

# Transcription of *Proteus mirabilis* *flaAB*

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*Proteus mirabilis*, a Gram-negative urinary tract pathogen, has two highly homologous, tandemly arranged flagellin-encoding genes, *flaA* and *flaB*. *flaA* is transcribed from a  $\sigma^{28}$  promoter, while *flaB* is a silent allele. Previous studies have demonstrated the presence of a family of hybrid flagellin genes, referred to as *flaAB*. These genes are composed of the 5' end of *flaA* and the 3' end of *flaB*, and are produced through excision of the intervening DNA between the two genes. Although the existence of *flaAB* DNA has been documented, it was not known if transcription of *flaAB* occurs in wild-type *P. mirabilis*. In this study, proof of *flaAB* transcription was obtained from a combination of RNA dot-blots and RT-PCR assays using specific primers and probes for *flaAB* and *flaA*. The RNA data were further supported by the demonstration of phenotypic switching of the locus using a FlaAB-detector strain. The results show that *flaAB* mRNA is transcribed and is 1/64 as abundant as *flaA* in the population of wild-type cells, suggesting that *flaAB* constitutes 1.0–1.5 % of the total flagellin message. Nucleotide sequence analysis of *flaAB* products produced by RT-PCR from the wild-type confirms previous reports of a variable fusion site between *flaA* and *flaB* resulting in a hybrid flagellin transcript. These data support the hypothesis that the production of FlaAB is integral to the physiology of *P. mirabilis*.

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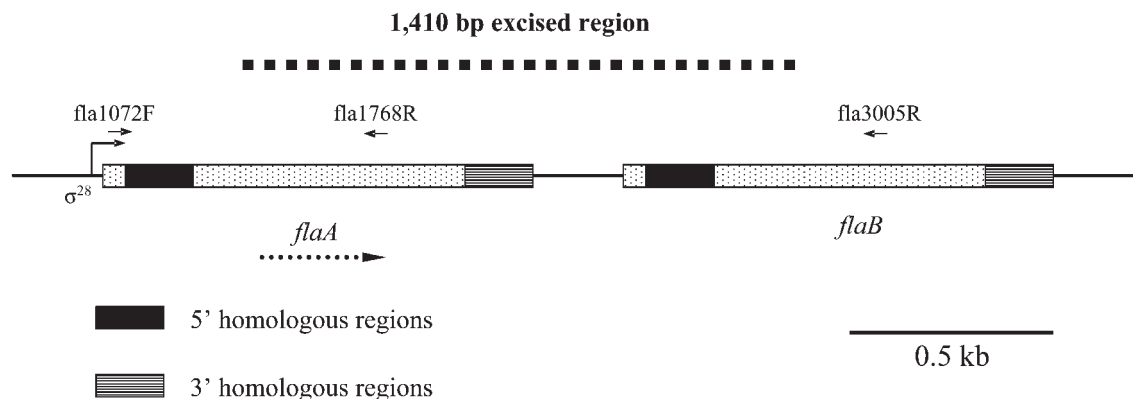
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## INTRODUCTION

*Proteus mirabilis* is an opportunistic pathogen of the human urinary tract (Coker *et al.*, 2000; Mobley & Belas, 1995). Infections of this bacterium persist despite the host response, which in part may be due to the ability of cells to differentiate from short swimmer forms into elongated, multinucleated and hyperflagellated swarmer cells (Allison & Hughes, 1991; Belas, 1996; Williams & Schwarzhoff, 1978). This differentiation takes place in response to growth on surfaces, with environmental cues such as flagellar tethering being implicated in its initiation (Gygi *et al.*, 1995). At the genetic level, differentiation involves the coordinate expression of a global regulon of 25–50 genes (Belas *et al.*, 1991a). The transformation of the swimmer cell with several peritrichous flagella, into the swarmer cell covered in thousands of flagella, is mirrored in an increase in expression of flagellin, the protein subunit of the flagellar filament (Belas *et al.*, 1991a). Hyper-expression of flagellin is a hallmark of the swarmer cell and requires a substantial expenditure in energy and carbon. Flagella are also highly immunogenic, further adding to the cost of producing these structures when the bacteria come under attack by host immune defences.

Our laboratory previously characterized (Belas & Flaherty, 1994) two tandemly arranged and highly homologous flagellin-encoding genes of *P. mirabilis*, *flaA* and *flaB* (Fig. 1). While *flaA* and *flaB* have an overall DNA sequence identity of about 80 %, domains adjacent to the 5' and 3'

ends contain regions of nearly 100 % identity, suggesting that they may be sites of homologous recombination between the two genes. Measurements of transcription and primer extension analysis have identified a  $\sigma^{28}$  promoter (Arnosti & Chamberlin, 1989; Helmann, 1991) upstream of *flaA* from which the gene is expressed (Belas, 1994). These studies also showed that *flaB* is not transcribed, i.e. it is a silent allele. In concert with the transcriptional data, FlaA<sup>−</sup> mutants (*flaA'*::cam::*flaA*) are non-motile, while disruption of *flaB* (*flaB'*::cam::*flaB*) has no effect on motility (Belas, 1994). One of the interesting findings from these studies is that *flaA'*::cam::*flaA* mutations are unstable and occasionally revert to a motile phenotype that displays wild-type swimming and swarming behaviour. The motile revertants possess flagella that are antigenically distinct from wild-type flagella and fail to bind anti-FlaA antisera. N-terminal amino acid sequencing of the flagellin obtained from these motile revertants confirmed the occurrence of changes in the amino acid sequence. These revertants are genotypically different from the parent and contain an in-frame fusion of the 5' end of *flaA* ( $\sigma^{28}$  promoter region plus coding region of *flaA*) and the 3' coding region of *flaB* (Belas, 1994). Murphy & Belas (1999) characterized several different revertants and found that the formation of the resulting 'hybrid' *flaAB* gene was the result of a conservative loss of a 1410 bp segment of intervening DNA containing the 3' end of the *flaA* coding region through to a site in the 5' end of *flaB*. Interestingly, the placement of the ends of the 1410 bp segment varied from revertant to revertant, yielding multiple *flaAB* variants



**Fig. 1.** The *flaA*–*flaB* locus and oligonucleotide primer sites. The *flaA* gene is transcribed from a  $\sigma^{28}$  promoter, indicated by the 90° angled arrow immediately 5' to the coding region of the gene. The non-transcribed *flaB* allele is downstream of *flaA*, in the same orientation as *flaA*, and it is separated from *flaA* by a short intervening segment. The production of *flaAB* results from the deletion or excision of 1410 bp of DNA between the 3' region of *flaA* and the 5' region of *flaB* (indicated by the dashed line above the locus). The oligonucleotide primer pairs fla1072F plus fla1768R and fla1072F plus fla3005R were used to detect and transcribe *flaA* and *flaAB* mRNA, respectively. The domains in the two genes having >95% identity are indicated by either black (5' end of each gene) or striped boxes (3' end).

and a potential suite of FlaAB proteins. Hybrid flagellin DNAs were also found in wild-type cells obtained from both laboratory cultures and experimental mouse urinary tract infections. An analysis of many such hybrid flagellin genes also confirmed the presence of numerous *flaAB* gene variants.

Thus, the data to date support the formation of *flaAB* at the DNA level, but do not shed light on whether the hybrid flagellin genes are transcribed in the wild-type. We show in this report that *flaAB* is transcribed in wild-type *P. mirabilis* and accounts for about 1.0–1.5% of the total flagellin message in the population.

## METHODS

**Strains and growth conditions.** *P. mirabilis* BB2000 is a wild-type strain containing the flagellin-encoding genes *flaA* and *flaB*, and is the parent of DF1003, which is a spontaneously derived *flaAB*-locked mutant (Belas, 1994). *P. mirabilis* was grown at 37 °C in Luria broth (LB; Sambrook *et al.*, 1989). To obtain isolated colonies, *P. mirabilis* was grown on LSW<sup>−</sup> agar (Belas, 1994).

**DNA manipulation.** Genomic DNA was extracted and PCR amplified using standard methods (Ausubel *et al.*, 1987) and previously described conditions (Belas, 1994; Murphy & Belas, 1999). Agarose gel electrophoresis was used to separate DNA by standard methods (Ausubel *et al.*, 1987). Gels were stained with SYBR Gold (Molecular Probes) or ethidium bromide (Sigma-Aldrich) and the DNA fluorescence was visualized using a Fluor Imager model 575 and the ImageQuant (version 4.1) analysis software (Molecular Dynamics). PCR products were inserted into the TA vector pCR2.1 (Invitrogen), which was then transformed into competent *Escherichia coli* DH5 $\alpha$  using established procedures (Ausubel *et al.*, 1987). Sequence editing and detection of ORFs was carried out using the computer programs Chromas (version 1.42) and DNAMAN (Lynnon BioSoft).

**RNA dot-blots.** Using the hot phenol technique (von Gabain *et al.*,

1983), total RNA was extracted from 50 ml LB cultures grown at 37 °C for 4 h, according to the method of Belas (1994). The extracts were checked for purity and concentration by formaldehyde gel electrophoresis (Ausubel *et al.*, 1987; Belas, 1994) and spectrophotometric measurements at 260 and 280 nm (Ausubel *et al.*, 1987). Samples of total RNA or chromosomal DNA were diluted tenfold and applied to consecutive slots in a dot-blot manifold (model SRC-96, Schleicher and Schuell). Labelled probes were added to the hybridization solution at a specific activity of 0.1  $\mu\text{Ci ml}^{-1}$  ( $3.7 \times 10^9$  Bq  $\text{ml}^{-1}$ ) of solution, and hybridization was carried out at 42 °C, followed by washes by established methods. Oligonucleotides were end-labelled with 6000 Ci [ $\gamma$ - $^{32}\text{P}$ ]dATP  $\text{mmol}^{-1}$  ( $2.2 \times 10^{14}$  Bq  $\text{mmol}^{-1}$ ; Amersham Pharmacia Biotech) using the KinAce-It kinasing kit (Stratagene) according to the manufacturers' instructions. Phospho-imaging analysis (Storm 840; Molecular Dynamics) was used to detect hybridization and the dot intensity was determined by the mean pixel intensity in equal-sized squares (ImageQuant version 1.2; Molecular Dynamics).

**RT-PCR.** RNA for RT-PCR was extracted from 2 ml overnight LB cultures that were subsequently diluted 1:100 and grown for 4 h to mid-exponential phase. Extraction was carried out using the RiboPure kit for bacterial RNA extraction (Ambion), with the following modifications. After initial chloroform extraction, the partially purified RNA was incubated with 10 U RNase-free DNase I (Roche Diagnostics) for 45 min at 37 °C to digest genomic DNA, before completion of final purification. The RNA was checked for purity and concentration as described above. Reverse transcription was carried out with 500 ng purified RNA in a 20  $\mu\text{l}$  reaction using the GeneAmp Gold RNA PCR kit, according to the manufacturer's instructions (Applied Biosystems). *flaAB* was detected using 0.4  $\mu\text{M}$  of the *flaAB*-specific primer fla3005R (5'-CCAGAGCGTTTGC-ATCGAT-3'), while *flaA* was detected with *flaA*-specific primer fla1768R (5'-GATGCTTTTAATCCAAGTTTAGTTTAGTACCT-3'). A 3  $\mu\text{l}$  aliquot of the reverse transcriptase mix was used as template in a 50  $\mu\text{l}$  PCR amplification reaction according to the manufacturer's instructions (Applied Biosystems), with 0.15  $\mu\text{M}$  each of the 5' primer fla1072F (5'-CTTAGGAAGTGCAATCGAGC-3') and the respective 3' primer. PCR cycling conditions were as follows: 10 min at 95 °C (for 'hot start' enzyme AmpliTaq Gold DNA polymerase;

Applied Biosystems), 34 cycles of 94 °C for 1 min, 62 °C for 2 min (a combined annealing/extension step), 1 cycle of 72 °C for 10 min. Gel analysis of products was carried out on a 5 µl sample diluted 1:4 in deionized H<sub>2</sub>O.

To confirm that the RNA preparations were free of contaminating DNA, control PCR amplifications with RNA samples plus oligonucleotide primers *fla1072F* and *fla3005R* were performed. Amplifications that produced a unique 1933 bp fragment, in addition to the expected 523 bp fragment, indicated the presence of genomic DNA and were not used.

**Phenotypic switching frequency of FlaAB.** The phenotypic switching frequency and relative abundance of FlaAB-producing cells in the population were determined using a *lacZ* transcriptional fusion strain that produces  $\beta$ -galactosidase when FlaAB is expressed. This strain, JM3006, was constructed as follows. A plasmid, pflaDAB (Belas, 1994), carrying the intact *flaA*–*flaB* genes was modified using site-directed mutagenesis (QuikChange, Stratagene) to introduce a *Bam*HI site between the *flaB* stop codon and the putative *flaB* terminator. A promoterless *lacZ/kan* cassette (Barcak *et al.*, 1991) was then inserted at the *Bam*HI site to produce the '*flaB*::*lacZ*' plasmid, pJM104. pJM104 was digested with *Eco*RV and the '*flaB*::*lacZ*' fragment was cloned into the suicide vector pGP704 (Miller & Mekalanos, 1988) in *E. coli* SM10  $\lambda$ pir and conjugally transferred to *P. mirabilis* wild-type (Belas *et al.*, 1991b). Colonies containing the mutated *flaB*::*lacZ* locus were identified by the acquired kanamycin resistance and verified by *Eco*RI restriction digests and sequence analysis of the mutated (*flaB*::*lacZ*) genomic locus. Swimming and swarming motility of six such kanamycin-resistant clones was measured as previously described (Belas, 1994). Stability of the mutant was determined by measuring kanamycin resistance after overnight growth of the cells in LB lacking the antibiotic, as well as by PCR analysis of the *flaA*–*flaB* locus. The frequency of FlaAB expression was measured by incubating JM3006 in LB plus kanamycin for 4 h at 37 °C and spreading a diluted sample of the cells on LSW<sup>−</sup> agar containing 40 µg X-Gal ml<sup>−1</sup>. Following overnight incubation at 37 °C, the numbers of Lac<sup>+</sup> (FlaAB; blue) and Lac<sup>−</sup> (FlaA; white) colonies were counted ( $n=1000$ ) as follows. Briefly, a digital image of the colonies was obtained and analysed using Adobe Photoshop, by selecting a blue-colour threshold value of R(ed) 115, G(green) 222 and B(lue) 230, which is a pale-blue colour. All colonies having this tint or a darker blue were identified using the Photoshop Replace Colour tool, with the settings of hue 180, saturation 100, lightness −28 and fuzziness 100. The FlaAB colonies in the digital image were thereby assigned a red colour, distinguishing them from FlaA colonies and allowing for quick manual enumeration. An estimate was thus generated of the percentage of the population expressing FlaAB.

## RESULTS

### *flaAB* mRNA is present in approximately 1.5% of wild-type *P. mirabilis*

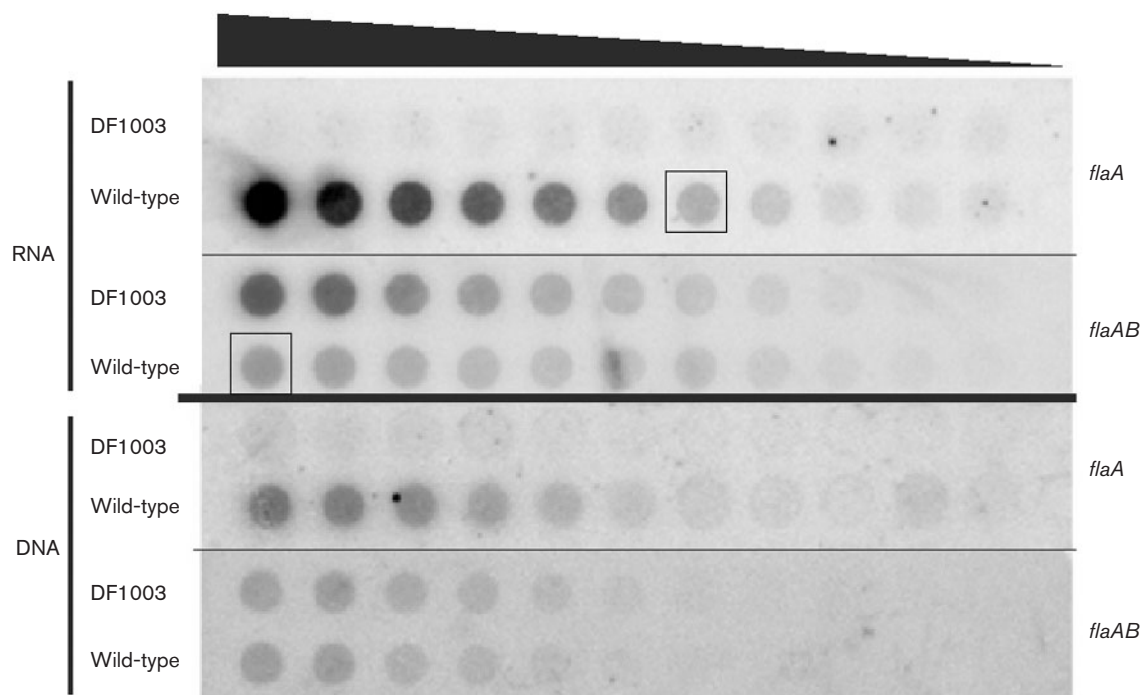
Oligonucleotides were used as gene-specific probes to detect *flaA* (oligonucleotide *fla1768R*) and *flaAB* (*fla3005R*) mRNA (Fig. 1) on RNA dot-blots of total RNA extracted from wild-type cells (Fig. 2). A spontaneous motile revertant strain, DF1003 (FlaAB<sup>+</sup>), locked in expressing *flaAB* only, served as a positive control for *flaAB* expression. RNA-free genomic DNA from the wild-type strain was used as a positive control for the *flaA* gene, while RNA-free DNA from the FlaAB-locked strain served as the negative control for *flaA* and as the positive control for *flaAB*.

As shown in Fig. 2, the *flaA* probe failed to hybridize to DF1003 RNA (Fig. 2, row 1), while the same oligonucleotide strongly hybridized to wild-type RNA (Fig. 2, row 2). This is expected, since DF1003 does not express *flaA*. RNA from DF1003 (Fig. 2, row 3) and from the wild-type (Fig. 2, row 4) hybridized to the *flaAB*-specific probe, indicating the presence of *flaAB* mRNA in both the wild-type and FlaAB-locked (positive control) strain.

An estimate of the ratio of *flaAB* mRNA to *flaA* transcript in the population of wild-type cells was obtained using densitometry analysis of the RNA dot-blot data presented in Fig. 2. The density ( $51 \pm 3$  pixels mm<sup>−2</sup>) of the 1:1 dilution of wild-type RNA hybridized to the *flaAB*-specific probe (Fig. 2, row 4, column 1) was nearly equal to the density, ( $49 \pm 5$  pixels mm<sup>−2</sup>) of the 1:64 dilution of wild-type RNA hybridized to the *flaA* oligonucleotide (Fig. 2, row 2, column 7). This means that there was about 64 times more *flaA* mRNA than *flaAB* mRNA present in the wild-type population, or put another way, *flaAB* mRNA constituted about 1.5% of the total flagellin mRNA present in the population. Since the same  $\sigma^{28}$  promoter driving *flaA* transcription was presumably used to transcribe the hybrid *flaAB* gene, the difference in concentration between *flaA* and *flaAB* mRNA was due mainly to differences in transcript abundance and not differential promoter efficiency.

### Analysis of *flaAB* mRNA

Confirmatory evidence of *flaAB* transcription in wild-type cells was also obtained by RT-PCR. A 500 ng quantity of DNaseI-treated total RNA extracted from the wild-type and the *flaAB*-locked strain (DF1003) was reverse-transcribed and the cDNA product was used as a PCR template. These results are shown in Fig. 3(a). Under the conditions of the assay, the RT-PCR reaction of wild-type RNA plus the *flaA*-specific primer *fla1768R* (Fig. 3a, left lane) produced the predicted *flaA* product (0.7 kbp), while RNA extracted from the *flaAB*-locked strain produced an RT-PCR product of 0.50 kbp (Fig. 3a, middle lane) when primed with the *flaAB*-specific primer. Significantly, RT-PCR of wild-type RNA plus the *flaAB*-specific oligonucleotide (Fig. 3a, right lane) resulted in a product that was an identical size (0.50 kbp) to that produced by the *flaAB*-locked RNA, strongly implying that it too is produced from *flaAB* mRNA. As can be seen in Fig. 3(a), much less *flaAB* product was obtained from wild-type cells compared to *flaA* product from the wild-type, or compared to *flaAB* product from the phase-locked strain. Although the percentage of *flaAB* to *flaA* in wild-type cells was not determined by RT-PCR, visual examination of the bands in Fig. 3(a) suggests that the difference in amount parallels the RNA dot-blot frequency of about 1.5%. The presence of contaminating DNA in all RNA samples was assessed by PCR amplification using *fla1072F* with *fla3005R*, *fla2333R* (5'-ACACTATCGTCATTAAATCGAAGGTA-3') or *fla1768R*. None of these reactions produced a positive result (data not shown), indicating that the RNA samples



**Fig. 2.** Detection and relative abundance of *flaA* and *flaAB* mRNA in the wild-type and *flaAB*-locked strain (DF1003). RNA dot-blot hybridizations are shown in the upper four rows, while DNA dot-blot hybridizations are displayed in the lower four rows. The dot-blot were hybridized to either *flaA*- or *flaAB*-specific oligonucleotide probes, as indicated on the right-hand side of the figure. The decreasing triangle over the dot-blot represents a twofold dilution of either RNA, for RNA dot-blot, or DNA, for the DNA dot-blot. Thus, the left-most spot is the initial concentration of 10 µg, followed by (left to right): 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64, 1 : 128, 1 : 256, 1 : 512 and 1 : 1024 dilutions of the initial sample. The probes fla1768R and fla3005R were specifically chosen (Fig. 1) to measure either *flaAB* or *flaA* mRNA, respectively, in the RNA dot-blot. They also serve as controls to detect concomitant flagellin DNA in the DNA dot-blot. RNA extracted from DF1003 serves as a positive control for *flaAB* mRNA in the RNA dot-blot (rows 1 and 3), and is also used as a control to confirm the absence of the excised '*flaA*-*flaB*' 1410 bp region in the DNA dot-blot of DF1003 genomic DNA (rows 5 and 7). Densitometry analysis was used to find concentrations of *flaA* and *flaAB* in the wild-type that produce equivalent pixel density. The results suggest that the 1 : 64 dilution of RNA hybridized with *flaA* probe (row 2, column 7) is equal in intensity to the 1x (initial) concentration of RNA when hybridized to the *flaAB*-specific oligonucleotide (row 4, column 1). These two dots are highlighted by squares.

were free of contaminating DNA. Therefore, the simplest interpretation of the RT-PCR data is that *flaAB* is formed and transcribed in wild-type *P. mirabilis* populations.

### Sequence analysis of *flaAB* transcripts

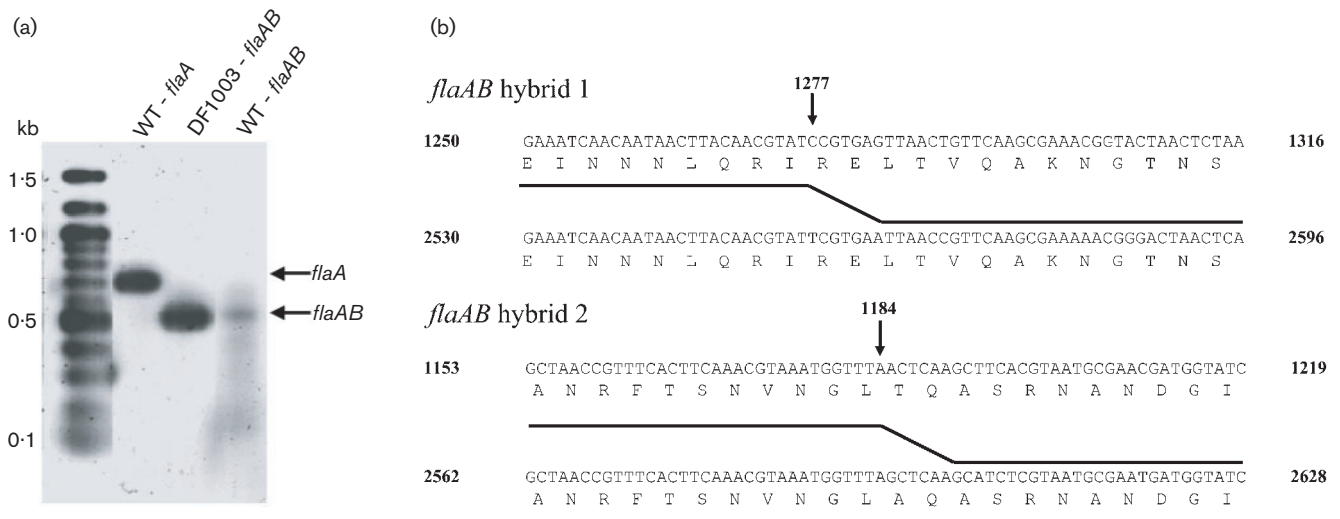
We determined the nucleotide sequence of two *flaAB* cDNA PCR products produced in separate RT-PCR amplifications of wild-type RNA with the *flaAB*-specific oligonucleotide. The results, shown in Fig. 3(b), identify these cDNA products as 523 bp *flaAB* molecules with junction sites upstream of *flaA*<sub>1277</sub> and *flaA*<sub>1184</sub> respectively, where the nucleotide position refers to the numbering originally used by Belas & Flaherty (1994). By coincidence, the junction in the first *flaAB* product is within the same region as the one identified in DF1003, while the second is at a previously unrecognized site. The sequences displayed 100 % identity to segments of *P. mirabilis* *flaA* and *flaB* in the respective regions upstream and downstream

of the junction forming the hybrid gene, with the exception of a single base substitution at *flaA*<sub>164</sub> (C to T) in the first product, though that did not alter the deduced amino acid sequence. Analysis of the nucleotide sequence of the two positive controls confirmed that they were wild-type *flaA* and DF1003 *flaAB* respectively (data not shown).

### Phenotypic analysis suggests FlaAB is expressed in approximately 1 % of the population

To corroborate the mRNA data, the percentage of bacterial colonies expressing FlaAB was measured through the use of a strain (JM3006) harbouring a stable *flaB*::*lacZ* transcriptional fusion. This strain was constructed so as to allow  $\beta$ -galactosidase to be produced only if *flaAB* was transcribed and translated. Indeed, JM3006 possesses wild-type swimming and swarming motility, suggesting that the *flaB*::*lacZ* fusion does not impair flagellar synthesis





**Fig. 3.** RT-PCR detection and sequence of *flaAB* mRNA in wild-type cells. (a) Products generated from RT-PCR amplification of RNA from either wild-type or *flaAB*-locked strains amplified with *flaA*- or *flaAB*-specific oligonucleotide primers. The first lane contains 100 bp size standards; the second lane contains the results from an amplification of wild-type RNA with the *flaA*-specific primer; the third lane is an RT-PCR of *flaAB*-locked RNA with the *flaAB*-specific primer; and the fourth lane product results when wild-type RNA is reverse transcribed and amplified with the *flaAB*-specific primer. RT-PCR reactions using the respective specific primer plus the forward primer *fla1072F* would generate either a 696 bp *flaA* product or a 523 bp *flaAB* DNA (resulting from the excision of 1410 bp from the intact *flaA*–*flaB* chromosomal locus). The primer pair *fla1072F* and *fla3005R* was also used to test for the presence of contaminating genomic DNA, and would produce a 1.93 kbp product if DNA was present in RNA samples (none was detected). As a positive control, *P. mirabilis* chromosomal DNA and this primer set amplified the 1.93 kbp product (not shown). (b) Sequence analysis of two of the *flaAB* products generated by separate RT-PCR reactions of wild-type RNA with *flaAB*-specific primers. The junction in the *flaAB* hybrid 1 occurs at nucleotide 1277, while the junction for *flaAB* hybrid 2 is located approximately 90 bases upstream of hybrid 1, at nucleotide 1184.

or function (data not shown). Fig. 4 shows a photograph of colonies of this FlaAB-expression-detection strain growing on XGal-containing agar. In this assay, blue colonies express varying levels of FlaAB, while white colonies express FlaA. Using Adobe Photoshop, a cut-off threshold of ‘blue’ was chosen to distinguish FlaAB<sup>+</sup> from FlaA<sup>+</sup> colonies, and an analysis of 1000 colonies from six separate experimental replicates was performed. The frequency of FlaAB expression was estimated to be approximately 1 %, which agrees with the estimated value (1/64 or 1.5 %) obtained by RNA dot-blot quantification.

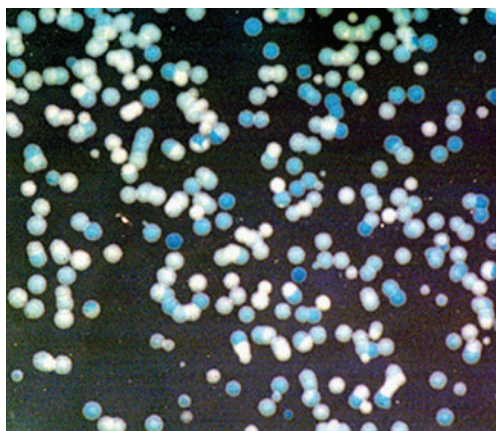
The stability of the FlaAB phenotype was measured by restreaking FlaAB<sup>+</sup> (Lac<sup>+</sup>) colonies and FlaA<sup>+</sup> (Lac<sup>−</sup>) colonies on LSW<sup>−</sup> agar plus X-Gal, and observing the resultant Lac phenotype. Each progenitor colony gave rise to the FlaAB phenotype at the same frequency as originally observed (approx. 1.5 %), suggesting that the frequency of FlaAB expression within the population was not affected by the progenitor colony phenotype.

## DISCUSSION

The data presented in this report support the hypothesis that *flaAB* is transcribed and expressed in wild-type

*P. mirabilis* populations. In assessing these results, we took advantage of both a spontaneously generated *flaAB*-locked strain, DF1003, previously shown to be stable and to express *flaAB* to produce hybrid flagellin as a positive control (Belas, 1994), and a *flaB*::*lacZ* FlaAB-detector strain (JM3006). Although they are estimates, both the densitometric analysis of the RNA dot-blot and the colony phenotypic analysis suggest that *flaAB* accounts for about 1.0–1.5 % of the flagellin message in these populations. The efficacy of this RNA as the *flaAB* message was established by nucleotide sequence analysis of the resulting RT-PCR products, which also confirmed previous reports that the sites of fusion between *flaA* and *flaB* to produce the hybrid are variable. This results in the production of a suite of *flaAB* DNAs and mRNAs within the population (Murphy & Belas, 1999). Furthermore, the frequency of FlaAB expression in the population is independent of the flagellin phenotype of the progenitor cells, and is the same in vegetative swimmer cells and differentiated swarmer cells (data not shown).

In interpreting these results, it is important to be mindful of the limitations associated with each method used. Indeed, it is due to these limitations that multiple means were sought to measure *flaAB* transcription and expression. One



**Fig. 4.** Phenotypic analysis of FlaAB-expressing colonies. The Lac phenotype of strain JM3006 (*flaB::lacZ*) is shown on LSW<sup>-</sup> agar medium containing the chromogenic substrate X-Gal. FlaAB-expressing colonies are Lac<sup>+</sup> (blue), while those colonies expressing FlaA are Lac<sup>-</sup> (white). A colony was scored as expressing FlaAB if it produced a blue tint of  $\geq$  R(ed) 115, G(reen) 222 and B(lue) 230, as measured by Adobe Photoshop, while all other colonies, including weak Lac<sup>+</sup>, i.e. very light blue colonies and Lac<sup>-</sup> colonies (white), were thus scored as expressing FlaA. The weak Lac<sup>+</sup> and segregated colonies are most likely to contain a mixture of FlaAB- and FlaA-expressing cells.

factor that could alter the interpretation of the results is the potential for selective amplification of templates during PCR. This could lead to a distortion in the amount of product generated. In control PCR amplifications using the *flaA*- and *flaAB*-specific oligonucleotides, with purified *flaA* or *flaAB* DNA alone, or in varying ratios mixed together, we found no evidence for selective amplification of either template DNA over a 10 000-fold range in concentration (data not shown). This suggests that selective amplification is not a complicating factor in the interpretation of the results. Similarly, the difference in wild-type *flaAB* mRNA abundance observed in RT-PCR analysis compared to the RNA dot-blot method may have been affected by the different RNA extraction methods used for each procedure. The estimate of FlaAB frequency in the phenotypic analysis may also have been affected by the 'blue' cut-off threshold value used to distinguish Lac<sup>+</sup> from Lac<sup>-</sup> colonies (see Methods). This conservative threshold may have resulted in an underestimate of the frequency of FlaAB-expressing cells.

The abundance of *flaAB* mRNA estimated from these data is relatively high when compared to the abundance of alternative flagellins produced by other known flagellar switches (Gillen & Hughes, 1991; Harris *et al.*, 1987). The possibility exists that selective degradation of either *flaA* or *flaAB* mRNA could affect the outcome of these experiments and skew the abundance estimate. In an earlier study (Belas, 1994), we found that *P. mirabilis* flagellin mRNA was

quite stable *in vitro*, suggesting that *flaA* mRNA, which would presumably represent the majority of flagellin transcript in the earlier work, is not readily degraded. The stability of *flaAB* mRNA is not known, and it remains a possibility that this factor could influence interpretation of the current estimate of *flaAB* abundance.

Another potential complication in estimating the abundance of *flaAB* mRNA may arise if the oligonucleotide primers used to detect *flaA* and *flaAB* respectively had different affinities for their respective targets. Indeed, the *flaAB* primer does show about 1.5- to 2-fold lower affinity for its target than does *flaA* for its cognate site, and this difference may lead to an overestimation of the abundance of *flaAB* by an equivalent factor. Given this, the estimated abundance of *flaAB* is best used as a general measurement and not a strict value. However, it is important to be mindful that none of these limitations directly affects the conclusion that *flaAB* is transcribed.

While the molecular mechanism responsible for generating *P. mirabilis* *flaAB* transcripts is not known, the existence and relative abundance of *flaAB* mRNA suggest that the mechanism used to produce it is not likely to be similar to the well-studied Hin recombinase switch responsible for *Salmonella enterica* serovar Typhimurium flagellar antigenic switching (Gillen & Hughes, 1991). Nonetheless, it is reasonable to speculate that the production of FlaAB flagellin must benefit *P. mirabilis* in some manner to warrant the large expenditure of energy and carbon required to produce the hybrid flagella.

What role might *flaAB* play? One possible role for *flaAB* could be as a generator of flagellar (H) antigen variation, as was originally suggested by us (Belas, 1994). FlaAB flagellar filaments are likely to have different amino acid residues exposed to the external environment, as compared to FlaA flagellin. Thus, the transcription of *flaAB* and expression of FlaAB protein during urinary tract infections may be an effective means used by the cells to evade the host immune defences.

Alternatively, transcription and expression of *flaAB* may serve another function: production of a flagellar filament whose morphology yields a more efficient propeller for swimming and swarming motility in 'extreme' conditions. This scenario is entirely plausible, since FlaAB flagellin is different from FlaA flagellin (Murphy & Belas, 1999), and even small changes in the amino acid residues composing the filament could lead to significant alterations in the quaternary structure of the resulting flagellar filament. We have recently analysed the swimming and swarming motility of the wild-type and DF1003 (FlaAB-locked) strains by cell motion analysis, and observed significant differences in the motility of DF1003 (compared to wild-type) at extremes of pH, salinity and viscosity, suggesting that in such conditions expression of FlaAB flagella offers the bacterium an advantage (Manos *et al.*, 2004). The persistence of *P. mirabilis* in human urinary tract infection may be due in

part to the expression of FlaAB flagella. High salinity, alkaline pH and increased viscosity are environmental features of the urinary tract, while acidic pH favours urinary tract infection (Raz & Stamm, 1993). The ability to survive in or rapidly move through such environments may offer the *P. mirabilis flaAB* phenotype an advantage during colonization and pathogenesis, and enhance its prospects for survival and increased virulence.

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## REFERENCES

- Allison, C. & Hughes, C. (1991). Bacterial swarming: an example of prokaryotic differentiation and multicellular behaviour. *Sci Prog* **75**, 403–422.
- Arnosti, D. N. & Chamberlin, M. J. (1989). Secondary sigma factor controls transcription of flagellar and chemotaxis genes in *Escherichia coli*. *Proc Natl Acad Sci U S A* **86**, 830–834.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates & Wiley.
- Barcak, G. J., Chandler, M. S., Redfield, R. J. & Tomb, J. F. (1991). Genetic systems in *Haemophilus influenzae*. *Methods Enzymol* **204**, 321–342.
- Belas, R. (1994). Expression of multiple flagellin-encoding genes of *Proteus mirabilis*. *J Bacteriol* **176**, 7169–7181.
- Belas, R. (1996). Sensing, response and adaptation to surfaces: swarmer cell differentiation and behavior. In *Molecular and Ecological Diversity of Bacterial Adhesion*, pp. 281–331. Edited by M. Fletcher. New York: Wiley-Liss.
- Belas, R. & Flaherty, D. (1994). Sequence and genetic analysis of multiple flagellin-encoding genes from *Proteus mirabilis*. *Gene* **148**, 33–41.
- Belas, R., Erskine, D. & Flaherty, D. (1991a). *Proteus mirabilis* mutants defective in swarmer cell differentiation and multicellular behavior. *J Bacteriol* **173**, 6279–6288.
- Belas, R., Erskine, D. & Flaherty, D. (1991b). Transposon mutagenesis in *Proteus mirabilis*. *J Bacteriol* **173**, 6289–6293.
- Coker, C., Poore, C. A., Li, X. & Mobley, H. L. (2000). Pathogenesis of *Proteus mirabilis* urinary tract infection. *Microbes Infect* **2**, 1497–1505.
- Gillen, K. L. & Hughes, K. T. (1991). Negative regulatory loci coupling flagellin synthesis to flagellar assembly in *Salmonella typhimurium*. *J Bacteriol* **173**, 2301–2310.
- Gygi, D., Rahman, M. M., Lai, H. C., Carlson, R., Guard-Petter, J. & Hughes, C. (1995). A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis*. *Mol Microbiol* **17**, 1167–1175.
- Harris, L. A., Logan, S. M., Guerry, P. & Trust, T. J. (1987). Antigenic variation of *Campylobacter* flagella. *J Bacteriol* **169**, 5066–5071.
- Helmann, J. D. (1991). Alternative sigma factors and the regulation of flagellar gene expression. *Mol Microbiol* **5**, 2875–2882.
- Manos, J., Artimovich, E. & Belas, R. (2004). Enhanced motility of a *Proteus mirabilis* strain expressing hybrid FlaAB flagella. *Microbiology* **150**, 1291–1299.
- Miller, V. L. & Mekalanos, J. J. (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.
- Mobley, H. L. & Belas, R. (1995). Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. *Trends Microbiol* **3**, 280–284.
- Murphy, C. A. & Belas, R. (1999). Genomic rearrangements in the flagellin genes of *Proteus mirabilis*. *Mol Microbiol* **31**, 679–690.
- Raz, R. & Stamm, W. E. (1993). A controlled trial of intravaginal estriol in postmenopausal women with recurrent urinary tract infections. *N Engl J Med* **329**, 753–756.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- von Gabain, A., Belasco, J. G., Schottel, J. L., Chang, A. C. Y. & Cohen, S. N. (1983). Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. *Proc Natl Acad Sci U S A* **80**, 653–657.
- Williams, F. D. & Schwarzhoff, R. H. (1978). Nature of the swarming phenomenon in *Proteus*. *Annu Rev Microbiol* **32**, 101–122.