**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 Δ*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 Δ*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 Δ*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 calcineurin 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 FlhD2C2, 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 (α28) 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 Δ*flhE* strain, using primers Δ*flhE*for (TCCGATAACGCTCATATCGCATGACGCGGAGACCATTGGAGGAAAATGTTGAGGCTGGAGCTGCTT) and Δ*flhE*rev (TCCGGCAACCTACCTCACATTATATTATTCATTAAAACACGGGTTCCTTTCATCTTAAAAATTCGTTGCGGATCGCTGCC). The pKD4 (KmR) 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 (TGAGGAAACATATGGTGAGGAGGTTGAGGAAAATGTTGAGGCTGGAGCTGCTT) and FlhErev (AACCCGCGAGGTAGTTCTACAATCTACC). And cloned (XbaI–HindIII) S’ of the arabinose-inducible promoter of expression vector pBAD18, to create pBAD18-FlhE. A derivative gene encoding N-terminally truncated FlhEΔN (lacking aa 1–16)
was cloned into pBAD18 after PCR using primers FlhE-T (AATTCTAGAATACTTTTTAACCTTTAAGAATATACCATGG-GGAAAGCGGCTGGCAG) and FlhERev to make pBAD18-FlhEAN.

**Fluorescence microscopy of cells.** Cells scraped from swarm plates were resuspended in saline (to an OD$_{600}$ of 0.05), and fixed onto glass slides using 4% paraformaldehyde (in 20 mM PIPES, pH 7.4) before blocking with PBS (50 mM NaPO$_4$/Na$_2$PO$_4$, 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 PBS, and diluted to an OD$_{600}$ of 1.0. Total extracellular FliC protein (internal to biofilm LB plus 0.5–2% glucose, and incubated overnight at 30 °C) was prepared by shearing (5 min vortex) of harvested cells, and the supernatant at 4 °C was separated into cytosolic and membrane fractions according to Auvray et al. (2001).

**Cell fractionation.** Harvested swarm cells were resuspended in PBS, and diluted to an OD$_{600}$ of 1.0. Total extracellular Fls 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 OD$_{600}$ 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 1−1, 5 g yeast extract 1−1) were inoculated in 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/BamHI cloning of fhlB (using primers FlhBpromEco, GATCTCAGCAGAGACCTTTCATATG; and FlhBPromEco, GAGTCGCCAACAACCTGGGATAG) and flic (primers FlhBpromEco, GATCTCAGCAGAGACCTTTCATATG; and FlhBPromEco, GAGTCGCCAACAACCTGGGATAG) promoter fragments into the lacZ fusion vector pGSP123 (Stafford et al., 2005), which is identical to pRS515 except that Km® is replaced by Gm® (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 fhlB and flic genes was synthesized using Mu-MLV reverse transcriptase (Promega), and primers fhlBrevRT (TTCGGCGCTGGGATATAATG) and flicrevRT (ATTGCTCAGACCTTTCATATG), resulting in cDNA originating within fhlB and flic, respectively. In the final reactions, primers fhlBrevRT/fhlForRT (internal to fhlB, ACCGCTCATCGGCGCGGCGGCGTGCG) and flicrevRT/flicForRT (flic internal, TGGCGCGCGCGCCGCTGGC) were used to amplify internal fragments of fhlB and flic. To assess transcripts spanning the fhlBA and flicE operons, intergenic regions, primer pairs fhlBForRT/flhARevRT (TCCGGAGGTACCCGCGGACCAGG) and flhARevRT (TCCGGATAACCCGCATATCC)/flhERTRev were used. All PCR reactions used Taq polymerase, and products were analysed on 1.5% agarose ethidium bromide (EtBr) gels.

**RESULTS AND DISCUSSION**

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

The fhlE gene in *Sal. typhimurium* is located immediately downstream of the flhBA genes, and the fhlE stop codon overlaps the fhlE start codon (Fig. 1). Nevertheless, a transposon insertion in *fhlE* 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 *fhlE* 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*, *fhlE* 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 *fhlA* gene). The *fhlE* 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 *fhlE*. 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*, *fhlE* is located downstream of the *flhFG* genes thought to be involved in flagellar assembly and gene regulation (McCarter 2001), while in *Ralstonia metallidurans*, *fhlE* is separated from *flhB* by the *flhA* gene that encodes the flagella-specific sigma factor σ$^2_8$. The *fhlE* 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*.

The *fhlE* genes are 400±25 bp and encode proteins of approximately 14 kDa, with a predicted N-terminal signal peptide I leader sequence, and a predicted periplasmic or outer membrane location; the *fhlE* sequences of the 10 genera in Fig. 1 contain between 7 and 13 divergent searches revealed no significant similarity of FlhE to any class of proteins. *fhlE* is transcribed in an *flhBAE* operon activated by FlhD$_2$C$_2$.

The *flhE* operon is transcribed from a class II (early) flagellar promoter upstream of *flhB*, and is therefore...
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 flhDC2 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 flhDC2 strains. Fig. 2 shows that flhE is transcribed as part of a polycistronic messenger RNA in an flhDC2-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...
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 arabino-inducible promoter (Fig. 3). In contrast, a truncated version of FlhE lacking the putative N-terminal 16 aa leader signal peptidase I sequence (MRKWALLLFPLTVQA), 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 Δ*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 (α28-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 *flhE* mutant compared with the wild-type (Fig. 4a), transcription of both genes was unaltered in the Δ*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 Δ*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 Δ*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 Δ*flhE* mutation, so we examined wild-type and Δ*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 Δ*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 Δ*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 EPS (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 Δ*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; Lat et al., 2005), and swarming by such mutants, and of the weakly swarming *Es. 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* Δ*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...
swarming defect of the ΔflhE strain was not due to the absence of a secreted surfactant, such as serawettin from *Serratia marcescens* (Matsuyama et al., 1992).

It therefore seemed possible that other unknown FlhE-related surface properties were influencing the ability to swarm. We incubated the wild-type, the ΔflhE mutant, and the ΔflhE 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 *Salmonella* extracellular matrix (Solano et al., 1998; Romling et al., 1998). Fig. 5(b) shows that the ΔflhE 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 ΔflhE mutant colonies were still able to make biofilm under similar conditions (i.e. glass in adherence test medium) (data not shown), suggesting that the ΔflhE 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 ΔflhE 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 (tafii, 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. *wzxE* and *wcaI*) (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 swimming and increased adherence to PVC have been reported in a *dldc* mutant (defective in O antigen synthesis) (Mireles *et al.*, 2001). We assessed biofilm formation by wild-type and ΔflhE 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 ΔflhE 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 ΔflhE 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 swimming 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 peptide 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.

**ACKNOWLEDGEMENTS**

We thank Jean Guard-Bouldin and Gillian Fraser for useful discussions, Johnathan Green and Lyndsey Brawn for assistance with fluorescence microscopy, and Ute Römling for anti-CsgA antisera. This work was supported by a Wellcome Trust Programme grant (C.H.).

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