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Genomic rearrangements in the flagellin genes of *Proteus mirabilis*

Catherine A. Murphy[†] **and Robert Belas**[*]
Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202, USA.

Summary

Molecular analyses have revealed that *Proteus mirabilis* possesses two genes, *flaA* and *flaB,* that are homologous to each other and to flagellin genes of many other species. Both swimmer and swarmer cells transcribe *flaA*, but not *flaB*. FlaA$^-$ mutants are non-motile and do not differentiate showing the essential role of *flaA* in swarmer cell differentiation and behaviour. At a low frequency, motile, differentiation-proficient revertants have been found in FlaA$^-$ populations. These revertants produce an antigenically and biochemically distinct flagellin protein. The revertant flagellin is the result of a genetic fusion between highly homologous regions of *flaA* and *flaB* that places the active *flaA* promoter and the 5′ coding region of *flaA* adjacent to previously silent regions of *flaB* generating a hybrid flagellin protein. Analysis of the *flaA–flaB* region of two such revertants reveals that a portion of this locus has undergone a rearrangement and deletion event that is unique to each revertant. Using a polymerase chain reaction (PCR) to amplify the *flaA–flaB* locus from wild-type swimmer cells, swarmer cells and cells obtained after urinary tract infection, we uncover at least six general classes of rearrangements between *flaA* and *flaB*. Each class of rearrangement occurs within one of nine domains of homology between *flaA* and *flaB*. Rearrangement of *flaA* and *flaB* results in a hybrid flagellin protein of nearly identical size and biochemical properties, suggesting a concerted mechanism may be involved in this process. The data also reveal that the frequency and distribution of *flaAB* rearrangements is predicated on environmental conditions. Thus, rearrangement between *flaA* and *flaB* may be a significant virulence component of *P. mirabilis* in urinary tract infections.

Received 24 July, 1998; revised 5 October, 1998; accepted 9 October, 1998. †Present address: Saint Vincent College, Department of Biology, 300 Fraser Purchase Road, Latrobe, PA 15650, USA. *For correspondence. E-mail belas@umbi.umd.edu; Tel. (410) 234 8876; Fax (410) 234 8896.

Introduction

Proteus mirabilis is an opportunistic pathogen frequently associated with urinary tract infections (UTIs) in hospital patients with complicated urinary tracts because of chronic urinary catheterization (Senior, 1983; Mobley and Warren, 1987). It is a dimorphic bacterium that undergoes dramatic morphological and physiological changes in response to growth on surfaces or in viscous environments (Allison and Hughes, 1991; Belas, 1996a). When *P. mirabilis* is transferred from liquid to solid medium, the cells differentiate by a combination of events that ultimately produce swarmer cells (Belas *et al*., 1991a; Belas, 1996a,b; 1997). The differentiation process leading to the development of a swarmer cell involves a global regulon of 25–50 genes that is co-ordinately expressed when the appropriate environmental signals are sensed (Belas *et al*., 1991a; Allison *et al*., 1993). This ability to differentiate into swarmer cells may aid in colonization of the surfaces of catheters and the urinary tract, because the expression of these genes is thought to be required for invasion of epithelial cells (Allison *et al*., 1992). Thus, the regulation of swarmer cell gene expression is crucial to understanding the pathogenesis of *P. mirabilis*.

The rate of synthesis of certain proteins (e.g. FlaA, the subunit of the flagellar filament) undergoes a remarkable increase during differentiation of the vegetative swimmer cell to the differentiated swarmer cell. Swimmer cells have only a few flagella, whereas the elongated swarmer cells are profusely covered by thousands of newly synthesized flagella (Belas *et al*., 1991a). We previously identified a cloned region of *P. mirabilis* genomic DNA containing three open reading frames (ORFs) that were identified based on their homology to other known flagellar genes (Belas and Flaherty, 1994). The region included two nearly identical, genetically linked copies of flagellin-encoding genes, called *flaA* and *flaB*. A third, unlinked copy of a potential flagellin-encoding gene, referred to as *flaC*, is also present on the chromosome (Belas and Flaherty, 1994). *flaA* has an upstream regulatory sequence with strong homology to the well-characterized σ^{28} promoter specific to flagellar genes (Arnosti and Chamberlin, 1989; Mirel and Chamberlin, 1989; Helmann, 1991). Measurement of the transcription of *flaA* and *flaB* clearly shows that only *flaA* is expressed in wild-type cells, whereas neither *flaB* nor *flaC* are expressed, being silent copies of flagellin-encoding genes (Belas, 1994). FlaA$^-$

mutants, constructed by insertion of a chloramphenicol (*cam*) gene into the *flaA* reading frame, are non-motile and do not differentiate, indicating the importance of this gene and its product (flagellin) in the initial sensing required for swarmer cell differentiation. Similar mutants constructed in *flaB* show no defects in motility or swarmer cell differentiation.

It has been observed that motile (Mot$^+$) revertants arise from *flaA′*::*cam*::*′flaA* mutants. Such Mot$^+$ revertants are able to swim through media to which anti-FlaA polyvalent antisera has been added (Belas, 1994), indicating that the flagella synthesized by the revertants are antigenically distinct from those produced by wild-type cells. In addition, Southern blot analysis of Mot$^+$ revertants revealed a deletion within the *flaA–flaB* locus apparently caused the reversion from Mot$^-$ to Mot$^+$.

In the current study, we sought to gain a better understanding of *P. mirabilis* flagellin antigenic variation and the molecular basis underlying this phenomenon. Through the use of a polymerase chain reaction (PCR) amplification method designed to detect rearrangements at the *flaAB* locus, we have found that these bacteria have the ability to produce many distinct hybrid flagellin proteins that may be antigenically distinct from the wild-type flagellin. These hybrid flagellins appear to be the result of homologous recombination between *flaA* and *flaB* involving a concerted mechanism of deletion. Our results highlight the potential importance of flagellin gene rearrangements both in the biology of *P. mirabilis* and in the development of vaccines for the prevention of UTIs caused by this uropathogenic bacterium.

Results

Revertant flagellin is antigenically distinct from wild-type flagellin because of a deletion and rearrangement at the flaAB *locus*

Previously, we showed that FlaA$^-$ mutants were unstable and frequently reverted to the wild-type motile phenotype (Belas, 1994). Moreover, the *flaA* Mot$^+$ revertants were capable of movement through medium amended with an anti-FlaA polyvalent antiserum, strongly suggesting that these revertants produced flagellin that was antigenically distinct from wild-type flagellin. Biochemical analysis of *flaA* Mot$^+$ revertant flagellin (referred to as FlaAB to distinguish it from wild-type FlaA flagellin) showed that wild-type FlaA and hybrid FlaAB have identical N-terminal ends but different C-termini (Belas, 1994). Southern blot DNA–DNA hybridization was carried out to determine the genetic cause for the difference in flagellin antigenicity between the wild type (BB2000) and the Mot$^+$ revertants, DF1002 and DF1003. The chromosomal DNA from BB2000, DF1001 (a *flaA′*::*cam*::*′flaA* mutant and parent to DF1002 and DF1003) and the Mot$^+$ revertants was isolated and digested with *Hin*dIII. *Hin*dIII provides a unique DNA fragment pattern for the *flaD–flaA–flaB* region and is therefore useful for diagnosing changes that might occur therein. Plasmid pfliDC1C2, harbouring *′flaD flaA flaB,* was radioactively labelled and used as a probe. The Southern hybridization (Fig. 1) shows that the fragments obtained from the two revertants are smaller than the wild type, indicating that a deletion or rearrangement has occurred. To determine where these changes occurred in the DNA of the revertants, oligonucleotide probes to the *flaD′–flaA–flaB* region were used to probe BB2000, DF1002 and DF1003 chromosomal DNA. A summary of the results are found in Fig. 2. For DF1002, the DNA from map position nt 40–2565 differed from that found in BB2000, whereas the region affected in DF1003 was from nt 1498–2565. In both DF1002 and DF1003 there

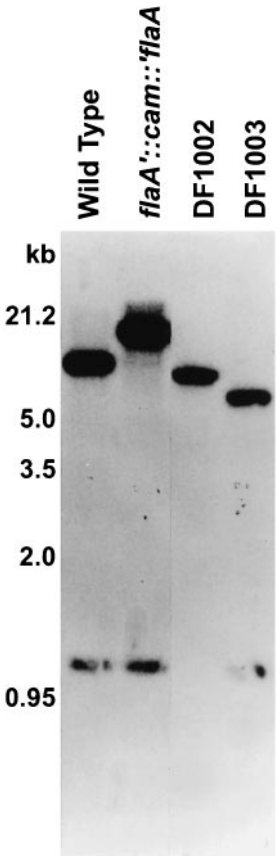

Fig. 1. Southern hybridization of chromosomal DNA isolated from the wild-type, *flaA′*::*cam*::*′flaA* parental and two *flaA* Mot$^+$ revertant strains. Chromosomal DNA was digested with *Hin*dIII and the resulting DNA fragments were separated on a 1% agarose gel before Southern blotting. The probe used to label the *flaA* region was a pfliDC1C2 (Table 1) containing part of *flaD* and the entire *flaA–flaB* region. From the physical map of the region, when cut with *Hin*dIII, a fragment of 1.2 kb and a larger fragment composed of flanking DNA are predicted. Lanes: 1, wild-type BB2000; 2, *flaA′*::*cam*::*′flaA* parent DF1001; 3, *flaA* Mot$^+$ revertant DF1002; 4, *flaA* Mot$^+$ revertant DF1003.

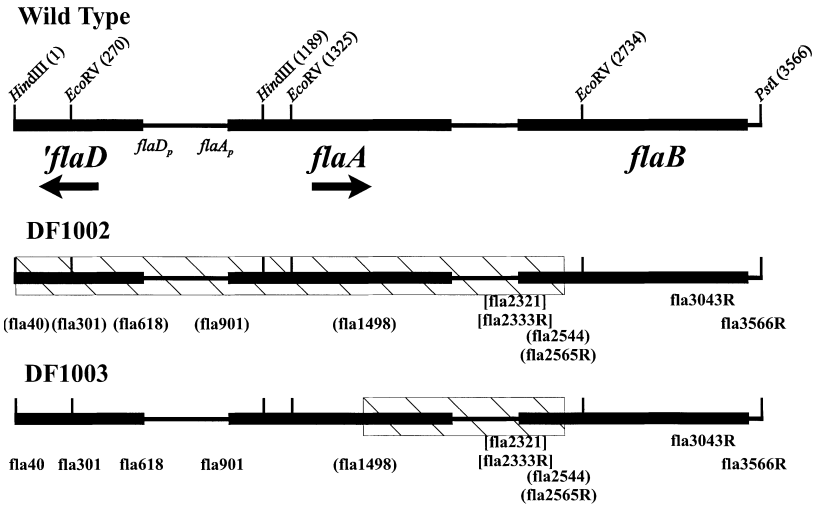

Fig. 2. Summary of the Southern hybridization data. Oligonucleotide probes to regions of the *'flaD–flaA–flaB* region were labelled and used to analyse the nature of the mutation in DF1002 and DF1003. The following notations are used: (i) If a probe hybridized to the same size fragment in both the mutant and wild-type strain it is shown without brackets. (ii) If a probe hybridized to a fragment, but the size of the DNA was not the same as the wild type it is enclosed in parentheses. (iii) If a probe failed to hybridize to the mutant DNA it is enclosed in square brackets. The general area affected in each revertant is shown as a hash-marked box.

were regions on the chromosome to which the probes would bind, but the size of the DNA was not the same as BB2000, and there was one region (nt 2308–2346) where the probes failed to hybridize. These results indicate that the *flaA–flaB* locus of each revertant is lacking a segment of DNA and that the missing DNA is different in each of the two strains examined.

Mot$^+$ revertant flagellin is the product of a hybrid flagellin gene

From the data obtained from Fig. 2, a PCR-based amplification scheme using oligonucleotide primers fla901 and fla3043R (Table 1) was designed whereby changes in the *flaA–flaB* locus could be easily detected. In this assay,

Table 1. Strains, plasmids and oligonucleotides used.

Strain, plasmid or oligonucleotide	Relevant characteristic(s) or sequence	Derivation or description	Source and/or reference
Escherichia coli			
INVαF′	F′ *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 gyrA96 relA1* ϕ*80 dLacZ*Δ*M15* Δ(*lacZY–argF*)*U169 deoR thi-1* λ^-		Invitrogen
Proteus mirabilis			
BB2000	Wild-type, Rf^r	Spontaneous from PRM1	Belas *et al.* (1991b)
DF1001	*flaA′*::*cam*::*'flaA*, $FlaA^-$ Cm^r Rf^r	*cam* gene from pUT/mini-Tn*5*-CM inserted at the *Eco*RV site in BB2000 *flaA* gene	Belas (1994)
DF1002	*flaA*, Mot^+ revertant, Cm^s Rf^r	Spontaneous from DF1001	Belas (1994)
DF1003	*flaA*, Mot^+ revertant, Cm^s Rf^r	Spontaneous from DF1001	Belas (1994)
Plasmids			
pfliDC1C2	'*flaD flaA*$^+$ *flaB*$^+$ Ap^r	pBluescript SK(−) with a 3.6 kb *Hin*dIII–*Pst*I fragment, nt 1–3567	Belas (1994)
pCR2.1	Ap^r Km^r	PCR TA cloning vector	Invitrogen
Oligodeoxyribonucleotides			
fla40	5′-ATCTGTACCTTCTTTACGAGA-3′	*flaAB*-specific DNA probe, nt 40–60	This study
fla301	5′-AGCATGGGCAAGCTCTGTTACAGAG-3′	*flaAB*-specific DNA probe, nt 301–325	This study
fla618	5′-GGTCTCCTTTCTGTGTTCGT-3′	*flaAB*-specific DNA probe, nt 618–638	This study
fla901	5′-AGGTTGTATCTGGGGTGCCGATAAA-3′	*flaAB*-specific DNA probe, nt 901–926	This study
fla1498	5′-ACGATACATTAGGTGTTGCTAGCGAT-3′	*flaAB*-specific DNA probe, nt 1498–1523	This study
fla2321	5′-ATGACGATAGTGTTTAGGCAACGTTA-3′	*flaAB*-specific DNA probe, nt 2321–2346	This study
fla2333R	5′-TACCTTCGATTTAATGACGATAGTGT-3′	*flaAB*-specific DNA probe, nt 2333–2308	This study
fla2544	5′-TTATTACAGGAGTGTAATAACA-3′	*flaAB*-specific DNA probe, nt 2544–2565	This study
fla2565R	5′-TAGCAATAGCTTGACCTGCCG-3′	*flaAB*-specific DNA probe, nt 2565–2545	This study
fla3043R	5′-TGAAATACCAGTCACATATTTTTTACTGC-3′	*flaAB*-specific DNA probe, nt 3043–3015	This study
fla3566R	5′-GTTGTTGCCATTGCTGCA-3′	*flaAB*-specific DNA probe, nt 3566–3549	This study

the unaltered *flaA–flaB* locus produced an amplicon of 2142 bp, whereas deletions or rearrangements within this locus would be predicted to generate a smaller product. For example, the size of the PCR amplicons from both DF1002 and DF1003 were both ≈740 bp. This 740 bp fragment was cloned and the nucleotide and deduced amino acid sequences of each revertant *flaAB* locus determined, comparing them with the wild-type *flaA–flaB* sequence. The nucleotide sequence of DF1002 revealed that DNA from nt 1148–2558 (as designated by Belas and Flaherty, 1994), which includes the 3′ end of *flaA* and the 5′ end of *flaB,* was deleted yielding a hybrid flagellin gene and an antigenically distinct protein, referred to as $FlaAB_{1002}$ (Fig. 3A). In DF1003 a similar deletion process occurred removing DNA from nt 1277–2687 that also produces an antigenically distinct hybrid flagellin, $FlaAB_{1003}$ (Fig. 3B). In both cases the deletion occurred between regions of homology on *flaA* and *flaB*. Interestingly, both deletions remove 1410 bp from the *flaA–flaB* region and resulted in proteins of nearly identical size (39292 Da for $FlaAB_{1002}$ and 39276 Da for $FlaAB_{1003}$). This suggests that a conservative mechanism may be acting to ensure that a functional hybrid flagellin protein results from such rearrangements, perhaps the consequence of the physiological and biochemical constraints that are necessary for export and assembly of a functional flagellin filament.

Analysis of the flaA–flaB *region of wild-type* P. mirabilis *reveals a naturally occurring family of hybrid flagellins*

To determine whether a deletion and/or rearrangement occurs in the *flaA–flaB* region of wild-type *P. mirabilis,* wild-type chromosomal DNA was PCR amplified using the same oligonucleotide primers (fla901 and fla3043R). The published nucleotide sequence (Belas and Flaherty, 1994) predicts that this reaction should produce a single product of 2142 bp, including the entire *flaA–flaB* region. If the population contains both wild-type and hybrid flagellin molecules, however, at least one additional smaller fragment would be expected. As the data show in Fig. 4, two DNA products were generated, a 2142 bp and a 740 bp fragment. These data suggest that wild-type *P. mirabilis* consists of a heterogeneous population containing both wild-type FlaA (2142 bp fragment) and cells that

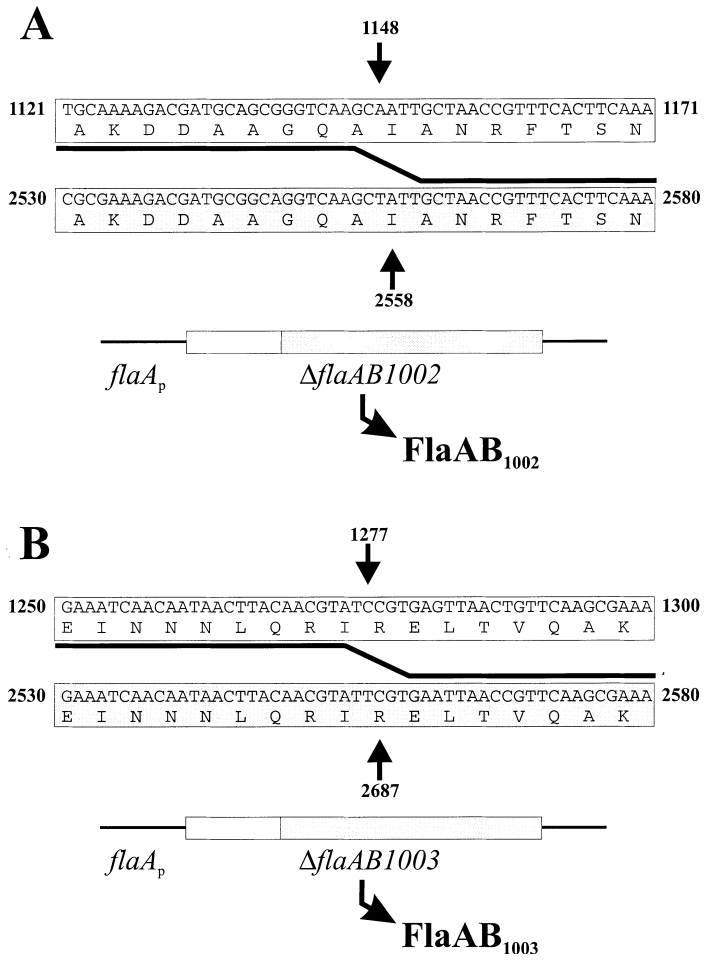

Fig. 3. Sequence analysis of the Mot^+ revertant strains. The nucleotide and deduced amino acid sequences of the wild type, DF1002, and DF1003 were aligned and compared.
A. The nucleotide sequence of DF1002 revealed that DNA from map position nt 1148–2558 was deleted, yielding a hybrid flagellin gene and an antigenically distinct protein, $FlaAB_{1002}$.
B. In DF1003, a similar deletion process occurred removing DNA from nt 1277–2687. This deletion also resulted in an antigenically distinct hybrid flagellin, $FlaAB_{1003}$. Both deletions remove 1410 bp segment of DNA from the *flaA–flaB* region.

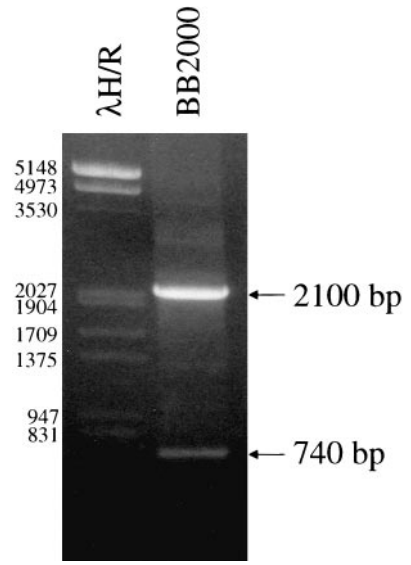

Fig. 4. PCR amplification of the DNA in the *flaA–flaB* locus of wild-type *P. mirabilis* BB2000. Two oligonucleotide primers, fla901 and fla3042R (Table 1), were used to amplify the *flaA–flaB* region using chromosomal DNA isolated from wild-type BB2000 as template. The resulting PCR products were separated by electrophoresis on a 1% agarose gel. Two fragments were observed; 2100 bp and 740 bp. Lanes: 1, lambda phage digested with *Eco*RI and *Hin*dIII (Promega Corp.); 2, BB2000 DNA isolated from swimmer cells.

have undergone a deletion and rearrangement in the *flaA–flaB* region of the chromosome (Δ*flaAB*, 740 bp fragment), producing a new hybrid protein.

RFLP analysis demonstrates heterogeneous ΔflaAB *hybrid genes*

Owing to the homology between the separate domains of *flaA* and *flaB*, we hypothesized that the 740 bp fragment may itself be heterogeneous, being made up of hybrid flagellin genes each with a different site of recombination. To test this, we developed an assay based on restriction fragment length polymorphism (RFLP) inherent to this locus. There are 12 *Rsa*I sites in the *flaA–flaB* region of wild-type *P. mirabilis*. We reasoned that if a deletion and rearrangement had occurred within the *flaA–flaB* region, different RFLP patterns would be expected after digestion with *Rsa*I. Figure 5 shows that four different RFLP patterns were observed when 15 wild-type swimmer cell clones, plus DF1002 and DF1003 were analysed. The *Rsa*I digest of the 740 bp fragment from clones SM1-5, SM3-3, SM4-50, SM5-3 and SM6-1 generated four fragments (420 bp, 180 bp, 100 bp and 50 bp), whereas clones SM1-2, SM2-4, SM3-2, SM5-1, SM5-5, SM6-4 and SM7-5, and the revertants DF1002 and DF1003 gave two fragments (560 bp and 185 bp), and clone SM7-2 resulted in three fragments (420 bp, 180 bp and 150 bp). Only one fragment of ≈750 bp was observed for clone SM3-1, suggesting that there may not be a *Rsa*I restriction site on this fragment. We extended these analyses to include a larger sample and, based on the *Rsa*I RFLP analysis of 45 wild-type swimmer cell clones, found five different groups could be described (Table 2).

The frequency and distribution of flagellin gene rearrangements is affected by external conditions

One possible reason why *P. mirabilis* may produce hybrid flagellin molecules is to escape detection during UTI. We asked whether, as a general rule, the external environment around the cells influenced the frequency and distribution of the types of hybrid flagellins produced. For this we chose three different conditions. The mildest condition was a non-inducing liquid nutrient broth that would encourage the development of swimmer cells. The surface of a nutrient agar medium (Luria agar) was considered to be a harsher environment due to lower humidity, greater oxygen tension, and other effects associated with the drier conditions. Cells in this environment would be induced to form

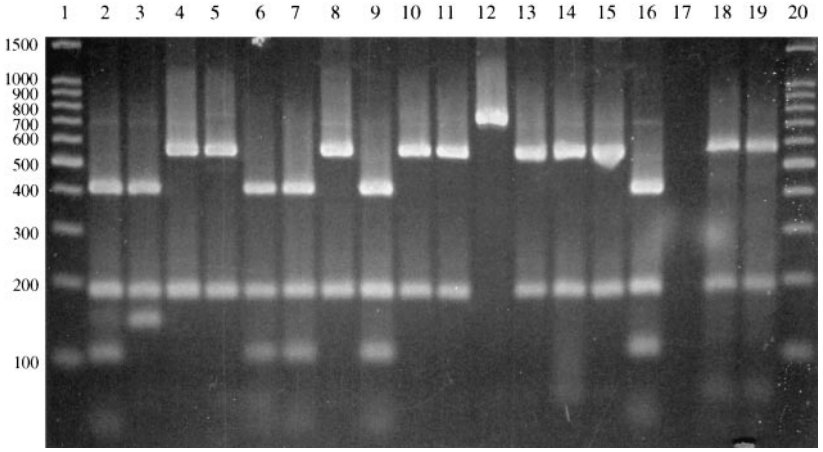

Fig. 5. Restriction fragment length polymorphism (RFLP) analysis of the 740 bp fragment of swimmer cell clones. The 740 bp fragment of the *flaA–flaB* region from BB2000 swimmer cells was amplified using PCR and cloned into pCR2.1. Clones were screened for the presence of the correct-sized fragment using colony PCR, then the PCR product was digested with *Rsa*I. Lanes: 1, 100 bp ladder; 2, SM1-5; 3, SM2-2; 4, SM3-2; 5, SM1-2; 6, SM6-1; 7, SM3-3; 8, SM5-1; 9, SM4-50; 10, SM2-5; 11, SM5-5; 12, SM3-1; 13, SM2-4; 14, SM6-4; 15, SM7-5; 16, SM5-3; 17, empty; 18, DF1002; 19, DF1003; 20, 100 bp ladder.

Table 2. Grouping of swimmer, swarmer and urinary tract clones based on the RFLP (fragment size) patterns generated by an *Rsa*I digest.

Group number	Number of clones	Per cent of total	Fragment sizes (bp)	Sum of fragments
Swimmers				
I	18	40	420, 180, 100, 50	750
II	19	42	560, 185	745
III	3	7	750	750
IV	3	7	560, 420, 180	1160[a]
V	2	4	420, 180, 150	750
VI	0	0		
Swarmers				
I	19	50	420, 180, 100, 50	750
II	14	37	560, 185	745
III	0	0		
IV	1	3	560, 420, 180	1160[a]
V	4	10	420, 180, 150	750
VI	0	0		
Urine				
I	0	0		
II	2	10	560, 185	745
III	15	75	750	750
IV	0	0		
V	2	10	420, 180, 150	750
VI	1	5	420, 180, 150, 100, 50	900[a]

a. The sum of the fragment sizes was 745–750 bp. The apparent disparity seen for group IV and group VI fragments is due to partial digestion products (data not shown).

swarmer cells as has been previously described (Belas, 1997). The third condition, infection in a mouse urinary tract, was considered the harshest, because host defences specifically against the flagellin H-antigen would place strong negative pressure on the bacterial survival.

The 740 bp PCR amplicons from swimmer cells, swarmer cells, and cells isolated post infection from the urine of mice infected with *P. mirabilis* were analysed for *Rsa*I RFLP, as described earlier. The resulting RFLP patterns for 38 swarmer and 20 urinary tract Δ*flaAB* products were determined and compared with the patterns obtained for swimmer cells (Table 2). This comparison showed that the majority of the swimmer and swarmer hybrid flagellin products fell into RFLP group I and group II (82% and 87% respectively), whereas most urinary tract flagellin hybrid products belonged to group III (75%). No group III hybrid flagellin products were produced from swarmer cells, whereas a sixth RFLP pattern (group VI) was only seen in cells obtained from UTI. A goodness-of-fit chi-squared test on these data revealed that the distribution and frequency of the hybrid flagellin groups is biased and correlated with the growth condition (broth medium, solid medium or mouse urinary tract) used, indicating that this was not a random process.

flaAB deletions result in a hybrid flagellin gene

The nucleotide sequence of the 740 bp fragment from PCR products representing each of the six groups was determined to verify the nature of the hybrid molecule. The nucleotide sequence revealed that for each PCR product (groups I–VI) analysed a deletion of 1410 nucleotides had occurred within *flaA–flaB*. This change is the same size as the deletion found in the Mot$^+$ revertant flagellin genes from DF1002 and DF1003. Not surprisingly, the cross-over point where the recombination event occurred was unique to each clone analysed.

Table 3 lists the nucleotide and amino acid junctions where *flaA* recombines with *flaB* to produce a new hybrid flagellin gene in representatives of each of the six RFLP groups. To provide a foundation to understand the data in Table 3, an alignment of *flaA* and *flaB* was carried out in which domains of homology were identified (Fig. 6). The nucleotide homology of the two flagellins is 80%, as previously reported (Belas and Flaherty, 1994). At least nine distinct highly homologous DNA domains were observed when *flaA* and *flaB* were aligned and their sequences compared (Table 4). The greatest divergence of the sequences occurs in the central domain of each protein corresponding to FlaA amino acid positions V193–A265 and

Table 3. Fusion junctions for the *flaA–flaB* hybrid flagellin of wild-type *P. mirabilis*.

Group	Representative clone	Nucleotide junction (*flaA*/*flaB*)	Amino acid junction (FlaA/FlaB)	Size of deletion (nt)
I	SM1-5	nt1512/nt2922	V171/A172	1410
II	SM2-5	nt1305/nt2715	G102/T103	1410
III	SM3-1	nt1122/nt2532	A41/K42	1410
IV	SM2-3	nt1524/nt2934	K175L176	1410
V	SM7-2	nt1347/nt2757	V116/N117	1410
VI	U9-24	nt1554/nt2964	G185/V186	1410

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FlaA      1 MAQVINTNYL   SLVTQNNLNK   SQGTLGSAIE   RLSSGLRINS   AKDDAAGQAI 50
FlaB        **********   ********R   **SA**N***   *****M****   **********

FlaA     51 ANRFTSNVNG   LTQASRNAND   GISIAQTTEG   ALNEINNNLQ   RIRELTVQAK 100
FlaB        *******I**   **********   ***VS*****   **********   **********

FlaA    101 NGTNSNSDIT   SIQNEVKNVL   DEINRISEQT   QFNGVKVLSG   EKSEMVIQVG 150
FlaB        *********N   ******NQR*   *****V****   **********   ***K*T****

FlaA    151 TNDNETIKFN   LDKVDNDTLG   VASDKLFDTK   TEKKGVTAAG   AGVTDAKKIN 200
FlaB        *****V*E**   ***I******   ********A*   **********   DAI.**NALG

FlaA    201 AAATLDMMVS   L.VKEFNLDG   KPVTDKFIVT   KGGKDYVATK   SDFEL..DAT 250
FlaB        ISGSKKYVTG   IS***YKV**   *VSS**VVLN   D*SD**IVS*   ***T*KSGT*

FlaA    251 GTKLGLKASA   TTEFKVDAGK   DVKTLNVKDD   ALATLDKAIN   TIDESRSKLG 300
FlaB        TGEVEFTG*K   **K*TA****   ***V******   ******N**S   KV********

FlaA    301 AIQNRFESTI   NNLNNTVNNL   SASRSRILDA   DYATEVSNMS   RGQILQQAGT 350
FlaB        ******Q***   **********   **********   **********   KN********

FlaA    351 SVLAQANQVP   QTVLSLLR 368
FlaB        A*********   ********
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Fig. 6. Amino acid sequence of FlaA and FlaB. The deduced amino acid sequences of wild-type FlaA and FlaB were aligned and compared. Direct residue-to-residue identity is indicated by an asterisk (*).

FlaB amino acid positions G190–F263. The amino acid fusion junctions for each of the six groups and nine domains of homology in the complete *flaA–flaB* region are represented in Fig. 7. The fusion junctions for groups I, IV and VI occurred in domain 6, whereas the junctions for group II and V were in domain 3, and the group III junction occurred in domain 2.

Interestingly, all FlaAB proteins are produced at splice junctions before the antigenically exposed central domains of the flagellin molecule (Table 3). These hybrid molecules would therefore be expected to possess new exposed antigenic sites on their surfaces. Also of note is that our analysis of the deduced amino acid sequence from representatives of each of the six RFLP groups of hybrid flagellins confirms our previous data emphasizing that the resulting hybrid flagellin is conserved in both molecular size and biochemical properties. For example, the average size of the FlaAB molecules from DF1002, DF1003 and the six RFLP groups is 39246 Da (SEM = 15 Da). All of the FlaAB proteins have the same predicted isoelectric point of pH 5.19, which is the same as that predicted for FlaB. Unlike the FlaB and FlaAB flagellins, the isoelectric point of FlaA is predicted to be slightly more basic at pH 5.39.

The nucleotide sequence analysis also confirmed the fragment sizes generated by the *Rsa*I digest. *Rsa*I sites were found at *flaA* nt 1307, nt 1412, nt 1451, and *flaB* nt 2860. The predicted digestion products based on *Rsa*I restriction sites for group I, group II and group V clones correlated well with what was observed for the actual *Rsa*I digestion. Incomplete digestion by *Rsa*I is responsible for group IV and VI product sums adding up to greater than the predicted 740 bp (Table 2). Also, it appeared that there are no *Rsa*I sites for group III clones based on the *Rsa*I RFLP analysis, even though the nucleotide sequence revealed that there is indeed one *Rsa*I site in this 740 bp fragment. Again, this is probably the result of incomplete cutting by the enzyme.

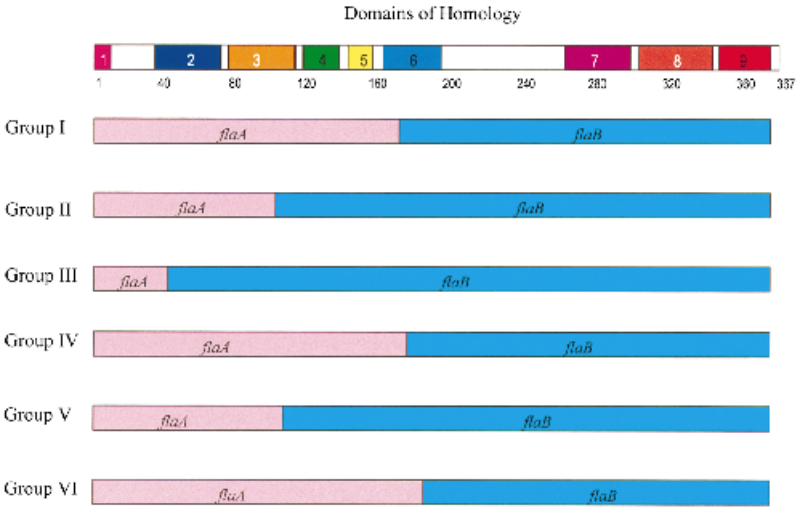

Fig. 7. Fusion junctions for the FlaAB hybrid flagellin species observed. The upper horizontal bar depicts the nine regions of *flaA*::*flaB* nucleotide homology (Table 3) with the corresponding deduced amino acid sequence residue numbers shown below it. The six groups of FlaAB hybrid flagellins identified through PCR–RFLP and sequence analyses are indicated with *flaA* sequences in pink and *flaB* sequences in blue.

Table 4. Regions of homology between *flaA* and *flaB*.

Domain	*flaA*	*flaB*	FlaA	FlaB
1	nt 1002–1033	nt 2411–2442	aa 1–11	aa 1–11
2	nt 1110–1219	nt 2519–2628	aa 37–73	aa 37–73
3	nt 1222–1354	nt 2631–2763	aa 75–118	aa 75–118
4	nt 1358–1411	nt 2767–2820	aa 120–137	aa 120–137
5	nt 1440–1466	nt 2849–2875	aa 147–155	aa 147–155
6	nt 1474–1577	nt 2883–2986	aa 159–192	aa 159–192
7	nt 1780–1858	nt 3195–3273	aa 261–286	aa 263–288
8	nt 1867–2011	nt 3282–3426	aa 290–337	aa 292–339
9	nt 2019–2096	nt 3428–3511	aa 340–365	aa 342–367

Discussion

As flagella and specifically flagellin (H-antigen) are extremely antigenic, changes in flagellin antigenicity may provide the bacteria with an effective means of avoiding the host immune response (Brunham *et al*., 1993). *Proteus mirabilis,* as well as many other bacterial species, possesses multiple flagellin genes whose function may enhance the survival of these pathogens. Studies on the transcriptional regulation of the *flaA* and *flaB* genes of *P. mirabilis* have demonstrated that *flaA* is the sole flagellin transcribed by wild-type cells (Belas, 1994). Biochemical studies of flagella isolated from *P. mirabilis* swimmer and swarmer cells (Bahrani *et al*., 1991; Belas *et al*., 1991a), along with genetic data from Tn*5* mutagenesis (Belas *et al*., 1991a,b), also show that *P. mirabilis* expresses only a single flagellin species, despite the presence of two flagellin-encoding genes. Interestingly, a similar situation appears in species of the unrelated bacterium *Campylobacter,* specifically *C. coli and C. jejuni*.

The flagellin subunit in *C. coli* and *C. jejuni* is encoded by two highly homologous tandem genes, *flaA* and *flaB*. Alm *et al*. (1993) have shown that *C. coli* VC167 mutants expressing only *flaA* form a full-length flagellar filament that confers slightly less than wild-type motility to the bacterium. This is similar to what we have seen in *P. mirabilis flaB* mutants (expressing only *flaA*) that retain wild-type motility. However, whereas *C. coli flaA* mutants are slightly motile, *P. mirabilis flaA* mutants are non-motile, again stressing the sole requirement of the *P. mirabilis flaA* gene product. This difference notwithstanding, in both *C. coli* VC167 (Alm *et al*., 1993) and *C. jejuni* (Harrington *et al*., 1997) there is strong evidence to suggest that recombination between *flaA* and *flaB* results in a chimeric flagellin protein that could potentially increase antigenic diversity. This observation in *Campylobacter* is identical to what is seen in *P. mirabilis* recombination between its *flaA* and *flaB* genes as described in this communication. Moreover, *C. coli* VC167 *flaA* mutants become highly motile over time by production of a full-length 'simple' flagellar filament composed solely of the hybrid FlaAB flagellin (Alm *et al*., 1993). The *C. coli* data may indicate that in addition to generating antigenic variation in the flagellar filament, *P. mirabilis flaA–flaB* recombination may be important for motility under adverse conditions, such as those encountered by the bacteria as they approach urinary mucosal epithelial surfaces.

Our analysis indicates that, although FlaA is the predominant flagellin species in the population, hybrid flagellin molecules are present in a significant portion (≈1–10%) of the cells. From previous studies (Belas and Flaherty, 1994) we had observed that a segment of DNA was apparently deleted from the *flaA–flaB* locus to form the hybrid flagellin. We have extended that analysis in the current research through detailed Southern blot analyses that revealed a deletion of *c.* 1300–1500 bp had occurred in the flagellin genes from two Mot^+ revertants, DF1002 and DF1003 (Figs 1 and 2). The Southern blot data affirmed other data, i.e. through PCR amplification (Fig. 4), RFLP grouping (Fig. 5 and Table 2) and sequence analysis of the Δ*flaAB* locus (Figs 6 and 7), that indicated that a genetic fusion had occurred between the 5′ end of *flaA* and the 3′ end of *flaB* (Figs 1, 2, and 3). The result of this fusion is the production of a new flagellin species that is distinct from FlaA.

In comparing the distribution, frequency and types of FlaAB molecules observed, it is important to emphasize that the six groups of hybrid flagellin classes determined from RFLP analysis are in fact an underestimate. We know this because DF1002 and DF1003 flagellins are in the same RFLP group (group II), yet their amino acid sequences are different because of recombination that occurred at different sites (Fig. 3). Thus, we may speculate that the heterogeneity of hybrid flagellin molecules in the population is far greater than predicted by the RFLP groups. In addition, when the FlaAB species obtained from both undifferentiated swimmer cells and differentiated swarmer cells are compared, subtle differences in the representation of the hybrid species can be seen. As an example, although both swimmer and swarmer cells possess FlaAB species predominantly of group I and II, group I hybrid molecules are more prevalent in the differentiated swarmer cells (50%) than in swimmer cells (40%). Further, group III FlaAB species are completely lacking in the swarmer cell population, although comprising 7% of the population of vegetative cells (Table 2).

More significantly, when cells were collected from the urine of mice 6–7 days after UTI, a unique distribution of hybrid flagellin groups was observed. In this population, group III hybrid flagellin was the most abundant and group I, which is most abundant under laboratory conditions, is absent (Table 2). There are several possible explanations for this observation, but we think that it is most likely that *P. mirabilis* with group I and group II FlaAB proteins may have been recognized by the immune systems of the mice, because *P. mirabilis* infections are common in juvenile

mice (D. Johnson, personal communication). Immune detection and response to group I and II FlaAB antigens would eliminate cells carrying these proteins from the population, skewing the frequency in favour of group III hybrid flagellins. It is also feasible that group III flagellins may confer certain physical or biochemical properties to the flagellar filament that enhances the survival of these cells during mouse UTI, perhaps in a similar manner to the multiple flagellin proteins of *Vibrio cholerae* (Klose and Mekalanos, 1998). We are exploring this possibility by constructing 'locked' mutants that only produce a single FlaAB type and that cannot undergo further rearrangements. These locked flagellin mutants will be compared with the wild type for their ability to invade and colonize our mouse model of ascending urinary tract pathogenesis.

The deduced amino acid sequence of FlaA predicts a protein of 365 amino acids, with a mass of 39 093 Da and an isoelectric point of pH 5.39 (Belas and Flaherty, 1994). If *flaB* were transcribed, FlaB is predicted to be a slightly larger molecule of 367 amino acids, with a mass of 39365 Da and possessing a slightly more acid isoelectric point (pH 5.19). Among the unexpected aspects to arise from our analysis of the FlaAB hybrid flagellin proteins is the observation of the conservation that exists between the hybrid flagellin proteins. All of the eight FlaAB proteins analysed in this report are 367 residues in length, have an isoelectric point of pH 5.19 and range in predicted molecular weight from 39206 to 39292 Da (mean = 39246 $\pm$ 15 Da). These data suggest that routine separation methods would be unlikely to detect the hybrid flagellin molecules because of their similar physical characteristics. The uniformity in flagellin protein size also points to the possibility that the mechanism producing a hybrid flagellin is conservative, such that only FlaAB proteins that possess the correct size and three-dimensional, folded conformation are permitted. These data indicate that selective forces predicated on producing a functioning hybrid flagellin protein also influence the distribution and frequency of the FlaAB population.

Although there is a marked conservation in hybrid flagellin molecules, two differences were noted between FlaA and the FlaAB flagellins. First, Chou–Fasman (Chou and Fasman, 1978) predictions of FlaA and FlaAB secondary protein folding patterns indicates that the two proteins have different three-dimensional conformations. The hybrid proteins more closely resembling FlaB, which is to be expected because FlaAB is composed of significant segments of FlaB.

The other difference between FlaA and the hybrid flagellins lies in the antigenicity of each protein. Examination of the FlaAB flagellins indicates that the fusion of FlaA and FlaB results in the greatest diversity around residues 125–175. This domain is midway in the protein and is thought to be exposed to the environment, whereas the N- and C-terminal domains are hidden as the flagellum is assembled from flagellin monomers (Macnab, 1992). Such diversity in this region is reasonable and predicted because revertants with hybrid flagella fail to be tethered by anti-FlaA antisera (Belas, 1994).

What is the molecular mechanism by which rearrangements occur between *flaA* and *flaB* domains? The homology between the domains in each gene suggests that RecA-dependent homologous recombination (Clark, 1973) may be involved. We are currently in the process of constructing a RecA defective strain of *P. mirabilis* and will use this mutant strain to determine the role of RecA in producing antigenically distinct hybrid flagellin proteins. It is also possible that additional proteins functioning in concert with RecA and through a site-specific mechanism may be required, perhaps to confer specificity to the *flaA–flaB* locus.

The data support two distinctly different models to explain the reported observations. Both models argue that antigenic variation is produced by homologous recombination between like domains in *flaA* and *flaB*. This homologous recombination results in the looping out and deletion of DNA. The fate of this deleted DNA is what separates the two models.

In the first model, the deleted segment of *flaA–flaB* is lost forever. This model predicts that the process of flagellar antigenic variation is a terminal event that, although resulting in a cell that expressed a new FlaAB species, is ultimately destined to not undergo further rearrangements. For such a model to be useful to the population requires an assumption that the recombination between *flaA* and *flaB* is a very infrequent event within the population, occurring in only a small fraction of the entire population. We do not have a precise way at present to predict this frequency, but analysis of the stoichiometry between the 2000 bp product and the 740 bp product produced from PCR amplification suggests that Δ*flaAB* products comprise at least 1–10% of the total population. Thus, rearrangements that produce FlaAB appear to be common.

The second model predicts that the DNA deleted from the *flaAB* locus is not lost but retained elsewhere on the chromosome, presumably at a silent locus. This type of silent locus cassette model for antigenic variation has been observed in *Neisseria* and *Borrelia* spp. (Deitsch *et al*., 1997; Finlay and Falkow, 1997). Three lines of evidence suggest that this model may best explain flagellar antigenic variation in *P. mirabilis*. First, we have reported on the existence of *flaC*, a genetic locus with homology to the *flaAB* locus, which is genetically unlinked to *flaAB* (Belas and Flaherty, 1994). This could be the site of the silent locus. Second, analysis of motile revertants from DF1001 (*flaA′*::*cam*::*′flaA*) has demonstrated two classes of Mot$^+$ revertants, one class that is chloramphenicol sensitive, e.g. DF1002 and DF1003, and a second class that

retains resistance to the antibiotic (Belas, 1994). We are currently examining these chloramphenicol-resistant Mot$^+$ revertants to determine the nucleotide sequence flanking the *cam* gene. If the second model is right, this sequence will correspond to *flaC*. Finally, the first model implies that cells locked in expressing only one hybrid flagellin should be relatively common in the population. It also suggests that, after time, populations might become composed entirely of cells expressing only a single FlaAB species. We have never been able to find such a cell that is locked in expressing only one flagellar antigen.

The data presented in this report also bring to light issues regarding the efforts of others to immunize high-risk patients against *P. mirabilis* UTI. Obviously, any approach towards immunization will have to take into consideration that flagellar antigenic variation may present a substantial obstacle towards successful immunization.

Experimental procedures

Bacterial cultures and transformation

The strains, plasmids and oligonucleotides used in this study are listed in Table 1. *P. mirabilis* BB2000 is wild-type for swimming and swarming behaviours. *E. coli and P. mirabilis* were cultured at 37°C in either LB broth or on LB agar (Sambrook *et al.*, 1989). 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, 1000 ml of distilled H_2O) was used to phenotypically inhibit swarming (Belas *et al.*, 1991a). *Escherichia coli* INVαF′ was used as the recipient for all plasmid transformations as described by the manufacturer (Invitrogen). Ampicillin (50 μg ml^{-1}) and rifampin (100 μg ml^{-1}) were used for antibiotic selection.

DNA purification and analysis

Chromosomal DNA was isolated from BB2000 by using a modification of the CTAB procedure (Ausubel *et al.*, 1987). Plasmid DNA was purified using the Qiagen Plasmid Mini Kit as described by the manufacturer. Restriction enzymes were used as specified by the enzyme suppliers. Oligonucleotide primers were obtained commercially (Genosys).

Southern blot analysis of flaA *Mot$^+$ revertants*

Chromosomal DNA from *P. mirabilis* BB2000, DF1001, DF1002, and DF1003 was digested with *Hin*dIII, and the resulting DNA fragments were separated on a 1.0% agarose gel in 1× Tris–acetate–EDTA buffer (Sambrook *et al.*, 1989). After ethidium bromide staining and photography, the DNA fragments were transferred to a Nytran membrane (Schleicher and Schuell) as recommended by the manufacturer. Plasmid pfliDC1C2 (Table 1) was used as a probe and labelled by nick translation (Sambrook *et al.*, 1989) using [α-^{32}P]-dCTP (New England Nuclear). Stringent conditions were maintained throughout hybridization and washing. After thorough washing of the membrane to remove unbound probe DNA, the membrane was autoradiographed, and the resulting film was examined.

Oligonucleotide probes to regions of the *flaD′–flaA–flaB* locus (Table 1) were fluorescently labelled using the Vistra System (Amersham) and used to analyse the nature of the mutation in DF1002 and DF1003. Chromosomal DNA was digested with *Eco*RV, *Hin*dIII and *Dra*I, and the resulting fragments were separated on a 1% agarose gel in 1× TAE buffer. After ethidium bromide staining, the DNA fragments were transferred to a Nytran membrane as described above.

Primary PCR amplification of chromosomal DNA

Chromosomal DNA isolated from BB2000 was amplified with primers fla901 and 3043R by combining 0.5 μM each primer, 0.8 ng of template (chromosomal DNA) μl^{-1}, 200 μM each dNTP (dATP, dCTP, dGTP and dTTP; Promega), 1× *Taq* Buffer (Perkin-Elmer), 4.5 mM $MgCl_2$, 0.05 U μl^{-1} *Taq* polymerase (Perkin-Elmer) and deionized water to bring the volume to 50 μl. The thermocycler protocol was 30 amplification cycles of 1 min at 94°C (denaturation), 1.5 min at 65°C (annealing), 2.0 min at 72°C (extension), followed by 5 min at 72°C. To ensure uniform distribution and sampling of template DNA, seven separate reactions were performed using the BB2000 chromosomal DNA as template. The products of PCR amplification were separated and analysed by agarose gel electrophoresis as described above.

Analysis of flaAB *rearrangements in swimmer cells*

As a clean-up procedure, a second PCR amplification was carried out using the gel-purified 740 bp fragments generated by the primary PCR amplifications as the template DNA. A small plug of agarose containing the 740 bp fragment was cut from the agarose gel using a sterile Pasteur pipette and placed in the PCR reaction mixture (reaction mixture and thermocycler protocol as described above). To confirm that the second PCR amplification generated one band of $\approx$740 bp, the amplicons from this PCR reaction were analysed by agarose gel electrophoresis. One microlitre of the PCR sample was used in a ligation reaction containing plasmid pCR2.1 (TA cloning kit; Invitrogen), then transformed into *E. coli* INVαF′. The transformants were plated on LB agar containing ampicillin and X-Gal (40 μg ml^{-1}) and incubated overnight at 37°C. White transformants, i.e. those harbouring pCR2.1 with a 740 bp insert, were restreaked to fresh LB agar with ampicillin and X-Gal and, after overnight incubation, analysed for the presence of the 740 bp fragment using colony PCR. The colony PCR amplification reaction contained: 200 μM each dNTP, 1× *Taq* Buffer, 4.5 mM $MgCl_2$, 0.05 U μl^{-1} *Taq* polymerase (Perkin-Elmer), 0.5 μM primer fla901, 0.5 μM primer 3043R, and deioinzed water to bring the volume to 50 μl. After the reaction mixture was set up, a toothpick was used to pick white colonies, and then the tip was swirled in the reaction mix for a few seconds. The thermocycler protocol was as described above, and the resulting reaction products were analysed by agarose gel electrophoresis. After the screen of the amplicons to ensure that a 740 bp band was present, positive reaction mixtures were digested with *Rsa*I and the fragments analysed on a

2% Trevigel (Trevigen, Gaithersburg, MD, USA) in 1× TAE, and RFLP patterns were analysed and groupings established.

Analysis of flaAB *rearrangements in swarmer cells*

BB2000 was incubated in LB broth at 37°C for 16 h, and 5 μl of this overnight culture was spotted onto the centre of LB agar and incubated at 37°C for 6 h. After 6 h, a toothpick was used to remove cells from the outer edge of the periphery of the swarming colony (≈3 cm from point of inoculation). The toothpick was swirled in the PCR reaction mixture, and amplification was carried out as described previously.

Analysis of flaAB *rearrangements during UTI*

The CBJ/JHSD mouse model for ascending urinary tract infection was used according to the method of Johnson *et al.* (1987) with an initial infection inoculum of 2.4×10^6 *P. mirabilis* BB2000 per mouse (6 mice total). Urine was collected from five mice after 6 and 7 days of infection and centrifuged at 12 000 r.p.m. for 2–3 min The supernatant was removed and 100 μl of digestion buffer (50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween-20) containing 200 μg proteinase K ml^{-1} was added to the pellet. The samples were incubated for 1 h at 55°C, then at 95°C for 8–10 min to inactivate the proteinase K (Zhao *et al.*, 1997). For further purification, samples were run through a Qiagen Mini prep column, washed, eluted and dried according to the manufacturer's protocol. The pellet was resuspended in 20 μl of water, then 10 μl was used in a PCR amplification reaction which contained: 200 μM dNTP, 1× *Taq* buffer; 4.5 mM $MgCl_2$, 0.05 U μl^{-1} *Taq* polymerase (Perkin-Elmer), 0.5 μM primer fla901, 0.5 μM primer 3043R and deioinzed water to bring the volume to 50 μl. Five separate PCR amplification reactions were carried out using cells obtained from the urine of five different mice and the PCR products processed as described above.

Sequence analysis of flagellar rearrangements

Nucleotide sequencing of double-stranded plasmid DNA was performed using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Reactions were run from the Prism Ready Reaction Dye Deoxy Termination Kit (Applied Biosystems) in conjunction with AmpliTaq-FS DNA polymerase. T7 forward primer and M13 reverse primer were used, and reactions were run on a model 373 A DNA sequencer with stretch upgrades (Applied Biosystems). Assembly of the intact nucleotide sequence, genetic reading frames, deduced amino acid sequence, repeated nucleotide sequences, DNA–DNA and amino acid homologies were conducted using the Genetics Computing Group suite of software (Devereux *et al.*, 1984).

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