations in *P. mirabilis flaD* failed to produce flagella and lacked all characteristics of swarmer cells when placed under inducing conditions. These findings lend credence to earlier reports (15) establishing this gene as essential for filament synthesis in *P. mirabilis*. *S. typhimurium fliD* mutants fail to make hook-associated protein 2, although flagellin is still synthesized (19, 29). In such *FliD*⁻ mutants, flagellin accumulates to a high level in the surrounding medium (30, 31). By using immunoblotting of SDS-polyacrylamide gels, FlaA has been found in the surrounding medium of *P. mirabilis flaD* cells (11). Moreover, the relative transcription rate of *flaD* to *flaA* in swimmer cells is 1:20, which compares favorably to the relative rates of *flaD* and flagellin-encoding genes measured in *S. typhimurium* and *B. subtilis* (19, 29). These data provide a strong argument for the idea that *flaD* is the *P. mirabilis fliD* homolog.

Interestingly, at a low frequency, Mot⁺ revertants were found emanating as flares from *flaA* colonies on semisolid Mot agar plates. Since mutations in *flaD* and *flaB* constructed in the same manner are extremely stable under all conditions tested (11), this reversion is not caused by the manner in which the mutation was made but rather is caused by the *flaA* gene itself. Although experiments are currently in progress, the frequency of this reversion has not been accurately determined. However, since only one or two flares are seen from a single inoculum after 24 h of growth, the frequency is probably less than 1 in 10⁷ (assuming that after 24 h of growth, a colony in Mot agar would contain at least 10⁷ cells).

Analysis of the reversion process. A significant observation of this study is that *flaA* Mot⁺ revertants are capable of movement through media amended with an anti-FlaA polyvalent antiserum (Fig. 4). This finding suggests that the flagella synthesized by the revertants are antigenically distinct from those produced by wild-type cells. Since the antiserum used is polyvalent and produced in response to whole flagella rather than denatured flagellin (14), it may be anticipated that it contains antibodies capable of binding to many different flagellin epitopes. During such binding, bacteria may be tethered together flagellum to flagellum, thus preventing them from achieving wild-type swimming or swarming motility. This is exactly what happens when anti-FlaA is added to cultures to wild-type *P. mirabilis*: the bacteria become tethered by flagellum-to-flagellum binding (11). In contrast, *flaA* Mot⁺ revertants are only loosely bound when the same antiserum is applied to cell suspensions, indicating that *flaA* Mot⁺ rever-

tants synthesize flagella containing only a subset of possible FlaA epitopes.

Analyses of Southern blots from *flaA* Mot⁺ revertants reveal that a deletion of variable size within the *flaA* locus apparently caused the reversion from Mot⁻ to Mot⁺. The extent of the deletion appears to go from the *EcoRV* site in *flaA* outwards into (and perhaps beyond) *flaB*. Many of the mutants thus screened have deletions of sufficient size to delete *flaB* completely; e.g., DF1003 (Fig. 5, lane 4) has a deletion estimated to be about 7 kb. The absence of *flaB* has been confirmed by probing DF1003 chromosomal DNA with a *flaB*-specific oligonucleotide, PflIC2 (11). Thus, for DF1003 at least, the deletion removes over 50% of *flaA* and all of *flaB*. Whether the mutation affects *flaC* cannot be ascertained because of lack of a *flaC*-specific probe. This observation may indicate that the reversion process is a one-way event in which a deletion occurs, permanently removing a portion of DNA downstream from *flaA* and spanning into *flaB* and further into genes downstream from *flaB*. Thus, the mutation in DF1003 may result in a hybrid gene fusion wherein the 5' end of *flaA* is fused to the 3' end of *flaC*, yielding a functional flagellin as a consequence. Moreover, *flaA* Mot⁺ Cm^s revertants have not been seen to revert to Cm^r Mot⁻, emphasizing the possibility that the process is a variably sized, one-way deletion.

Biochemical analysis of *flaA* Mot⁺ revertant flagellin (FlaR). The data gathered from SDS-PAGE, immunoblotting of V8 protease-digested FlaR, N-terminal amino acid sequencing, and amino acid composition analyses show that FlaA and FlaR have identical N-terminal ends but different C termini. For example, FlaR is 900 Da larger, or about eight amino acid residues longer, than FlaA. The hypothetical model (Fig. 7) used to explain the events producing FlaR predicts that any splice between the 5' end of *flaA* and either *flaB* or *flaC* that produces a functional flagellum will result in a motile revertant phenotype. This model is in keeping with the data from anti-FlaA tethering experiments and SDS-PAGE Western blots. The FlaA epitopes of FlaR are at the N-terminal end of the hybrid protein which is buried and not exposed to the antibody in the native flagellar filament (33). Denaturing the flagellin during SDS-PAGE exposes these FlaA epitopes, which are then free to react with the FlaA antibodies during Western blotting. The slightly larger size of FlaR than of FlaA obviously is still within the range of the size for the flagellin protein to permit the formation of a functional flagellar filament. The flagellin from other revertants is currently being studied to determine the minimum and maximum sizes allowable for a functioning flagellum.

The regions of similarity and uniqueness of each flagellin may also be observed through V8 protease partial digestion and immunoblotting. Partial proteolysis of FlaA and FlaR shows unique peptide products for each flagellin (Fig. 6), yet immunoblots also demonstrate that the two flagellin molecules have similarities, since anti-FlaA recognizes some epitope(s) on FlaR. This finding confirms earlier observations in studies using the same antiserum to tether flagella (Fig. 4). The idea that some of FlaA domains are shared by FlaR is also supported by N-terminal amino acid sequence data establishing that the first 28 residues of each protein are identical (15). This is totally in keeping with the hypothetical model depicted in Fig. 7. On the basis of this model, the splice site joining FlaA with FlaC (in the case of DF1003) is thought to be located at or near the *flaA EcoRV* site, or nearly 107 amino acid residues past the N terminus of FlaA.

Finally, there are significant differences seen in the amino acid compositions of the flagellins. The mole percent values of four residues, in particular Thr, Pro, Val, and Met, are

significantly different between the two flagellins. These data support the idea that FlaR is a hybrid protein composed of the N-terminal end of FlaA and the C-terminal end of some other protein, most probably the gene product of *flaC*.

PAGE analyses of FlaR preparations show the presence of two additional proteins (Fig. 6) not apparent in FlaA samples prepared with identical methods. The identities of these two proteins are not known, but their presence may be the result of the transcriptional fusion between *flaA* and *flaC*. Such a fusion could generate a multicistronic mRNA molecule composed of transcripts from the hybrid flagellin-encoding genes as well as other downstream genes that are activated as part of this transcriptional fusion. Conversely, the reversion process may have caused other (unknown) mutations such as the truncation of a protein. The nucleotide sequence of the region immediately adjacent to the putative fusion of *flaA* and the silent flagellin-encoding gene is currently being analyzed and will be the subject of subsequent communications.

Significance to pathogenesis and differentiation. An important question to consider is whether in vitro flagellin antigenic variation such as described here can occur in vivo, e.g., during colonization of the urinary tract. The inoculation of Mot⁻ bacterial cells in semisolid media or on the surface of hard agar places a strong selection for active motility (44, 49–51). This is due to the reduction of nutrients around the cells combined with the accumulation of waste products at the point of inoculation. Spontaneous mutations, occurring at random points around the chromosome, could randomly cause the deletion and genetic fusion seen in the *flaA* Mot⁺ revertants within the large population of Mot⁻ cells. Such spontaneous Mot⁺ mutants would have a tremendous selective advantage over the bulk of nonmotile cells because of their enhanced survival by being able to actively translocate to areas of greater nutrients and lower waste products. Such spontaneous Mot⁺ mutants would also be very easy to detect because of the very evident flare of motility that they produce.

Spontaneous mutations giving rise to an antigenically distinct flagellin would be as likely to occur in vitro as in vivo and may very well occur in both FlaA⁺ and Mot⁻ cells. Such antigenic changes could potentially increase the survivability of a urinary pathogen. For example, urinary pathogens are confronted by secretory immunoglobulin A as the bacteria attempt to colonize the bladder. Since flagellin is strongly antigenic, a major binding epitope for the immunoglobulins could be the flagellum. Tethering of the bacteria via their flagella would effectively prevent their motility and impose the same selective pressures in this case as those occurring in vitro. A spontaneous deletion that gave rise to an antigenically distinct flagellum would thus provide the bacteria with an avenue of escape from immobilization by the immunoglobulins. Additionally, deletions may not be the only way in which antigenic variation can arise. Indeed, such deletions would ultimately lead to irreversible loss of genomic sequences. Rather, it is predicted that deletions and (more importantly) other rearrangements of chromosomal DNA within the *flaA* locus may lead to a variable flagellar antigen without the irreversible loss of DNA. This would be a successful survival strategy, prolonging the chances of colonization by the bacteria. This premise is currently being tested in vitro and through the use of a mouse model of urinary tract infection.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant MCB-9206127 and Public Health Service grant AI27107 from the National Institutes of Health.

I thank David Flaherty for technical assistance, Mark Flocco and Sam Morris (Beckman Instruments) for their gift of N-terminal sequence analyses, David Maneval for assistance with Western blots, and Allen Place, Harold Schreier, Michael Silverman, and Frank Zappa for advice and encouragement.

REFERENCES

1. Alberti, L., and R. M. Harshey. 1990. Differentiation of *Serratia marcescens* 274 into swimmer and swarmer cells. *J. Bacteriol.* **172**:4322–4328.
2. Allison, C., N. Coleman, P. L. Jones, and C. Hughes. 1992. Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect. Immun.* **60**:4740–4746.
3. Allison, C., and C. Hughes. 1991. Bacterial swarming: an example of prokaryotic differentiation and multicellular behaviour. *Sci. Prog.* **75**:403–422.
4. Allison, C., H. C. Lai, D. Gygi, and C. Hughes. 1993. Cell differentiation of *Proteus mirabilis* is initiated by glutamine, a specific chemoattractant for swarming cells. *Mol. Microbiol.* **8**:53–60.
5. Allison, C., H. C. Lai, and C. Hughes. 1992. Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. *Mol. Microbiol.* **6**:1583–1591.
6. Alm, R. A., P. Guerry, M. E. Power, and T. J. Trust. 1992. Variation in antigenicity and molecular weight of *Campylobacter coli* VC167 flagellin in different genetic backgrounds. *J. Bacteriol.* **174**:4230–4238.
7. Arnosti, D. N., and M. J. Chamberlin. 1989. Secondary sigma factor controls transcription of flagellar and chemotaxis genes in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**:830–834.
8. Atsumi, T., L. McCarter, and Y. Imae. 1992. Polar and lateral flagellar motors of marine *Vibrio* are driven by different ion-motive forces. *Nature (London)* **355**:182–184.
9. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman, and K. Struhl. 1987. Current protocols in molecular biology. Greene Publishing Associates and John Wiley & Sons, Inc., New York.
10. Bahrani, F. K., D. E. Johnson, D. Robbins, and H. L. Mobley. 1991. *Proteus mirabilis* flagella and MR/P fimbriae: isolation, purification, N-terminal analysis, and serum antibody response following experimental urinary tract infection. *Infect. Immun.* **59**:3574–3580.
11. Belas, R. Unpublished data.
12. Belas, R. 1992. The swarming phenomenon of *Proteus mirabilis*. *ASM News* **58**:15–22.
13. Belas, R., D. Erskine, and D. Flaherty. 1991. Transposon mutagenesis in *Proteus mirabilis*. *J. Bacteriol.* **173**:6289–6293.
14. Belas, R., D. Erskine, and D. Flaherty. 1991. *Proteus mirabilis* mutants defective in swarmer cell differentiation and multicellular behavior. *J. Bacteriol.* **173**:6279–6288.
15. Belas, R., and D. Flaherty. 1994. Sequence and genetic analysis of multiple flagellin-encoding genes from *Proteus mirabilis*. *Gene* **148**:33–41.
16. Belas, R., M. Simon, and M. Silverman. 1986. Regulation of lateral flagella gene transcription in *Vibrio parahaemolyticus*. *J. Bacteriol.* **167**:210–218.
17. Birnboim, H. C., and J. Doly. 1979. Rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
18. Brunham, R. C., F. A. Plummer, and R. S. Stephens. 1993. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. *Infect. Immun.* **61**:2273–2276.
19. Chen, L., and J. Helmann. 1994. The *Bacillus subtilis* σ^D -dependent operon encoding the flagellar proteins FliD, FliS, and FliT. *J. Bacteriol.* **176**:3093–3101.
20. De Lorenzo, M., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
21. Donnelly, C., and A. Sonenshein. 1984. Promoter-probe plasmid for *Bacillus subtilis*. *J. Bacteriol.* **157**:965–967.

22. Fairbanks, G., T. L. Steck, and D. F. H. Wallace. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606–2617.
23. Guerry, P., R. A. Alm, M. E. Power, S. M. Logan, and T. J. Trust. 1991. Role of two flagellin genes in *Campylobacter* motility. *J. Bacteriol.* **173**:4757–4764.
24. Guerry, P., S. M. Logan, and T. J. Trust. 1988. Genomic rearrangement associated with antigenic variation in *Campylobacter coli*. *J. Bacteriol.* **170**:316–319.
25. Harrington, M. 1990. Elution of protein from gels. *Methods Enzymol.* **182**:494.
26. Helmann, J. D. 1991. Alternative sigma factors and the regulation of flagellar gene expression. *Mol. Microbiol.* **5**:2875–2882.
27. Henrichsen, J. 1972. Bacterial surface translocation: a survey and a classification. *Bacteriol. Rev.* **36**:478–503.
28. Hoeniger, J. F. M. 1965. Development of flagella by *Proteus mirabilis*. *J. Gen. Microbiol.* **40**:29–42.
29. Homma, M., D. J. DeRosier, and R. M. Macnab. 1990. Flagellar hook and hook-associated proteins of *Salmonella typhimurium* and their relationship to other axial components of the flagellum. *J. Mol. Biol.* **213**:819–832.
30. Homma, M., H. Fujita, S. Yamaguchi, and T. Iino. 1984. Excretion of unassembled flagellin by *Salmonella typhimurium* mutants deficient in hook-associated proteins. *J. Bacteriol.* **159**:1056–1059.
31. Hughes, K., K. Gillen, M. Semon, and J. Karlinsey. 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* **262**:1277–1280.
32. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
33. Macnab, R. M. 1992. Genetics and biogenesis of bacterial flagella. *Annu. Rev. Genet.* **26**:131–158.
34. Mandel, M., and A. Higa. 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol.* **53**:159–162.
35. McCarter, L., M. Hilmen, and M. Silverman. 1988. Flagellar dynamometer controls swarmer cell differentiation of *V. parahaemolyticus*. *Cell* **54**:345–351.
36. McCarter, L., and M. Silverman. 1989. Iron regulation of swarmer cell differentiation of *Vibrio parahaemolyticus*. *J. Bacteriol.* **171**:731–736.
37. McCarter, L., and M. Silverman. 1990. Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus*. *Mol. Microbiol.* **4**:1057–1062.
38. Miller, J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
40. Mirel, D. B., and M. J. Chamberlin. 1989. The *Bacillus subtilis* flagellin gene (*hag*) is transcribed by the σ^{28} form of RNA polymerase. *J. Bacteriol.* **171**:3095–3101.
41. Mobley, H. L. T., and J. W. Warren. 1987. Urease-positive bacteriuria and obstruction of long-term urinary catheters. *J. Clin. Microbiol.* **25**:2216–2217.
42. O'Rear, J., L. Alberti, and R. M. Harshey. 1992. Mutations that impair swarming motility in *Serratia marcescens* 274 include but are not limited to those affecting chemotaxis or flagellar function. *J. Bacteriol.* **174**:6125–6137.
43. Ozols, J. 1990. Amino acid analysis. *Methods Enzymol.* **182**:587–601.
44. Quadling, C., and B. A. D. Stocker. 1957. The occurrence of rare motile bacteria in some non-motile *Salmonella* strains. *J. Gen. Microbiol.* **17**:424–436.
45. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. Sar, N., L. McCarter, M. Simon, and M. Silverman. 1990. Chemotactic control of the two flagellar systems of *Vibrio parahaemolyticus*. *J. Bacteriol.* **172**:334–341.
47. Scott, T. N., and M. I. Simon. 1982. Genetic analysis of the mechanism of the *Salmonella* phase variation site specific recombination system. *Mol. Gen. Genet.* **188**:313–321.
48. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. Silverman, M., and M. Simon. 1973. Genetic analysis of flagellar mutants in *Escherichia coli*. *J. Bacteriol.* **113**:105–113.
50. Silverman, M., and M. Simon. 1974. Assembly of hybrid flagellar filaments. *J. Bacteriol.* **118**:750–752.
51. Silverman, M. R., and M. I. Simon. 1972. Flagellar assembly mutants in *Escherichia coli*. *J. Bacteriol.* **112**:986–993.
52. Szekely, E., and M. Simon. 1983. DNA sequence adjacent to flagellar genes and evolution of flagellar-phase variation. *J. Bacteriol.* **155**:74–81.
53. von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang, and S. N. Cohen. 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. *Proc. Natl. Acad. Sci. USA* **80**:653–657.
54. Zieg, J., M. Silverman, M. Hilmen, and M. Simon. 1977. The metabolism of phase variation, p. 25–35. In G. Wilcox (ed.), *Molecular approaches to eucaryotic genetic systems*. Academic Press, New York.