Salmonella typhimurium flhE, a conserved flagellar regulon gene required for swarming

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The *Salmonella typhimurium* gene *flhE* is located at the end of a large flagellar locus in at least 10 peritrichously flagellated Gram-negative bacterial genera, but it shares no significant similarity with other genes. This study shows that *flhE* is transcribed as part of an *flhBAE* flagellar operon, under the control of the flagellar master regulator FlhD₂C₂. Deletion of the chromosomal *flhE* gene did not affect swimming motility, but it abolished swarming motility across solid agar. Swarming was restored to the $\Delta flhE$ mutant by the 130 aa putative envelope protein FlhE, but not by a truncated version lacking the N-terminal signal peptidase I recognition sequence. The $\Delta flhE$ mutant was indistinguishable from the wild-type parent in number and distribution of flagella, secretion of flagellin subunits, and flagellar gene expression, and there were no obvious differences in cell-surface LPS and extracellular polysaccharide. The $\Delta flhE$ mutant was able to swarm when non-ionic surfactant was included in agar medium, and it showed differences to the wild-type in binding calcofluor and Congo red dyes, and in biofilm production. The data show that the *flhE* gene is part of the flagella regulon but that it has no role in flagella biogenesis. It appears, nevertheless, to act at the cell envelope to influence flagella-dependent swarming.

INTRODUCTION

Motile bacteria like *Escherichia coli* and *Salmonella typhimurium* are able to swim through liquid by rotation of peritrichous helical flagella extending from their cell surface (Macnab, 1996). Flagella biogenesis and chemotaxis require over 50 genes transcribed in an ordered programme (Macnab, 1996). At the apex of this regulon is the master regulator *flhDC*, which activates genes encoding the flagella early structures, the type III flagella subunit secretion apparatus (Kutsukake *et al.*, 1990; Soutourina & Bertin, 2003), and a flagellar sigma-factor (σ^{28}) that activates late genes encoding the multicomponent flagellar filament, motor and chemotaxis apparatus (Chadsey *et al.*, 1998; Karlinsey *et al.*, 2000). Assembly of the flagella is strictly ordered, and coupled to this expression programme. The FlhD₂C₂ activator also controls non-flagellar genes in an extended motility regulon (Stafford *et al.*, 2005; Soutourina & Bertin, 2003). Multicellular swarming migration over solid surfaces (Allison & Hughes, 1991) requires increased flagella production via upregulation of *flhDC* (Fraser & Hughes, 1999; Dufour *et al.*, 1998; Hay *et al.*, 1997; Givskov *et al.*, 1995), and other cell surface components that facilitate assembly of swarm cell rafts (Hay *et al.*, 1999; Allison *et al.*, 1994), and surface lubrication by LPS (Toguchi *et al.*, 2000; Belas *et al.*, 1995), exopolysaccharide (EPS) (Gygi *et al.*, 1995) and secreted surfactant (Toguchi *et al.*, 2000; Gygi *et al.*, 1995; Lai *et al.*, 2005).

Motility genes are clustered within three loci around the chromosome of *Sal. typhimurium* and related bacteria (Macnab, 1996), and their approximate function has, in virtually all cases, been established. In this paper, we re-examine the gene *flhE*. This gene was given a flagellar nomenclature due to its location at the end of a large flagellar and chemotaxis gene locus, but an early report has indicated that it is not involved in motility (Minamino *et al.*, 1994).

METHODS

Bacterial strains and plasmids. Bacterial strains were grown at 37 °C in LB, unless stated otherwise. Swarm cells were isolated after 6 h incubation on swarm agar (0.6 % Bacto agar plus 0.5 % glucose; Wang *et al.*, 2004). Wild-type *Sal. typhimurium* SJW1103 (Yamaguchi *et al.*, 1984) is motile, and the isogenic *flhDC* mutant SJW1368 is non-motile (Ohnishi *et al.*, 1994). Deletion of *flhE* was achieved by the method of Datsenko & Wanner (2000) to create the $\Delta flhE$ strain, using primers $\Delta flhE$ For (TCCGATAACCGTCAT-ATCCGCATGCACGGCGACCATTTGGAGGAAAATAATGGTGTAG-GCTGGAGCTGCTTC) and $\Delta flhE$ Rev (TCCGGCAACCTACCTCAC-TTTATAAAACAGCGTTTCTATTATTCAAATTCGGGGGATCCG-TCGACC), and the pKD4 (Km^R) plasmid as a template (Datsenko & Wanner, 2000). Deletion of the *flhE* gene was verified by PCR. The entire *flhE* gene was amplified by PCR using primers FlhEfor (TGGAGGAAACATATGCGTAAATGGCTGGCGTT-G) and FlhErev (AACCCTCGAGGCGGTAGTTCACAATCACC), and cloned (*Xba*I–*Hind*III) 5' of the arabinose-inducible promoter of expression vector pBAD18, to create pBAD18-FlhE. A derivative gene encoding N-terminally truncated FlhEAN (lacking aa 1–16)

Abbreviations: EPS, exopolysaccharide; EtBr, ethidium bromide.

was cloned into pBAD18 after PCR using primers FlhE-T (AATTCTAGAAATAATTTTGTAACTTTAAGAAGATATACCATGGCGGAAGGCGCGTGGCAG) and FlhERev to make pBAD18-FlhEAN.

Fluorescence microscopy of cells. Cells scraped from swarm plates were resuspended in saline (to an OD₆₀₀ of 0.05), and fixed onto glass slides using 4% paraformaldehyde (in 20 mM PIPES, pH 7.4) before blocking with PBS (50 mM NaPO₄/Na₂PO₄, pH 7.4, 150 mM NaCl) plus 3% (w/v) BSA for 1 h at 25 °C. Primary anti-flagellin antibody (1/1000, v/v, in PBS) was added for 2 h before washing (2 × 10 min, PBS), incubation with AlexaFluor-488/594-conjugated anti-rabbit secondary antibody (1/1000, v/v, in PBS; Molecular Probes) (2 h, 25 °C), and further washing (3 × 10 min, PBS). Cell membranes were stained for 10 min with SynaptoRed (in the dark), and coverslips were mounted using ProLong Anti-fade reagent (Molecular Probes), and visualized using a fluorescence microscope (Leica DM IRBE). Images were captured by a CCD digital camera (Hamamatsu) and processed using OpenLab software (Improvision).

Cell fractionation. Harvested swarm cells were resuspended in PBS, and diluted to an OD₆₀₀ of 1.0. Total extracellular FlhC protein was prepared by shearing (5 min vortex) of harvested cells, and TCA precipitation (10%, v/v, final concentration) of cell-free supernatant at 4 °C for 1 h. Extracellular protein was centrifuged for 1 h at 300 000 g to separate filament (pellet) from monomeric flagellin (soluble fraction, precipitated with 10% TCA, 4 °C, 1 h). Cells were separated into cytosolic and membrane fractions according to Auvray *et al.* (2001).

LPS and EPS extraction. Crude LPS was prepared from swarm cells (number of cells equivalent to 1 ml culture at an OD₆₀₀ of 1), according to Hitchcock & Brown (1983). LPS was also extracted by a hot-phenol method for analysis by urea (high molecular mass) and deoxycholate-SDS (low molecular mass) PAGE, and visualized using silver staining (Guard-Petter *et al.*, 1995). EPS was isolated and visualized according to Gygi *et al.* (1995).

Biofilm assay. Overnight cultures grown in biofilm LB (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹) were inoculated at a 1 in 10 dilution into 96-well PVC microtitre plate wells (Falcon) containing fresh biofilm LB plus 0.5–2% glucose, and incubated overnight at 30 °C. Biofilm was washed twice with distilled water, air-dried for 30 min, and stained for 15 min with 1% crystal violet before washing with water and air drying. Biofilm was quantified as absorbance at 550 nm, following extraction with 95% ethanol (Mireles *et al.*, 2001).

In vivo assay of transcription. Transcription was assessed as cell β-galactosidase activity (Miller, 1972) of gene fusions created by *EcoRI*/*Bam*HI cloning of *flhB* (using primers FlhBPromEco, GAATTCACACGAGACTTTCTTTATC; and FlhBPromBam, GGA-TCCGCAACCCCTGGATAG) and *flhC* (primers FliCpromEco, GAATTCCTTTTGCAAAAATAATGC; and FliCpromBam, GGATCC-TCAATTACAACCTTGATG) promoter fragments into the *lacZ* fusion vector pGPS123 (Stafford *et al.*, 2005), which is identical to pRS551 except that Km^R is replaced by Gm^R (Simons *et al.*, 1987). For RT-PCR, RNA was extracted from swarm cells using hot acidic phenol. After removal of contaminating DNA by using Rq1 DNase (Promega), cDNA specific for the *flhB* and *flhE* genes was synthesized using Mu-MLV reverse transcriptase (Promega), and primers *flhB*RevRT (TTGGCGTGGCGATATAATG) and *flhE*RevRT (ATTGCTCCGCACCTTTTAACG), resulting in cDNA originating within *flhB* and *flhE*, respectively. In the final step, primers *flhB*RevRT/*flhB*ForRT (internal to *flhB*, ACCGCTCATCGCGGCGTGG) and *flhE*RevRT/*flhE*ForRT (*flhE* internal, TGGCG-TTGTGCTCTTTCC) were used to amplify internal fragments of *flhB* and *flhE*. To assess transcripts spanning the *flhBA* and *flhAE*

intergenic regions, primer pairs *flhB*ForRT/*flhA*RevRT (TCGCGAA-GTTACCGCCGACCAGG) and *flhA*ForRT (TCCGATAACCGTC-ATATCC)/*flhE*Rev were used. All PCR reactions used *Taq* polymerase, and products were analysed on 1.5% agarose ethidium bromide (EtBr) gels.

RESULTS AND DISCUSSION

The *flhE* gene in the flagellar loci of peritrichously flagellated bacteria

The *flhE* gene in *Sal. typhimurium* is located immediately downstream of the *flhBA* genes, and the *flhA* stop codon overlaps the *flhE* start codon (Fig. 1). Nevertheless, a transposon insertion in *flhE* has indicated that the gene is not essential for swimming motility, casting doubt on its flagellar gene nomenclature (Minamino *et al.*, 1994). Our renewed interest in *flhE* was prompted by its presence in the flagellar gene loci of over 10 genera of peritrichous Gram-negative bacteria (Fig. 1). In the *Enterobacteriaceae* *Esc. coli*, *Serratia marcescens*, *Erwinia carotovora*, *Yersinia pestis*, *Citrobacter rodentium* and *Shigella flexneri*, *flhE* is located as in *Sal. typhimurium*, i.e. immediately distal to *flhBA* encoding the integral membrane flagellar export proteins FlhB and FlhA (except in *Shigella*, which contains no *flhA* gene). The *flhE* genes from the human pathogens *Esc. coli*, *Y. pestis*, *Cit. rodentium* and *Shi. flexneri* are similarly located at the end of motility gene locus, with non-flagellar genes downstream, and they are apparently transcribed independently from *flhE*. In *Erwinia* and *Serratia*, the *flhBA(E)* genes lie immediately adjacent to the chemotaxis genes *cheBYZ*, and within a still larger flagellar gene cluster containing the divergently transcribed *flgAMN* and *flgBCDEFGHIJKL* genes. In the free-living soil microbes *Azotobacter vinelandii* and *Chromohalobacter salexigens*, *flhE* is located downstream of the *flhFG* genes thought to be involved in flagellar assembly and gene regulation (McCarter 2001), while in *Ralstonia metallidurans*, *flhE* is separated from *flhG* by the *flhA* gene that encodes the flagella-specific sigma factor σ²⁸. The *flhE* gene is thus always linked to flagella genes, and has not been located separately from flagellar gene loci. The sequence identity between the deduced amino acid sequence of *Sal. typhimurium* FlhE, and those of other *Enterobacteriaceae*, ranges from 37 to 83%, while it is lower (28–38%) for *A. vinelandii*, *R. metallidurans* and *Chr. salexigens*. All the *flhE* genes are 400 ± 25 bp and encode proteins of approximately 14 kDa, with a predicted N-terminal signal peptidase I leader sequence, and a predicted periplasmic or outer membrane location; the FlhE sequences of the 10 genera in Fig. 1 contain between 7 and 13 apparently randomly distributed proline residues. BLAST searches revealed no significant similarity of FlhE to any class of proteins.

flhE is transcribed in an *flhBAE* operon activated by FlhD₂C₂

The *flhBA* operon is transcribed from a class II (early) flagellar promoter upstream of *flhB*, and is therefore

Fig. 1. Location of *flhE* gene in peritrichous flagellated eubacteria. The *flhE* gene is shown in black, flagellar genes are in grey and non-flagellar genes are in white. Using the predicted amino acid sequence of the *Sal. typhimurium* LT2 *flhE* gene (AAL20828.1) with the BLASTP algorithm (Altschul *et al.*, 1997) and the colibase website (Chaudhuri *et al.*, 2004), *flhE* was identified in *Y. pestis* (GenBank accession no. NP_669822), *Esc. coli* (AAC74948), *Cit. rodentium* (colibase GL096266), *Shi. flexneri* (AAN43476), *Ser. marcescens* (colibase GL076148), *Erw. carotovora* (colibase CAG74604), *R. metallidurans* (YP_585844), *A. vinelandii* (ZP_00417385) and *Chr. salexigens* (ABE59363).

activated by the flagellar master regulator FlhD₂C₂ (Fig. 2). To assess whether expression of *flhE* is flagellar-like, we purified RNA from wild-type and *flhDC*⁻ strains, and performed RT-PCR using primers targeted within the *flhE* gene and the class II *flhB* gene. The results (Fig. 2) show that transcription of *flhE* was dependent on FlhD₂C₂, i.e. it mirrored that of *flhB*. We assessed whether *flhE* was transcribed as part of a contiguous polycistronic messenger RNA molecule by measuring transcription across the *flhBA* and *flhAE* intergenic regions in the wild-type and *flhDC*⁻ strains. Fig. 2 shows that *flhE* is transcribed as part of a polycistronic messenger RNA in an *flhDC*-dependent manner, and it indicates that there is no post-transcriptional processing of the messenger RNA *in vivo*. These expression data establish *flhE* as part of the FlhD₂C₂ regulon, and indeed as part of an *flhBAE* operon. Together with the conserved location of *flhE* in the flagellar loci of peritrichously flagellated bacteria, this gives renewed validity to its designation as a flagellar gene. We investigated the possible function of *flhE* in motility.

Loss of *flhE* attenuates swarming but not swimming

We set out to re-examine a possible role for *flhE* in motility by constructing an *flhE* deletion strain using the method of Datsenko & Wanner (2000). We confirmed the report by

Minamino *et al.* (1994) that the swimming phenotype of such a mutant is at most only marginally reduced from the wild-type (Fig. 3). However, in common with several

Fig. 2. Transcription of *flhE* as part of the *Sal. typhimurium* *flhBAE* operon. RT-PCR reactions were performed on RNA extracted from swarm cells of wild-type (WT) and *flhDC*⁻ strains, and products (sizes and corresponding locations are indicated in the top panel) within the *flhB* and *flhE* coding sequences, and spanning junctions between *flhB*-A and *flhA*-E were visualized by UV illumination of 1.5% agarose EtBr gels.

Fig. 3. Effect of *flhE* deletion on *Sal. typhimurium* motility. Wild-type (WT), the $\Delta flhE$ mutant and the $\Delta flhE$ mutant complemented with pBAD18-FlhE (E) and FlhE $^{\Delta N}$ pBAD18-FlhE $^{\Delta N}$ (E $^{\Delta N}$) were incubated on media for (a) swimming (0.35 % agar, 6 h) and (b) swarming (0.6 % agar + 0.5 % glucose, overnight).

Gram-negative species, *Salmonella* is also capable of swarming motility, which is a form of flagella-dependent mass migration that is assayed as movement across the surface of denser 0.6 % agar, rather than the standard 0.35 % agar. The ability of *Sal. typhimurium* to swarm was severely attenuated by *flhE* loss, and was restored by a plasmid expressing the *flhE* gene *in trans* from the pBAD18 arabinose-inducible promoter (Fig. 3). In contrast, a truncated version of FlhE lacking the putative N-terminal 16 aa leader signal peptidase I sequence (MRKWLALLFPLTVQA), and representing the mature form of the protein (aa 17–130), did not complement the swarming defect, even at high induction levels, indicating the importance of its secretion (Fig. 3). These data suggest that FlhE is a cell envelope protein that does have a role in flagellar-dependent motility, i.e. not cell swimming motility, but swarming migration.

Loss of *flhE* does not impair flagellar gene expression or assembly

To test if transcription of flagella genes is altered in the $\Delta flhE$ mutant, plasmid-borne transcriptional *lacZ* fusions were constructed to the flagellar class II promoter controlling the FlhD₂C₂-dependent *flhBAE* operon, and to the flagellar class III (σ^{28} -dependent) *fliC* promoter. The activity of these promoter fusions during growth in liquid culture revealed that while transcription of the class II *flhB(AE)* and class III *fliC* promoters was reduced 122- and 421-fold, respectively, in an *flhDC* mutant compared with the wild-type (Fig. 4a), transcription of both genes was unaltered in the $\Delta flhE$ strain.

Loss of swarming motility could be due to attenuated post-transcriptional expression, or assembly of flagellar structural subunits. To examine this, levels of FliC protein were analysed by immunoblotting whole-cell, cytosolic, membrane-associated, extracellular and filament-incorporated fractions in the $\Delta flhE$ strain, and compared with the wild-type (Fig. 4b). These assays showed that the intracellular

level of FliC was unaltered, as was external flagellin in the filaments. The stability of the flagella to shearing in the $\Delta flhE$ mutant was also unchanged (data not shown). It remained possible that the number or distribution of flagella on the cell surface was changed by the $\Delta flhE$ mutation, so we examined wild-type and $\Delta flhE$ mutant cells harvested from swarm agar, and fixed to glass slides. Fig. 4(c) shows representative merged fluorescence microscopy images highlighting flagella (visualized using anti-flagellin primary antibody and FITC-labelled secondary antibody) and cell membranes (stained with SynaptoRed). The images indicate no obvious change in flagellar number (approximately 15 per cell) or distribution. The combined data establish that the $\Delta flhE$ mutation does not reduce flagella gene expression, assembly or stability, or differentiation into swarm cells. The attenuation of flagellar-dependent swarming must have a non-flagellar cause.

Altered surface and biofilm properties of the $\Delta flhE$ strain

Transposon mutations attenuating swarming motility of flagellated bacteria have been mapped to genes involved in the biosynthesis, not only of cell-free surfactants (Nakano *et al.*, 1992; Eberl *et al.*, 1999), but also of LPS (Toguchi *et al.*, 2000; Belas *et al.*, 1995) and EPS. Fig. 4(d) shows that representative samples of crude LPS from the wild-type and $\Delta flhE$ strains, extracted according to Hitchcock & Brown (1983), failed to highlight any obvious differences. Furthermore, low-molecular-mass (Fig. 4d) and high-molecular-mass LPS (data not shown) were analysed by silver staining (Guard-Petter *et al.*, 1995), and, again, no changes between the two strains were evident. In common with LPS, some components of the EPS are thought to reduce surface resistance, and aid in swarming migration; for example, a mutation in the *cmfA* gene of the strongly swarming *Proteus mirabilis* abolished swarming migration due to loss of an EPS rich in galacturonic acid and galactosamine (Gygi *et al.*, 1995). However, when crude acid hydrolysable EPS was assessed according to Gygi *et al.* (1995), again no differences were observed between the mutant and the wild-type (data not shown). This is unsurprising, since the biosynthetic pathways for LPS and several types of EPS are well characterized, and FlhE shares no motifs with their enzymes.

Such transposon mutations attenuating swarming motility commonly reduce the 'wettability' of the bacterial cell surface (Toguchi *et al.*, 2000; Gygi *et al.*, 1995; Belas *et al.*, 1995; Lai *et al.*, 2005), and swarming by such mutants, and of the weakly swarming *Esc. coli* K-12, can be recovered by the addition of external surfactants such as Tween 80 (Niu *et al.*, 2005; Toguchi *et al.*, 2000). The *Sal. typhimurium* $\Delta flhE$ strain was incubated on 0.6 % agar plates containing the non-ionic detergent Tween 80 to increase wetting and reduce the surface tension of the agar. As shown in Fig. 5(a), swarming was recovered to almost the wild-type level. However, this could not be restored by addition of spent medium from a wild-type culture, indicating that the

Fig. 4. Flagellar and LPS production in the wild-type and $\Delta flhE$ mutant. (a) Activity ($10^{-2} \times$ Miller units) of plasmid *lacZ* gene fusions to the promoter regions of *flhB* (class II) and *fliC* (class III) in *Sal. typhimurium* wild-type (WT) and $\Delta flhE$ and $flhDC^-$ mutants. (b) Flagellin (FlhC) in whole swarm cells (wc), and derived membrane (mem) and cytosolic (cyt) fractions. Total extracellular flagellin (ex) was separated into filament (fil) and soluble monomeric (mon), and analysed by SDS-PAGE and immunoblotting. (c) Swarm cells were stained using SynaptoRed and rabbit-anti-flagellin fluorescent antibodies, and viewed at 540 and 366 nm, respectively, by fluorescence microscopy. (d) Crude and low-molecular-mass LPS (LMM) from wild-type and $\Delta flhE$ strains was visualized by SDS-PAGE (12%) and silver staining.

swarming defect of the $\Delta flhE$ strain was not due to the absence of a secreted surfactant, such as serawettin from *Ser. marcescens* (Matsuyama *et al.*, 1992).

Fig. 5. Recovery of $\Delta flhE$ swarming and changes in $\Delta flhE$ mutant colony morphology. (a) The $\Delta flhE$ mutant incubated (14 h) on swarm agar with and without Tween 80 (0.02 % w/v) surfactant. (b) Wild-type (WT), the $\Delta flhE$ mutant and the $\Delta flhE$ mutant carrying pBAD18-FlhE were grown on agar containing calcofluor (CF; $200 \mu\text{g ml}^{-1}$) for 72 h, agar containing Congo red ($40 \mu\text{g ml}^{-1}$) and Coomassie blue ($20 \mu\text{g ml}^{-1}$) (CR) for 96 h, and in LB in PVC wells for 16 h before visualization of the biofilm with crystal violet (1 %).

It therefore seemed possible that other unknown FlhE-related surface properties were influencing the ability to swarm. We incubated the wild-type, the $\Delta flhE$ mutant, and the $\Delta flhE$ mutant complemented with FlhE, on LB agar containing calcofluor, an LB agar containing Congo red and Coomassie blue, which have been used to highlight altered sugar composition (binding to β -glucans, particularly cellulose) and expression of thin aggregative filaments (curli) in the *Sal. typhimurium* extracellular matrix (Solano *et al.*, 1998; Rmmling *et al.*, 1998). Fig. 5(b) shows that the $\Delta flhE$ mutant colonies have altered colony morphology on both media, and that this phenotype reverted to wild-type when FlhE was provided *in trans*. Such changes in calcofluor-binding properties of colonies have been shown to correlate with mutations in the *bcs* operons responsible for biosynthesis of cellulose (Solano *et al.*, 2002). This change is concomitant with defects in biofilm formation on a glass surface (Solano *et al.*, 1998). However, the $\Delta flhE$ mutant colonies were still able to make biofilm under similar conditions (i.e. glass in adherence test medium) (data not shown), suggesting that the $\Delta flhE$ change in calcofluor binding was not due to alteration in cellulose production. Despite the *Salmonella* wild-type SJW1103 not displaying an rdar phenotype on Congo-red-containing medium, it did display a lacy edged colony morphology, while the $\Delta flhE$ colonies did not (Fig. 5b); this is another indicator of altered extracellular matrix composition. Altered colony

morphology on Congo red plates can be associated with loss of thin aggregative filaments (tafi, also known as curli), encoded in *Salmonella* by the *agf/csg* operon (Römling *et al.*, 1998; Solano *et al.*, 2002; Guard-Petter, 2001). Nevertheless, there are examples in the literature of many variations in Congo red colony morphology, depending not only on curli expression, but also on expression of other factors, such as LPS and polysaccharide biosynthesis genes (e.g. *wzx*E and *wca*I) (Solano *et al.*, 2002).

The extracellular matrices of *Salmonella* and *Esc. coli* are also involved in biofilm formation on other inert surfaces, such as PVC and polystyrene (Mireles *et al.*, 2001; Römling *et al.*, 1998), and reduced swarming and increased adherence to PVC have been reported in a *ddhC* mutant (defective in O antigen synthesis) (Mireles *et al.*, 2001). We assessed biofilm formation by wild-type and Δ *flhE* strains growing on the PVC surface of microtitre wells. After crystal violet staining (Fig. 5b), quantification according to Mireles *et al.* (2001) confirmed the visual impression that the Δ *flhE* mutant formed approximately fivefold more biofilm than wild-type under all conditions tested (0.5–2 % glucose). Altered biofilm formation on PVC surfaces can also be associated with altered curli expression levels, but this is not the case for the Δ *flhE* strain, since assessment of curli levels using anti-CsgA antisera indicated unchanged curli expression (data not shown). Nonetheless, the extracellular matrix is complex, and new components continue to come to light (Wang *et al.*, 2004; Branda *et al.*, 2005).

Conclusion

The data suggest that *flhE* belongs to the flagellar regulon, but is not required for individual cell motility, or any aspect of flagellar production. The data suggest that it nevertheless has a role in the swarming motility of peritrichously flagellated Gram-negative bacteria, possibly influencing the composition of the extracellular matrix, and increasing surface lubrication or wettability. The protein sequences deduced from the *flhE* genes cited in Fig. 1 are short sequences of 138–158 aa that have no significant similarity with any protein in the current sequence databases. All FlhE proteins have a putative signal peptidase I leader sequence, indicative of a periplasmic or outer-membrane location, and removal of this N-terminal sequence (aa 1–16) apparently results in a loss of function. FlhE proteins have 7–13 proline residues, and proline-rich regions are often involved in protein–protein interactions (Seifert *et al.*, 2004; Larsen *et al.*, 1993). The *flhE* gene is not associated in the genome with other unknown genes, suggesting that it is not part of a pathway, but rather that it may encode a structural protein that acts alone on the surface, or contributes to a matrix-specific biofilm; for example, a protein that influences interaction with other cells in raft formation, or lubrication for surface movement. These possibilities are as yet unsupported by data, and it remains to be seen what this motility protein does.

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