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Enhanced motility of a *Proteus mirabilis* strain expressing hybrid FlaAB flagella

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Proteus mirabilis has two tandemly arranged flagellin-encoding genes, *flaA* and *flaB*. *flaA* is transcribed from a σ^{28} promoter, while *flaB* is silent. *flaA* and *flaB* can undergo reversible rearrangement to produce a set of hybrid genes referred to as *flaAB*. Flagellins composed of FlaAB protein have a different amino acid sequence and are antigenically distinct from flagellin composed of FlaA, implicating flagellin gene conversion as a putative virulence mechanism for *P. mirabilis*. The change in amino acid sequence is also hypothesized to alter the filament helix and, hence, affect the motility of FlaAB-expressing strains. To test this hypothesis, the motility of wild-type *P. mirabilis* was compared with that of a strain, DF1003, locked into the FlaAB$^+$ hybrid phase, under conditions of altered ionic strength, pH and viscosity. Cell motion tracking analysis showed that DF1003 has wild-type swimming velocity at physiological conditions, but moves significantly faster and travels further compared to the wild-type at NaCl concentrations greater than 170 mM. DF1003 is also significantly faster than the wild-type at pH 5·2, 5·8 and 8·2, and at 5 and 10 % polyvinylpyrrolidone. Measurements of amplitude and wavelength for isolated flagella subjected to pH 5·8 or 425 mM NaCl showed a loss of helical structure in FlaA flagella compared to FlaAB filaments, a feature that could significantly affect motility under these conditions. These results support a hypothesis that FlaAB flagellin imparts a motile advantage to *P. mirabilis* in conditions that otherwise may impede bacterial movement. In a broader context, flagellar antigenic variation, commonly thought to serve as means to avoid host defences, may also enhance motility in other bacterial species, thus aiding in the adaptation and survival of the cells.

INTRODUCTION

Bacteria are propelled by rotating flagellar filaments having a length of up to 15 μm and a diameter of 120–250 Å. In *E. coli*, the flagellar filament is helical in shape and made up of tens of thousands of subunits of the protein flagellin, with approximately 11 subunits per two turns of the helix (Burge & Draper, 1971; O'Brien & Bennett, 1972). Flagellin is a three-domain protein, with the highly conserved N- and C-terminal domains being responsible for the quaternary interactions between subunits and a central domain performing no obvious structural role, but projecting outwards and likely to contain surface-exposed antigenic epitopes (Fischer & Nachamkin, 1991).

Proteus mirabilis swimmer cells generally have six to ten peritrichous flagella. Studies by Belas and others have shown that the flagellin locus contains two flagellin-encoding genes, *flaA* and *flaB*, which have domains of high homology at both the nucleotide and amino acid level (Belas & Flaherty, 1994; Murphy & Belas, 1999). *flaA* possesses an upstream regulatory sequence with consensus homology to the flagellar class III σ^{28} promoters (Helmann, 1991). Biochemical analysis of isolated flagella indicates that they are composed of a sole flagellin species (Belas, 1994). Indeed, transcriptional *lacZ* fusions of *flaA* and *flaB* suggest that, while *flaA* expression is co-ordinately upregulated with swarmer cell differentiation, *flaB* remains silent and is not expressed in either swimmer or swarmer cells (Belas, 1994). This finding is corroborated by Northern blot analysis, with *flaA* yielding a single 1·2 kb mRNA, while several *flaB*-specific primers failed to yield a product. Furthermore, mutations constructed in *flaB* by allelic exchange were wild-type, while *flaA* mutants were defective in motility (Belas, 1994).

In earlier studies, *flaA* mutants were found to be unstable and occasionally produced flares of motile cells on semisolid motility (Mot) agar (Belas, 1994). Twelve *flaA* Mot$^+$ revertants (DF1002 to DF1013) were analysed and shown to behave like wild-type cells with respect to swarming and swimming, and other phenotypes (Belas & Flaherty, 1994). Southern blot analysis of DF1002 and DF1003 revealed that each revertant was missing a section of the *flaA-flaB* region, though the missing region was different in each case. PCR amplification using primers specific to either *flaA*

Abbreviation: PVP, polyvinylpyrrolidone.

or *flaB* revealed a 740 bp amplicon, as opposed to a 2142 bp amplicon for the full *flaA-flaB* locus. Sequencing of the cloned 740 bp amplicon showed a hybrid flagellin gene composed of the 5′ end of *flaA* and the 3′ end of *flaB*. While the junction creating the hybrid *flaAB* gene was different for both strains, the size of the deleted region was constant at 1410 bp and the resulting *flaAB* gene, and its deduced amino acid product, were nearly identical in size to *flaA* and FlaA, respectively (Murphy & Belas, 1999).

While originally hypothesized to involve evasion of host defences, the production of hybrid FlaAB filaments may also result in a change in the helical structure of the flagellar filament. Recent analysis of a *Salmonella enterica* serovar Typhimurium flagellin fragment known as F41 has demonstrated that the N and C termini form the densely packed core of the filament and are necessary for the axial intersubunit interactions between flagellin monomers in the protofilament (Samatey *et al.*, 2001). The shape of the filament depends upon the arrangement of the flagellin monomers, which depends in turn upon the amino acid sequence of the protein, temperature, pH, ionic strength and torsional load, i.e. an increased load on the filament causing changes in shape (Kamiya & Asakura, 1974, 1976a, b; Macnab, 1987). In *S. typhimurium*, the flagellar filament undergoes changes in helical pitch, amplitude and handedness in response to pH changes, switching from a long-pitch, left-handed corkscrew to a shorter-pitch, right-handed one (Macnab & Ornston, 1977). Changes in shape in turn affect motility, and hence a change in environment may in fact benefit a strain possessing helically different flagella.

In this study we examined the motility of DF1003 ($FlaAB^+$) and the wild-type ($FlaA^+$), and compared the swimming speed of each with the morphology of their respective flagella.

METHODS

Strains, media and growth conditions. *P. mirabilis* BB2000 is wild-type for swimming and swarming behaviours. *P. mirabilis* DF1003 is a spontaneous Mot^+ revertant isolated from a Mot^- (*flaA*::cam::*flaA*) parent and subsequently identified by sequence analysis to express only FlaAB hybrid flagellin (Belas, 1994). *P. mirabilis* strains were cultured from isolated colonies on LSW^- agar (Belas, 1994) and grown initially in 2 ml L-broth (10 g tryptone, 10 g NaCl, 5 g yeast extract in 1000 ml dH_2O) and 2 ml Tryptone-broth (T-broth) (10 g tryptone, 10 g NaCl in 1000 ml dH_2O) overnight at 37 °C in a shaking water bath. Overnight cultures were pelleted at 5000 ***g*** for 5 min and washed in 1× PBS (8 g NaCl, 0·2 g KCl, 1·44 g Na_2HPO_4, 0·24 g KH_2PO_4 in 1000 ml dH_2O, pH 7·2) diluted 1 : 100 into L-broth or T-broth and grown as above to mid-exponential phase (OD_{600} ~ 0·5). All subsequent measurements were conducted on T-broth cultures amended with either NaCl or polyvinylpyrrolidone (PVP), or buffered to the required pH using either phosphate or acetate buffers as described below.

Growth curves of wild-type and DF1003 strains were carried out as follows. T-broth (2 ml) was inoculated in triplicate from isolated colonies on L-agar and incubated overnight with shaking at 37 °C. Cultures were diluted 1 : 100 in 10 ml T-broth in 125 ml conical flasks and initial OD_{600} readings were taken to equalize inoculum densities. Cultures were incubated as above and OD_{600} readings, beginning at 2 h, were recorded until stationary phase was detected.

Measurement of swimming velocity – preparation of modified media

NaCl. T-broth (170 mM NaCl) was adjusted to a final NaCl concentration of 255, 340, 425 or 510 mM NaCl by addition of 5 M NaCl. Cultures were adjusted to 85 mM NaCl by addition of an equal volume of T-broth containing no NaCl.

pH. Aliquots of T-broth were adjusted to the required pH using 0·2 M stock sodium acetate solutions for pH 4·6 and 5·2, and 0·2 M stock potassium phosphate for pH 5·8–7·6 (Sigma-Aldrich). Tris buffer (1 M, pH 8·5) was used to buffer T-broth to pH 8·2 (final concentration 25 mM).

Viscosity. PVP MW 360 000 (Sigma-Aldrich) was dissolved to 50 % (w/v) in dH_2O and dialysed overnight in 25 mm diameter dialysis tubes (The Spectrum Companies) against several changes of dH_2O. The total volume of dialysed PVP was measured and adjusted to a final concentration of 20 % (w/v) with dH_2O. The PVP was autoclave-sterilized (20 min, 121 °C) and aliquots were mixed with appropriate volumes of either 2× or 4× T-broth to give the final concentrations of PVP (15, 10 and 5 %, w/v).

Microscopy and motility measurement. For each motility measurement, 2 ml overnight cultures of FlaA- and FlaAB-expressing cells were diluted 1 : 100 in fresh T-broth in 125 ml conical flasks and incubated for about 3 h until the OD_{600} was 0·5 (mid-exponential phase) for each strain. The cell suspensions were then diluted in the respective medium, and 10 µl of the diluted cell suspension was removed, placed onto a microscope slide and covered with a grease-gasketed coverslip. The cells were immediately viewed with phase-contrast at ×400 using an Olympus BX60 upright microscope. After choosing a field of view containing about 25–50 actively motile cells, video images were acquired using a Canon Elura (40MC) digital camera attached to the microscope. Five minutes of cell motion from two separate fields of view was acquired per strain for each condition measured. Following review of the entire video period, a 1 min segment starting within the first 20 s was selected for detailed motion analysis using Adobe Premiere, VirtualDub (version 4.1; Avery Lee 1998–2001) and Adobe Photoshop. The result was a series of still images taken every 0·33 s depicting the location of each cell in the field of view. This series of digital images was subsequently used to locate and measure the position of randomly chosen cells by recording their pixel coordinates then converting to a micrometre scale. Individual cells ($n=20$ for NaCl, $n=10$ for others) were tracked across nine consecutive series for a total of 2·66 s and the coordinates recorded. Statistical reproducibility of the measurements was ensured by repeating each experiment a minimum of five times over subsequent days.

Data analysis. The coordinates of each tracked cell were recorded onto Microsoft Excel spreadsheets and equations for total distance, mean distance, mean velocity, mean angle and tumble frequency were used to provide final measurements of these parameters for each cell. Representative cell tracks from each series were checked for accuracy by plotting the calculated coordinates and comparing them to the video track of the individual cell. Students' *t*-test (pairwise, independent, at $P<0·05$) was used to compare values obtained from FlaA- and FlaAB-expressing cells.

Measurement of filament length and the number of filaments per cell. Samples of the standardized dilutions of cells for cell motion analysis were taken and stained with AlexaFluor 594 (Molecular Probes) following the method described by Turner *et al.*

(2000). Fluorescently stained filaments were visualized with an Olympus BX60 upright microscope at an excitation of 480 nm and emission of 535 nm. Representative images were acquired using a Quantix model 1400 CCD camera (Photometrics) and analysed using the image-processing program IPlab 4.1a (Scanalytics) and Adobe Photoshop 7.0.

Measurement of flagellar helical parameters and preparation of isolated flagella. Flagella were prepared using the method described by Belas (1994). Briefly, *P. mirabilis* strains were incubated on L-agar to produce swarmer cells. Flagella were sheared from cells and harvested by centrifugation, then purified through a sucrose step gradient. The protein concentration of each fraction was determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemicals) and an aliquot from each was run on a 4–10 % gradient SDS-PAGE gel and stained with Coomassie blue (Sigma) to determine purity and the relative amounts of flagellin in each fraction. Fractions were stored at 4 °C and those showing the highest and cleanest yield of flagella for FlaA- and FlaAB-expressing cells were used for analysis.

Alexafluor staining of isolated flagella. Isolated flagella were stained with AlexaFluor 594 (Molecular Probes) following the method described by Turner *et al.* (2000) with the following modifications. One vial of AlexaFluor free dye was dissolved in 1 ml of a 1 : 1 solution of dichloromethane/2-propanol and divided into 100 µl aliquots in screw-cap microcentrifuge tubes. A flagellar suspension containing 1 µg protein was added to 100 µl of the dye, together with 25 µl Na_2HCO_3 to shift the pH to 7·8. Dye binding was achieved by shaking the suspension on a flat bed rotary shaker (100 r.p.m.; RT) in the dark for 1 h. Samples were then dialysed overnight in several changes of 1× PBS using a microdialyser as per the manufacturer's instructions (Spectrum). The concentrations of FlaA- and FlaAB-expressed flagella were equalized by diluting aliquots of dialysed samples 1 : 10, 1 : 20 and 1 : 50, and checking 10 µl of each dilution to give about 20–25 flagella per field of view. A volume of 6–10 µl of the appropriate dilution was then placed on glass coverslips and appropriate amounts of solutions or buffers designed to alter NaCl or pH levels to that required (85 mM NaCl, 425 mM NaCl, pH 5·8; prepared beforehand as described above), were added to the sample on the coverslip before inversion onto the slide.

Measurement of helical parameters. Measurements of amplitude (α) and wavelength (λ) ($n=100$), were made using the methods described by Kamiya & Asakura (1976b). Data analysis was carried out as described above, with significant difference between wild-type and DF1003 (FlaAB$^+$) flagella calculated for a 95 % confidence interval ($P<0{\cdot}05$).

RESULTS

FlaAB-expressing cells swim better than wild-type at high NaCl concentrations

The motility of the FlaA- and FlaAB-expressing strains may be affected by physiological factors other than flagellar shape, such as differences in their respective growth rates. Growth curves were therefore determined for the FlaA- and FlaAB-expressing strains grown under physiological (pH 7·0; 170 mM NaCl) and high salt (425 mM NaCl) conditions (Fig. 1a and b, respectively). Both strains grew at identical rates under both normal and high salt conditions. An estimation of the generation times for the two strains was obtained from the OD_{600} values at 0·3 and 0·6.

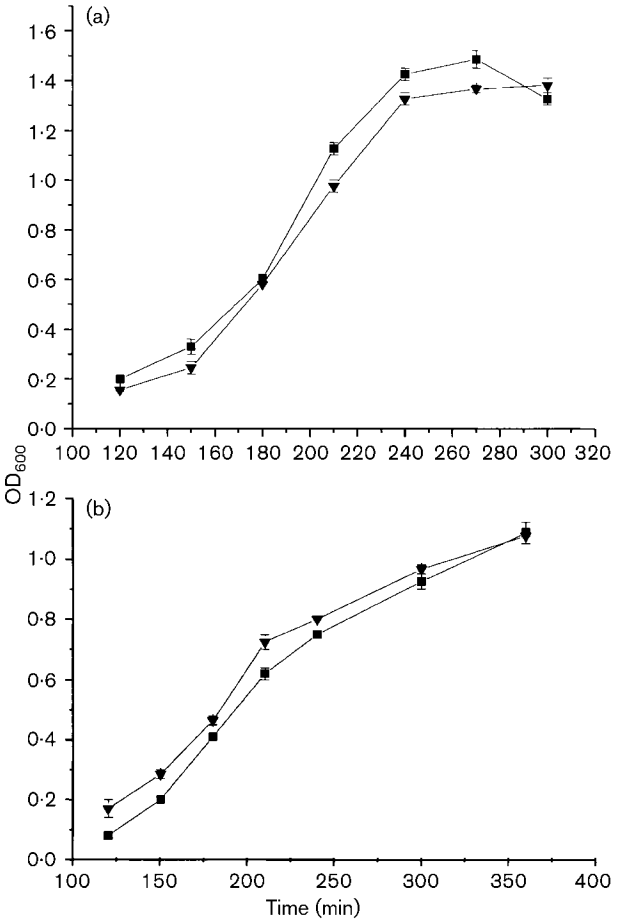

Fig. 1. Comparative growth curves for FlaA- (squares) and FlaAB-expressing (triangles) strains at (a) 170 mM NaCl, pH 7·0 (normal L-broth growth conditions) and (b) at 425 mM NaCl, pH 7·0 (three times L-broth level) carried out in triplicate, to assess possible differences in growth characteristics between the two strains. The results indicate the two strains grow at the same rate in both normal and high NaCl conditions. Generation time (between OD_{600} 0·3 and 0·6) was calculated for both strains to be about 30 min.

Both strains had a generation time of about 30 min. Thus, changes in motility observed between the wild-type cells and DF1003 (FlaAB$^+$) are unlikely to be the result of gross differences in the physiology of these two strains.

The concentration of NaCl in the growth medium affects motility (Kamiya & Asakura, 1976a). For example, when grown in 85 mM NaCl, the FlaAB-expressing strain was significantly less motile than the wild-type (Table 1; $19{\cdot}13 \pm 0{\cdot}38$ µm s^{-1} for FlaA$^+$ vs $9{\cdot}05 \pm 0{\cdot}29$ µm s^{-1} for FlaAB$^+$). At 170 mM NaCl, the physiological norm, the two strains did not exhibit a statistically significant difference in mean velocity ($22{\cdot}83 \pm 0{\cdot}54$ µm s^{-1} for BB2000 and $24{\cdot}84 \pm 0{\cdot}49$ µm s^{-1} for DF1003). At NaCl levels above 170 mM, the mean velocity of the FlaAB-expressing strain was significantly higher at all measured concentrations, i.e.

Table 1. Mean swimming velocity measurements of wild-type (FlaA$^+$) and DF1003 (FlaAB$^+$) in a range of NaCl, pH and viscosity levels

Condition	Mean velocity (μm s^{-1})		Pairwise comparison (Students' *t*-test *P* value)
	P. mirabilis wild-type FlaA$^+$	*P. mirabilis* DF1003 FlaAB$^+$	
85 mM NaCl	19·13±0·38	9·05±0·29	<0·000*
170 mM NaCl	22·83±0·54	24·84±0·49	0·109*
255 mM NaCl	19·73±0·55	22·05±0·45	0·006*
340 mM NaCl	11·38±0·31	17·87±0·50	<0·000*
425 mM NaCl	2·70±0·26	12·36±0·40	<0·000*
510 mM NaCl	2·04±0·10	2·49±0·14	0·025*
pH 4·6	1·93±0·11	2·49±0·17	0·260†
pH 5·2	6·60±0·20	10·10±0·32	0·003†
pH 5·8	11·77±0·27	16·33±0·40	<0·000†
pH 6·4	19·96±0·44	23·14±0·49	0·025†
pH 7·0	23·14±0·54	25·93±0·45	0·086†
pH 7·6	16·54±0·46	19·02±0·51	0·668†
pH 8·2	11·08±0·38	15·64±0·41	0·003†
5 % PVP	25·18±0·62	31·45±0·66	0·006†
10 % PVP	20·37±0·53	27·45±0·44	0·005†
15 % PVP	16·19±0·43	15·82±0·50	0·734†

*$n=20$.

†$n=10$.

255, 340, 425 and 510 mM NaCl (Table 1 and Fig. 2a). These data suggest that, while growth rates remain identical, FlaAB-expressing cells swim more efficiently at the high NaCl concentrations.

FlaAB$^+$ is more motile at extreme pH

The pH of the medium also affects flagellar morphology and function (Kamiya & Asakura, 1976a), thus the motility of wild-type and DF1003 were compared under acidic and basic pH conditions (Table 1 and Fig. 2b). The FlaAB-expressing strain was significantly more motile, i.e. had a higher mean velocity, than wild-type at either pH extremes, 5·2 or 8·2 (Fig. 2b), but its velocity was not statistically different from wild-type when measured at pH 7·0 (physiological norm). In acidic medium (pH 4·6), both strains exhibited reduced motility due to the adverse effects of the extreme acidity presumably on the flagellar motors and other physiological processes. These data were reproducible over multiple repetitive assays. The mean velocity in each case varied only slightly (between 4 and 12 %, dependent on assay), while the statistical difference in velocity between FlaA- and FlaAB-expressing cells remained unchanged and is statistically significant throughout the replicates (results not shown).

FlaAB$^+$ cells are motile in viscous medium

The addition of the viscosifying agent PVP to liquid medium has the effect of slowing down the rotation of the flagella and thus reducing the velocity of peritrichously flagellated bacteria at levels above 2 % (w/v) (Greenberg & Canale-Parola, 1977; Schneider & Doetsch, 1974). This effect was clearly exhibited by both strains as the concentration of PVP was increased from 5 to 10 % (w/v) (Table 1). However, the FlaAB-expressing strain was less affected overall, with its mean velocity at both 5 and 10 % PVP being significantly higher than that of the FlaA-expressing strain (Table 1). At higher PVP concentrations, i.e. 15 % PVP, both strains had almost equal swimming speeds, suggesting that there is an upper end to any advantage in viscous medium conferred by expression of FlaAB$^+$ filaments.

FlaAB$^+$ flagellar helix retains structure in high salt and acidic pH

We chose the three conditions that showed the greatest differences in swimming velocity between DF1003 and wild-type cells: 85 mM NaCl (pH 7·0; low NaCl concentration), 425 mM NaCl (pH 7·0; high NaCl concentration) and pH 5·8 (170 mM NaCl; low pH), and measured the amplitude and wavelength of the flagellar filaments of each strain. Filaments in 170 mM NaCl pH 7·0 (physiological norm) were used as a control for comparison. The results are presented in Table 2 and Figs 3 and 4. There is a significant difference between wild-type and FlaAB$^+$ filaments, in terms of both amplitude (Fig. 3a) and wavelength (Fig. 3b) under all conditions tested, including the physiological norm (control). Examples of individual flagella in pH 5·8 and 425 mM NaCl from both strains are compared

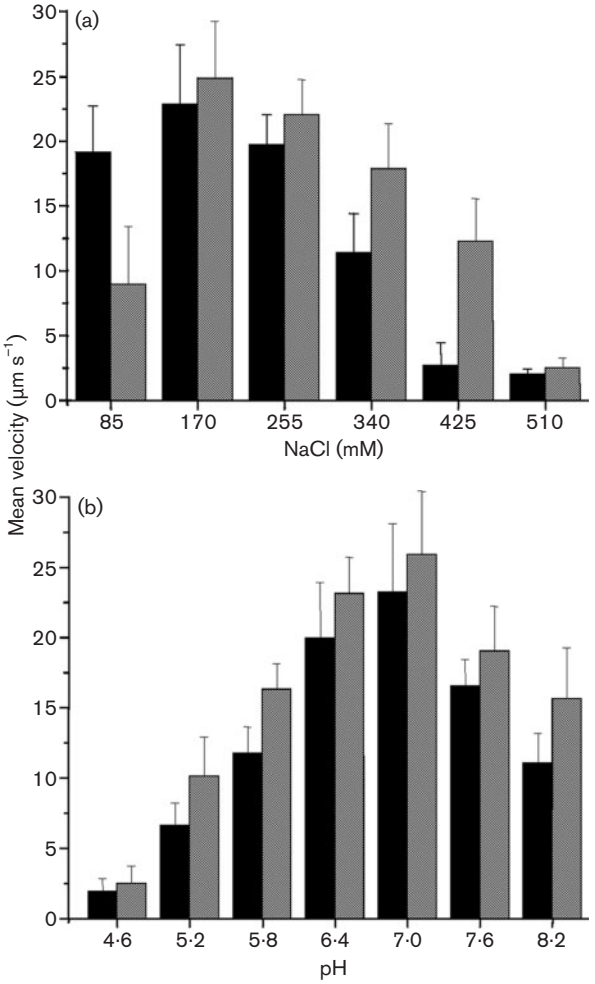

Fig. 2. A comparison of the mean velocity of *P. mirabilis* wild-type (FlaA$^+$; black bars) and DF1003 (FlaAB$^+$; shaded bars) measured in a range of NaCl and pH environments. (a) Velocity of the two phenotypes measured in T-broth amended with NaCl to a final concentration of 85 to 510 mM NaCl (0·5 to 3 times physiological levels). The mean velocity in each case consists of the velocity of 20 individual cells tracked for 2·66 min, with measurements taken every 0·3 min. (b) Comparison of the mean velocity of *P. mirabilis* FlaA$^+$ and FlaAB$^+$ measured in T-broth buffered with acetate or phosphate to give a range of pH from 4·6 to 8·2. The mean velocity in each case consists of the velocity of 10 individual cells tracked for 2·66 min, with measurements taken every 0·3 min.

side by side in Fig. 4(a) and (b), respectively, with the changes in amplitude and wavelength illustrated in Fig. 4(c). Either an increase in the NaCl concentration or a change in the acidity/alkalinity of the medium led to a greater structural change in FlaA$^+$ filaments as compared to FlaAB$^+$ filaments. Conversely, a reduction in NaCl concentration to 85 mM (50 % of physiological norm) resulted in a greater change in the structure of FlaAB$^+$ filaments compared to wild-type FlaA$^+$ filaments. Thus FlaAB$^+$ filaments appear to retain their helical structure at both high salt (425 mM NaCl) and acidic pH (5·8) when compared to wild-type filaments, but lose that structure at lower NaCl concentrations (85 mM NaCl). This phenomenon is illustrated in Fig. 5, where the difference between the filaments of each strain is shown as a percentage change in wavelength from the physiological norm (set to 100 %). While percentage change in FlaAB$^+$ remains near zero at 425 mM NaCl and pH 5·8, that for FlaA$^+$ rises sharply to +12 % at pH 5·8. On the other hand, both strains undergo a similar level of change (about 6 %) when the salt concentration is halved to 85 mM.

A comparison was made of the mean number of filaments per cell, as well as the length of individual filaments, on FlaA- and FlaAB-expressing cells. FlaA$^+$ cells possessed on average 4·44 ± 2·12 flagella per cell, while FlaAB$^+$ cells had 3·72 ± 2·21 flagella per cell. Statistical analysis of these values indicates that the difference in flagellar filament abundance between FlaA- and FlaAB-expressing strains is marginally significant ($P=0·03$, $n=94$). More significant ($P<0·001$), however, the mean length of the FlaA filaments was 6·51 ± 0·84 μm, compared to 5·08 ± 0·69 μm for the filaments of FlaAB-expressing cells.

Flagellin structure may be affected by amino acid substitutions

Kanto *et al.* (1991) identified key mutations in strains of *S. typhimurium* compared to the wild-type SJW1103 that were responsible for changes in flagellar shape in that

Table 2. Mean amplitude and wavelength measurements of wild-type (FlaA$^+$) and DF1003 (FlaAB$^+$) flagella

The Students' *t*-test *P* values were <0·00 in each case ($n=100$).

Amended condition	Mean amplitude (μm)		Mean wavelength (μm)	
	FlaA$^+$	FlaAB$^+$	FlaA$^+$	FlaAB$^+$
Normal conditions: pH 7·0, 170 mM NaCl	0·194 ± 0·003	0·210 ± 0·003	1·44 ± 0·01	1·47 ± 0·01
pH 7·0, 85 mM NaCl	0·240 ± 0·003	0·203 ± 0·003	1·51 ± 0·01	1·57 ± 0·01
pH 7·0, 425 mM NaCl	0·215 ± 0·003	0·251 ± 0·003	1·52 ± 0·01	1·46 ± 0·01
pH 5·8, 170 mM NaCl	0·170 ± 0·003	0·226 ± 0·003	1·62 ± 0·01	1·46 ± 0·01

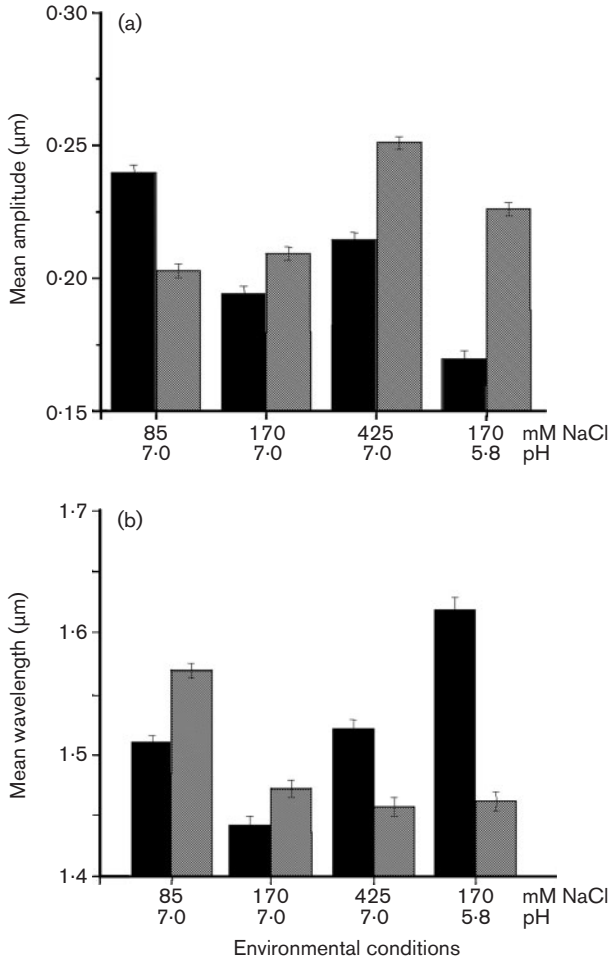

Fig. 3. Mean amplitude (α) and wavelength (λ) of isolated stained FlaA$^+$ (black bars) and FlaAB$^+$ (shaded bars) flagella in a range of environmental conditions ($n=100$). (a) From left to right, amplitude at 85, 170, 425 mM NaCl and at pH 5·8. The results for amplitude show a correlation between this parameter and motility for FlaA$^+$ and FlaAB$^+$. FlaAB$^+$ has a significantly greater mean amplitude, and hence a tighter helix, possibly leading to better motility, than FlaA$^+$ at 425 mM NaCl and pH 5·8. The reverse is true at 85 mM NaCl, where the reduced motility of FlaAB$^+$ compared to FlaA$^+$ (Fig. 2a) is reflected in significantly lower mean amplitude compared to FlaA$^+$. (b) Mean wavelength of FlaA$^+$ and FlaAB$^+$ flagella measured under the same conditions as for (a). Wavelength is inversely proportional to amplitude, thus the increase in wavelength seen in FlaA$^+$ at 425 mM NaCl and pH 5·8 is indicative of a more relaxed helix and probably reflects the significantly reduced motility of this strain compared to FlaAB$^+$, which does alter significantly in wavelength under these conditions.

species. We aligned the deduced amino acid sequences of the wild-type FlaA protein and FlaAB from DF1003 with *S. typhimurium* SJW1103 to look for substitutions at these sites. The CLUSTAL W alignment (Thompson *et al.*, 1994) revealed a substitution between FlaA and FlaAB at one of these sites (K414N) located in the conserved C-terminal end

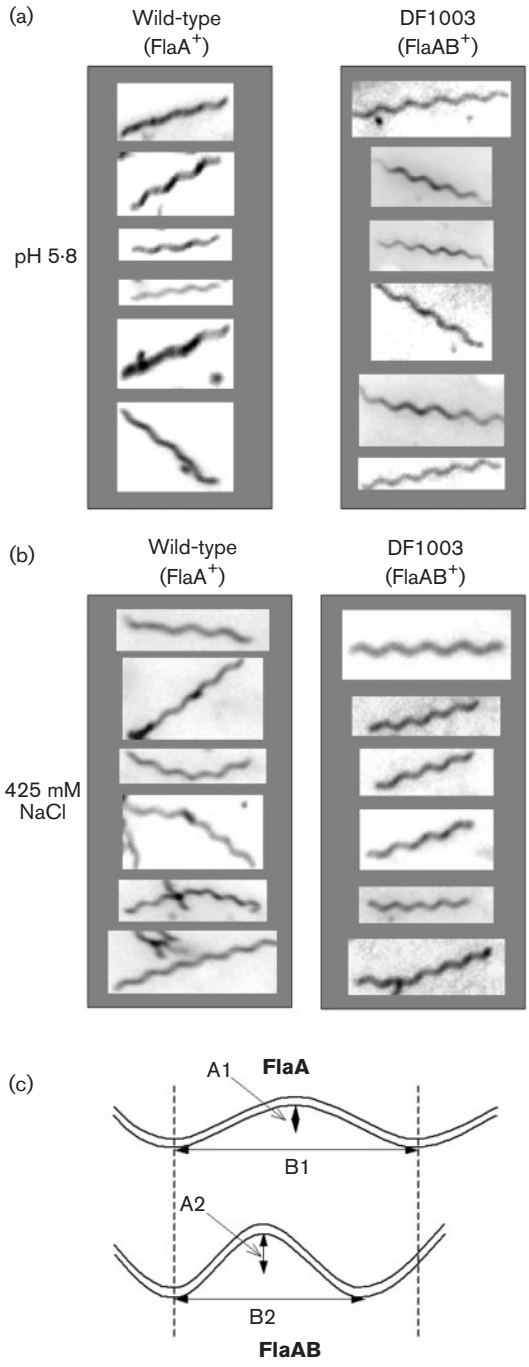

Fig. 4. Examples of isolated, Alexafluor-stained flagella from the *P. mirabilis* FlaA$^+$ and FlaAB$^+$ strains subjected to acidic pH and high salt conditions. (a) FlaA$^+$ and FlaAB$^+$ flagella subjected to pH 5·8. (b) FlaA$^+$ and FlaAB$^+$ flagella subjected to 425 mM NaCl (2·5 times normal). The FlaAB$^+$ flagella retain a tighter helical structure, while FlaA$^+$ flagella are more relaxed. The schematic diagram (c) illustrates how the shape of the FlaA flagellum was observed to change with respect to FlaAB at pH 5·8 and in 425 mM NaCl, and thus how this change affects amplitude and wavelength. The amplitude is reduced in FlaA (A1) with respect to FlaAB (A2), while the wavelength (B1) increases compared to FlaAB (B2).

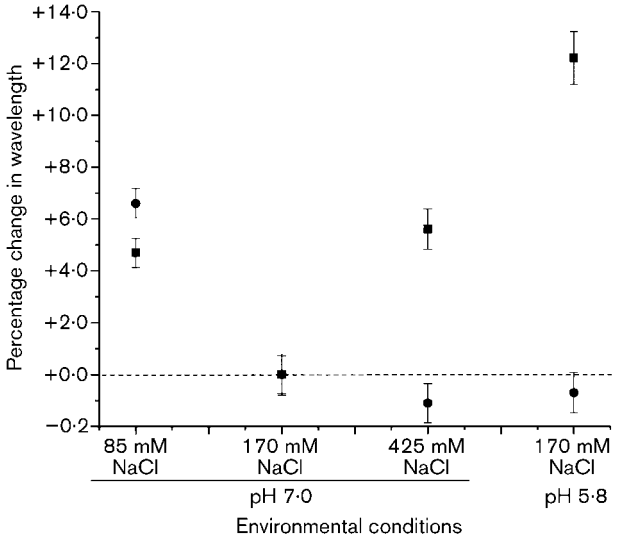

Fig. 5. Percentage change in mean wavelength of FlaA$^+$ (squares) and FlaAB$^+$ (circles) flagella in 85 and 425 mM NaCl and pH 5·8, compared to flagella subjected to normal conditions. Normal (170 mM NaCl, pH 5·8) was set at 100 % (dotted line) and the other wavelength measurements were calculated as a percentage of this value. For FlaA$^+$, the increase in wavelength is between about 5 and 13 %. FlaAB$^+$ flagella maintain their wavelength at 425 mM NaCl and pH 5·8, even undergoing a slight decrease (tightening of the helix), but are more affected than FlaA$^+$ at low salt (85 mM NaCl).

and shown underlined in Fig. 6. This substitution renders the site more compact and acidic in FlaAB due to the size and composition of the side chain. Analysis of the central domain (not shown) also revealed numerous substitutions between G-190 and D-266, including a 21 aa region containing 14 non-conserved substitutions between G-190 and V-212. At least three of these non-conserved substitutions (K198L, N200I and S210I) lead to replacement of hydrophilic with hydrophobic side chains, while a further three (A191D, T194D and A196N) introduce acidic side chains. Only one substitution (L205K) replaces a hydrophobic side chain with a hydrophilic one. Thus it is possible that while this region is not as critical to flagellin structure as the conserved termini, these resulting differences in secondary structure of the FlaAB protein may help it retain the helix morphology when exposed to high salt and acidic pH.

DISCUSSION

The data demonstrate that FlaAB-expressing *P. mirabilis* cells have enhanced swimming efficiency at extremes of pH and NaCl concentration, and in viscous medium, and this enhancement coincides with structural changes in the shape of the flagellar helix itself. Interestingly, we have also observed enhanced swarming motility of the FlaAB-expressing strains on nutrient agar at extremes of pH and NaCl (unpublished data), suggesting that enhanced motility of the FlaAB-expressing strain is not limited to swimming locomotion. These results support the hypothesis that the hybrid FlaAB flagellin imparts an advantage to *P. mirabilis* in conditions that may impede motility, including high salt (up to three times the physiological norm), acidic and alkaline pH, and a viscosity of up to 10 % (w/v) PVP. The hypothesis does not hold at the physiological norm (170 mM NaCl, pH 7·0) where no difference in motility was observed, nor at low salt levels (0·5 times normal) where the FlaA-expressing strain was significantly more motile. This may help explain why FlaA$^+$ is the dominant phenotype in wild-type BB2000 under normal laboratory growth conditions, while FlaAB is expressed by wild-type at frequency levels of 0·1–1·0 % (J. Manos & R. Belas, unpublished observation). However in the urinary tract, a prime site for colonization in humans, alkaline conditions may arise as a result of *P. mirabilis* infection. For example, in catheterized patients, *P. mirabilis* infections are accompanied by the expression of a potent urease whose activity can increase the pH of the urine (Morris & Stickler, 1998). Under such conditions, based on our data, it could be envisaged that the increase in pH during *P. mirabilis*

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S. typhimurium FliC     ENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSN
P. mirabilis FlaA       DDALATLDKAINTIDESRSKLGAIQNRFESTINNLNNTVNNLSASRSRILDADYATEVSN
P. mirabilis FlaAB      DDALATLDNAISKVDESRSKLGAIQNRFQSTINNLNNTVNNLSASRSRILDADYATEVSN
                        ::.* .:* *:  :*  **.***:****:*:*.**.******:::**** *:********

S. typhimurium FliC     MSRAQILQQAGTSVLAQANQVPQNVLSLLR
P. mirabilis FlaA       MSRGQILQQAGTSVLAQANQVPQTVLSLLR
P. mirabilis FlaAB      MSKNQILQQAGTAVLAQANQVPQTVLSLLR
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Fig. 6. CLUSTAL W alignment of the C-terminal end of wild-type *S. typhimurium* SJW1103 FliC, wild-type *P. mirabilis* FlaA$^+$ and *P. mirabilis* DF1003 FlaAB$^+$. An analysis of amino acid sequence in wild-type *S. typhimurium* FliC and 16 flagellar-shape mutants by Kanto *et al.* (1991) found that shape depended on single amino acid mutations localized to the N- and C-terminal regions. One of those mutations (A414V) leads to the formation of curly filaments in *S. typhimurium* and may have parallels in *P. mirabilis* FlaA and FlaAB, which also has a mutation at this site (K414N, underlined). The change replaces a long basic side chain in FlaA with a short acidic one in FlaAB and may lead to retention of FlaAB shape under acidic conditions.

infection would enhance the motility and survival of FlaAB-expressing cells compared to FlaA-expressing ones and provide a means for the population to adapt to the new conditions.

How might the expression of FlaAB$^+$ flagellin lead to enhanced motility in some environments? Amplitude and wavelength are inversely related; therefore a decrease in amplitude leads to a concomitant increase in wavelength and vice versa. In physical terms the former leads to a 'flattening out' or 'relaxation' in the helical flagellar structure, as portrayed in Fig. 4(a) and (b) and illustrated in the accompanying drawing (Fig. 4c). This change in flagellar filament morphology occurs concomitant with the changes in motility observed for FlaA$^+$ and FlaAB$^+$ cells. To emphasize this relationship, the increase in helical wavelength of FlaA$^+$ filaments from 1·44 to 1·62 μm at pH 5·8 is accompanied by a decrease in amplitude from 0·194 to 0·170 μm, producing a relaxed filament. However, at the same pH, the wavelength of FlaAB$^+$ flagella remains almost unchanged (from 1·47 to 1·46 μm), while the amplitude increases slightly (from 210 to 226 μm), indicating a tightening of the FlaAB$^+$ flagellar helix. In step with these morphological changes to the filament, the mean velocity of FlaA$^+$ cells decreased by 50 % (from 23·14 to 11·77 μm s^{-1}), while that of FlaAB$^+$ cells decreased by only 37 % (from 25·93 to 16·33 μm s^{-1}). Similarly, at 425 mM NaCl, the amplitude of the FlaAB$^+$ filament increases to a greater extent than FlaA$^+$ (210 to 251 μm against 194 to 215 μm). This structural change in the flagellum is reflected in greater retention of motility (50 % of normal velocity compared to only 12 % for FlaA$^+$). Thus, a tightening of the filament is correlated with a smaller decrease in swimming velocity and vice versa. Interestingly, when the FlaAB-expressing strain is subjected to 85 mM NaCl, it is significantly less motile (9·05 against 19·13 μm s^{-1}) and measurements of the amplitude and wavelength of the FlaA$^+$ filament show that it has tightened significantly, compared to the FlaAB$^+$ filament. Thus, in this environment the FlaA$^+$ filament is able to maintain a tighter helix than the FlaAB$^+$ filament and it is this helix morphology that apparently helps FlaA-expressing cells gain a motility advantage at this salt level, rather than the relaxation of helical structure in FlaAB$^+$. Therefore, environmental conditions, such as NaCl concentration and pH, alter flagellar filament morphology differently depending on whether a FlaA$^+$ or FlaAB$^+$ filament is produced and the tighter filament helix of FlaAB flagellin appears to be a better propeller under these conditions.

Measurements of the mean length of the filaments and the number of flagella per cell indicate that FlaA$^+$ cells produce longer filaments and may possess slightly more flagella per cell than the FlaAB-expressing cells. It is therefore feasible that possession of more, longer flagella might be a factor in determining the swimming speed of FlaA$^+$ cells. While a tantalizing hypothesis, it is unlikely for at least two reasons. First, for this hypothesis to be true, the length and number of flagella would have to change with the different environmental conditions tested. This is not the case, as the data indicate that both the number of flagella and length of the filaments were constant for each strain under the conditions tested. Second, the hypothesis predicts that FlaA-expressing cells would be better swimmers than cells expressing FlaAB$^+$. This is not the case, as having fewer and shorter flagella would be expected to inhibit FlaAB$^+$ motility rather than enhance it; yet the data show that under normal conditions the two strains have similar mean velocities, while under most amended conditions FlaAB-expressing cells are significantly faster than FlaA$^+$ cells, which have longer filaments and slightly more flagella. It appears far more feasible based on these data that the differences in swimming speed are due to alterations in the flagellar filament helix shape rather than the length or abundance of flagella on the cell.

Altered helicity can result from single amino acid substitutions in the flagellin sequence. For example, about a dozen point mutations have been identified in *S. typhimurium* SJW1103 that affect filament morphology and all except one are found within the conserved terminal regions of flagellin (Hyman & Trachtenberg, 1991; Kanto *et al.*, 1991; Samatey *et al.*, 2001). These single residue changes resulted in straight, coiled and two types of curly flagella. Hydrophobic interactions between residues in different subunits of the filament have also been found to play a role in filament supercoiling. Yokekura *et al.* (2003) examined intersubunit interactions in two mutations in the atomic model of the *S. typhimurium* SJW1655 flagellar filament. Residue 449, located in the C-terminal α-helix of domain D1 (CD1), and responsible for the R-type straight filament in the A to V mutation, is surrounded by F53 and F131 located on domain ND1a, with the mutation F53V destabilizing the R-type filament in favour of an L-type straight filament through a reduction in hydrophobic interactions.

While exact homologues of these *S. typhimurium* FliC amino acid changes are not apparent in FlaAB, an alignment of *S. typhimurium* FliC, *P. mirabilis* FlaA and FlaAB (Fig. 6) does reveals at least one amino acid substitution in the FlaAB protein that may play a role in the morphological changes observed in the different environmental conditions. This substitution is in a homologous site to one of the residues identified by Kanto *et al.* (1991) as responsible for flagellar shape in *S. typhimurium* FliC. While it is not the same substitution (A414V in *S. typhimurium*, K414N in *P. mirabilis*), the replacement amino acid residue (N) in the FlaAB protein has an acidic and less bulky side chain, which may contribute to its ability to maintain its shape in acidic environments.

Moreover, larger deletions can also affect flagellin filament structure through changes in intersubunit interactions. Two spontaneous mutant strains arising from SJW1103, SJW46 and SJW61 have deletions of 88 and 96 aa, respectively, in regions of the outermost subdomain, possibly

contributing to the mechanical instability and high level of polymorphism in this strain (Mimori-Kiyosue *et al.*, 1998). The absence of whole flagellin domains can also positively affect motility. Cohen-Krausz & Trachtenberg (2003) found that the largest and most variable domain, D3, is totally absent in the flagellin subunits forming the complex filament of *Rhizobium lupini* and suggest that the consequence of this may be a better hydrodynamic performance in viscous environments (Cohen-Krausz & Trachtenberg, 2003). While not strictly in the conserved terminal ends of FlaAB, several other substitutions (A191D, T194D, A196N, K198L, N200I and S210I) render the FlaAB protein more acidic and hydrophobic overall and may also play a role in retention of the helical structure in the FlaAB$^+$ strain under high salt and acidic pH conditions.

The prevailing view of bacterial flagella has been one of a highly immunogenic structure that plays a substantial role in evasion of the host response during infection through either phase variation or switching. While this stance is well supported by research, the results of the current study suggest that flagellar 'antigenic' variation may also result in mechanistic changes in the flagellar filament's helicity that aid in bacterial survival by improving the efficiency of the flagellar filament propeller.

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