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Loss of FlhE in the flagellar Type III secretion system allows proton influx into *Salmonella* and *E. coli*

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Summary

flhE belongs to the *flhBAE* flagellar operon in Enterobacteria, whose first two members function in Type III secretion (T3S). In *Salmonella enterica*, absence of FlhE affects swarming, but not swimming, motility. Based on a chance observation of a 'green' colony phenotype of *flhE* mutants on pH indicator plates containing glucose, we have established that this phenotype is associated with lysis of flagellated cells in an acidic environment created by glucose metabolism. The *flhE* mutant phenotype of *Escherichia coli* is similar overall to that of *S. enterica* but is seen in the absence of glucose and, unlike in *S. enterica*, causes a substantial growth defect. *flhE* mutants have a lowered cytoplasmic pH in both bacteria, indicative of a proton leak. GFP reporter assays indicate that the leak is dependent on the flagellar system, is present before the T3S system switches to secretion of late substrates, and gets worse after the switch and upon filament assembly, leading to cell lysis. We show that FlhE is a periplasmic protein that co-purifies with flagellar basal bodies. FlhE may act as a plug or a chaperone to regulate proton flow through the flagellar T3S system.

Introduction

Bacterial flagella enable individual swimming motility through liquid or group swarming motility over a surface (Macnab, 1996, Harshey, 2003, Kearns, 2010). Flagellar biogenesis has been best studied in *Salmonella enterica* (Macnab, 2003, Chevance & Hughes, 2008, Harshey, 2011). The function of most genes involved in this process is now largely understood. In this work, we report on the function of *flhE*, one of the few flagellar genes with an unassigned role. This gene is found mainly in Enterobacteria (Liu & Ochman, 2007), where it is co-expressed with *flhBA* genes, which encode two major components of the Type III secretion (T3S) system. Absence of FlhE does not affect swimming motility in *S. enterica* (Minamino *et al*., 1994), but has been reported to eliminate swarming motility (Stafford & Hughes, 2007).

Flagellum assembly requires over 60 proteins. The flagellum consists of three major parts: a membrane-embedded basal body, an external hook, and a filament (Fig. 1; see farthest structure on the right). The basal body is composed of a stationary stator and a moving rotor. Force generating units called Mot proteins comprise the stator, and the basal rotor is made of proteins that constitute the cytoplasmic C-ring mounted on a membrane MS ring that is continuous with a periplasmic rod (Fig. 1; see inset). The rod penetrates the peptidoglycan layer to exit through a pore formed by two rings - P and L. Externally, the rod connects to the hook and filament. Two hook-associated proteins, or HAPs, form a short hook-filament junction. The filament is several cell lengths long, is composed of thousands of subunits of a single protein, and is held in place by a capping protein at the tip.

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Flagellar genes are expressed in a hierarchy of three temporal classes (Chevance & Hughes, 2008) (Fig. 1; flow diagram at bottom). The class I *flhDC* operon encodes two transcriptional regulators that direct σ^{70}-dependent transcription from class II promoters, which control genes for assembly of the hook-basal body (HBB) substructure as well as two regulators - σ^{28} (FliA) and its inhibitor FlgM. Completion of the HBB signals export of FlgM, freeing σ^{28} to transcribe class III promoters, which control genes for the filament, the stator proteins, as well as the chemosensory pathway. The P ring (FlgI) and L ring (FlgH) proteins are secreted by the general (Sec) secretion pathway, but a T3S apparatus located at the base of the MS ring uses PMF-dependent secretion for export of all proteins that make up the rod, hook and filament (Minamino & Namba, 2008, Paul *et al.*, 2008) (Fig. 1, inset). This apparatus consists of six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ, FliR), three soluble components that form the ATPase complex for substrate delivery (FliI, FliH, FliJ), and four cytoplasmic proteins that act as substrate-specific chaperones (FlgN, FliA, FliS, FliT). FlhA and FlhB have substantial cytoplasmic domains, which along with the C-ring play an important role in docking the FliI ATPase (Erhardt & Hughes, 2010, Konishi *et al.*, 2009). FlhA is involved in the early stage of export, and FlhB is important for the late stage. A recent mutagenesis study of FlhA has assigned it a PMF-driven export function (Hara *et al.*, 2011). FlhB also acts as an export switch to control late flagellar protein export, as described below. The roles of FliOPQR are not yet understood.

Among proteins that are required for the structure and assembly of the rod and hook, two key players – FliK and FlhB - determine the rod-hook length and the switch to late secretion, respectively (Fig. 1). Absence of FliK abrogates hook-length control, resulting in polyhooks (Hirano *et al.*, 1994). FliK is secreted intermittently to serve as a molecular ruler (Ferris & Minamino, 2006, Shibata *et al.*, 2007, Erhardt *et al.*, 2011). Interaction of the FliK N-terminus ($FliK_N$) with the assembled hook cap (FlgD) is proposed to elicit a pause in FliK secretion. If the C-terminus of FliK ($FliK_C$) is near the C-terminus of FlhB ($FlhB_C$) when the pause occurs, which happens only when the hook reaches a length of 55 nm, their interaction causes auto-cleavage of FlhB, flipping the switch to a late secretion mode. FliK-dependent proteolytic cleavage of FlhB first promotes FlgM secretion, relieving the repression of σ^{28}. Efficient secretion of all late protein substrates requires dedicated chaperones. During this process, the hook cap is discarded and successively replaced first by the HAPs FlgK and FlgL, and then by the capping protein FliD. Filament subunits (either FliC or FljB) are added beneath FliD. In the absence of either the HAP proteins or FliD, filament assembly fails, and flagellin subunits are secreted into the medium (Homma *et al.*, 1984).

FlhE was identified in our laboratory as a protein whose absence reduces swarming motility in *S. enterica* (Butler *et al.*, 2010). Prior to this, there was a report that a null mutation in *flhE* abolishes swarming, with no apparent effect on flagellar assembly or swimming behavior (Stafford & Hughes, 2007). FlhE contains an N-terminal signal sequence for export into the periplasm via the Sec pathway (Minamino *et al*., 1994, Stafford & Hughes, 2007). This sequence was shown to be essential for its swarming phenotype (Stafford & Hughes, 2007). A plausible role for FlhE might be related to the secretory functions of FlhA and/or FlhB, because *flhE* is co-transcribed with *flhBA* (Stafford & Hughes, 2007). In a study hinting at such a role, PL-ring-defective mutants, which are arrested for rod growth, were observed to switch to late secretion in suppressors bearing null alleles of *flhE* (Hirano *et al.*, 2009). We show in the present study that absence of FlhE lowers the cytoplasmic pH in both *S. enterica* and *Escherichia coli*, impairs growth in *E. coli*, and causes filament assembly-dependent cell lysis in both bacteria. Our data implicate a role for FlhE in regulating proton flow through the PMF-driven flagellar secretion system.

Results

Absence of FlhE affects swarming, but not swimming, motility in S. enterica

The swimming and swarming phenotypes of a *flhE* mutant compared to wild-type *S. enterica* are shown in Fig. 2A. As noted earlier (Minamino *et al*., 1994), there was no effect of the mutation on swimming; however, there was ~50% inhibition of swarming. An earlier report found complete inhibition of swarming in a *flhE* mutant (Stafford & Hughes, 2007). The difference in the extent of swarming inhibition could be due to differences in the parental strains used or in the growth conditions; swarming is also sensitive to parameters such as humidity and the commercial source of the agar (Harshey, 2003). Consistent with the earlier report (Stafford & Hughes, 2007), however, the swarming bacteria were not defective in flagella synthesis (see below).

FlhE is a periplasmic protein detectable in flagellar basal body preparations

The *flhE* gene encodes a 130-amino-acid protein. The N-terminal 16 amino acids of FlhE display features typical of a cleavable signal sequence. Full-length and cleaved forms of the protein were identified in maxicell labeling experiments in *S. enterica*, consistent with secretion of FlhE into the periplasm by the general secretory pathway (Minamino *et al*., 1994).

To determine the location of FlhE in flagellated *S. enterica* cells, FlhE-FLAG, a functional epitope-tagged variant of FlhE, was used. When the tagged protein was expressed from its chromosomal location, the *flhE* defect was fully complemented but the FLAG signal was weak, as expected due to the low mRNA levels of the *flhBAE* operon (Wang *et al*., 2004). When FlhE was expressed from a plasmid, cell fractionation experiments showed that it was present in the cytoplasmic and periplasmic fractions, but not in the membrane fraction (data not shown). Isolated basal body preparations showed the presence of FlhE using FLAG antibodies when the plasmid expressed FlhE-FLAG, but not in the vector control (Fig. 2B). The recovery of intact basal bodies was confirmed by detection of two proteins integral to it - the cytoplasmic C-ring protein FliG and the rod protein FlgG (see cartoon on right). Cross-contamination of the flagellar preparation from membrane or periplasmic fractions was assessed by probing for the presence of the cytoplasmic membrane protein TonB and the periplasmic maltose-binding protein (MBP) in these preparations. These proteins were not detected. In summary, these data show that the periplasmic protein FlhE is also included within the basal body.

flhE mutants are green on pH indicator plates, a phenotype associated with cell lysis *Salmonella* geneticists routinely use ‘green’ plates in transduction experiments to distinguish phage P22-carrying or P22-free transductants as green versus yellow colonies, respectively. Green plates contain LB media with ~0.8% glucose and the pH indicator dyes aniline blue and alizarin yellow. In the case of P22 lysogens, lysis is required to observe the green color (Smith & Levine, 1967). Other mutants that show a green phenotype, for example *recA* strains, are also associated with cell death and lysis. Although the molecular basis of the color-lysis association is not known, it is generally believed that anything that weakens the membrane integrity to cause solute leakage and cell lysis, gives green colonies.

We observed that even in the absence of exposure to P22, *flhE* mutant colonies were green on these plates (Fig. 3A). When propagated on green swarm plates, the whole-cell (WC) suspension of the *flhE* mutant is clearly green compared to wild-type (Fig. 3B). When centrifuged (3000 *g*, 5 min), the green material forms a layer on top of the cell pellet. The yellow and green layers in the *flhE* mutant pellet were both seen to consist of cells under the microscope. Aniline blue is known to bind to different macromolecules: nucleic acids, glucans, and hydrophobic proteins (Evans, 1984, Hough *et al.*, 1985, Kippert & Lloyd,

1995). We surmise that the green color is the result of binding of the dyes to some cellular fraction either released from lysed cells or bound by dye that can permeate the dead cells (likely peptidoglycan, because spotting this macromolecule on the plates gave rise to green spots; not shown). Several tests for cell lysis were conducted and are shown in panels C-E. First, there was a 10% reduction in colony forming units (CFUs) in the *flhE* mutant compared to wild-type (Fig. 3C). Second, live-dead staining, where live cells are stained green and dead cells red, revealed a similar proportion (10%) of dead cells in the *flhE* mutant (Fig. 3D). Finally, agarose gel electrophoresis of supernatants of cultures, prepared as in Fig. 3B, showed the presence of released genomic DNA only in the *flhE* mutant (Fig. 3E). We conclude that the green color on the dye-containing plates arises from cell lysis within the *flhE* mutant colony. We surmise that cells in the green layer in Fig. 3B are less dense because of loss of their cytoplasmic content.

Cell lysis in S. enterica flhE mutants requires glucose

Glucose is a common component of both green plates and swarm plates. To test if glucose is needed for the cell-lysis phenotype, *S. enterica* wild-type and *flhE* mutant strains were grown on green swarm plates with and without glucose. The genomic DNA-release assay showed cell lysis only in the presence of glucose in the *flhE* mutant (Fig. 4A). Similar results were observed when arabinose was substituted for glucose.

Sugar metabolism in bacteria generates acids (Stokes, 1956), which lowers the pH of the growth medium, as seen in a broth culture in Fig. 4B. To test if it is the lowered pH that is responsible for cell lysis, the medium was initially buffered with HEPES to prevent pH changes. Increasing buffer concentration resulted in decreasing cell lysis (Fig. 4C), supporting this notion (HEPES concentrations higher than 100 mM were toxic, in that they induced lysis in wild-type cells). We conclude that FlhE provides a protective function against acidic pH in *S. enterica*. In experiments described below, we have used the green colony phenotype and/or genomic DNA release assay to dissect the timing and requirements for FlhE function.

Filament assembly is essential for cell lysis in flhE mutants

The green colony phenotype of the *S. enterica flhE* mutant provides a convenient handle for isolating second-site color suppressor mutants. A large-scale Tn*10d*Cm transposon mutagenesis of the *flhE* strain yielded 3.4% yellow suppressors (i.e. wild-type colony color; see Experimental Procedures). A majority of these suppressors were non-motile, suggesting that the flagellar system was important for the green phenotype. Of those that were motile, none had wild-type levels of motility. Suppressor mutants displaying ~ 40–80% of parent motility were sequenced (Table S1). Of these, a majority (16/27) mapped between *lrhA* and *yfbQ* genes. LrhA is a positive regulator of *flhDC* in *E. coli* (Lehnen *et al.*, 2002). Given that the mutants had diminished motility, we assume that the insertion diminished *lrhA* expression. When complemented with *flhDC* on a plasmid (p*flhDC*), these mutants regained full motility and turned green (data not shown), as did the *mdo* and *rcs* mutants known to downregulate flagellar gene expression in *S. enterica* (Toguchi *et al.*, 2000, Wang *et al.*, 2007). A consensus emerged that the green color depended on increased numbers of flagella.

To determine whether FlhE function is associated with a particular step of flagellar biogenesis, we systematically examined mutations in key flagellar genes in the *flhE* mutant background, only a subset of which is shown in Fig. 5. *flhDC, fliK, fliA, flgK/L, fliC-fljB,* and *fliD* mutants were yellow (Fig. 5A), and showed no lysis (genomic DNA release) on agarose gels (Fig. 5B), whereas the *motA* mutant was green and showed lysis. *fliK* and *fliA* mutants are defective in class 3 gene expression (see Fig. 1), whereas *flgK/L, fliC-fljB and fliD* mutants are proficient in both class 3 transcription and protein export but cannot

assemble an intact filament (see Fig. S1). The *motA* mutant can assemble flagella but cannot power their rotation. We conclude that filament assembly, but not rotation, is essential for cell lysis.

We had noticed that when p*flhDC* was introduced into yellow suppressor mutants shown in Table S1, they not only regained higher levels of motility, but their colonies became darker green compared to the *flhE* mutant parent. Also, the green color of the *flhE* mutant (but not the wild-type parent) turned darker green in the presence of p*flhDC* (Fig 5A, compare last two panels on row 2). This darkening of the green color was accompanied by dramatically increased cell lysis as measured by both the genomic DNA-release assay (Fig. 5B) and the CFU assay (Fig. 5C). Lysis depended on filament assembly under these conditions as well i.e. all of the yellow mutants shown in Fig. 5A remained yellow in the presence of p*flhDC* (not shown). Under the inducing conditions used, p*flhDC* increases flagella numbers ~ 2–3 fold. These results show that not only were filaments important for cell lysis, but that higher numbers of flagella caused more lysis.

To explore this result further, we introduced a *fliS* mutation eliminating the filament-protein chaperone into the *flhE* strain. This mutant is reported to make short filaments (Yokoseki *et al.*, 1995, Auvray *et al.*, 2001) (Fig. 5D). The *flhE fliS* double mutant was yellow (Fig. 5A), and showed no lysis (Fig. 5B) unless the strain also contained p*flhDC*, in which case the number of short filaments doubled (Fig. 5D). Under those conditions lysis increased modestly compared to the *flhE* strain with p*flhDC* (Fig. 5C). We conclude that filament length is important for lysis and that increased filament numbers increase cell lysis.

Absence of FlhE shows filament assembly-dependent cell lysis even when flagella grow in the periplasm

In order to gain insight into FlhE function by a different method, we monitored differences in gene expression profiles between wild-type and *flhE* mutant strains by microarray experiments. The data showed a slight upregulation of colanic acid biosynthesis genes in the *S. enterica flhE* mutant compared to wild-type (Table S2). Upregulation of these genes can be indicative of stress on the cell envelope activating, via the outer membrane protein RcsF, the Rcs signaling pathway (Laubacher & Ades, 2008). In gram-negative bacteria, stress sensors monitor permeability of the outer membrane, folding of envelope proteins, and energization of the inner membrane (Raivio, 2005, Rowley *et al.*, 2006). To test whether the filament-dependent cell lysis was due to stress on the outer membrane from the point of exit of the filament, we repeated the experiments described above in a *S. enterica* PL-ring mutant (*flgHI*) background. Flagella do not exit the outer membrane in this situation, and they grow inside the periplasm when combined with a *flhE* mutation (Chevance *et al.*, 2007) (Fig. 6A). Here too, the green color and lysis depended on absence of FlhE and the presence of an intact filament (Fig. 6B, C). *flgK* and *flgL* (HAP) mutants, which lack the hook-filament junction proteins, secrete flagellin subunits but fail to assemble a flagellum (see Fig. S1). The *flhE flgHI* mutant was observed to be more susceptible to lysis compared to the *flhE* mutant alone (Fig. 6C); indeed, induction of p*flhDC* in the *flhE flgHI* background was lethal. We conclude that filament-dependent lysis is independent of either external or periplasmic location of the flagella.

Tsr-dependent tumbling response indicates lowered cytoplasmic pH in the flhE mutant

Experiments in Fig. 4 show that the cell lysis phenotype of the *flhE* mutant depends on a lower external pH. Because *flhA*, which is co-transcribed in *flhE*, is implicated in PMF-driven protein export (Hara *et al*., 2011), we wondered if these proteins function in a common pathway such that absence of FlhE might cause a proton leak. Acidification of the cytoplasm is perceived as a repellent (tumble-inducing or CW rotation-producing) signal by

the Tsr chemoreceptor (Kihara & Macnab, 1981, Repaske & Adler, 1981). We therefore used this assay to assess whether the cytoplasm was acidified in the *flhE* mutant when the external pH was lowered. Because cells adapt rapidly to attractants and repellents, our experimental design incorporated our observation that upregulation of *flhDC* increases cell lysis (Fig. 5). p*flhDC* is under P_{BAD} promoter control, and is thus induced by arabinose. Addition of arabinose has the dual effect of lowering the external pH and increasing flagella. Wild-type and *tsr* strains with and without the *flhE* mutation and harboring p*flhDC* were videotaped and their run-tumble bias monitored at 0 min (no inducer) and 30 min after arabinose addition, as described in Experimental Procedures. The data are shown in Fig. 7. In the *flhE* mutant, tumble frequency increased in the tsr^+ (compare 7A and B) but not in the *tsr* strain (compare Fig. 7C and D). Based on this assay, we conclude that absence of FlhE leads to acidification of the cytoplasm.

Intracellular pH monitored by GFP reporter plasmids in S. enterica and E. coli

The chemoreceptor-based assay described above can only be performed after the switch to late or class 3 flagellar gene expression has taken place, because both the filament and the chemoreceptors are class 3 gene products (see Fig. 1). To test cytoplasm acidification by a second method, as well as to determine if the acidification is seen prior to the class 3 switch, a GFP reporter plasmid (GFPmut3b) that reports effectively on pH changes in the cytoplasm was used (Kitko *et al.*, 2009). The data are shown in Fig. 8. The fluorescence emission spectra at various intracellular pHs, when equilibrated to the external pH using sodium benzoate, respond to changes in pH, as previously reported (Fig. 8A). These data were used to generate a standard curve correlating internal pH with fluorescence intensity (Fig. 8B). To test whether the internal pH of the *flhE* mutant was lower than that of its wild-type parent, the cultures were suspended in LB buffered to pH 5.5, similar to the pH measured during growth in glucose (Fig. 4B). Under these conditions, the cytoplasmic pH of the *flhE* mutant was seen to be lower than wild-type (Fig. 8C), supporting the results from the chemoreceptor/repellent assay shown in Fig. 7. However, attempts to measure pH differences in mutants stalled at various steps in the flagellar biogenesis pathway, gave variable results (showing differences between the mutants on some days and not on others), and were inconclusive.

Salmonella and *E. coli* have somewhat different acid resistance mechanisms (Foster, 2004). We therefore turned to *E. coli*, both to verify the *S. enterica* results and to test if the GFP pH reporter gave more reliable data in *E. coli*. In contrast to *S. enterica*, the *E. coli flhE* mutant grew poorly (Fig. 9A), and formed tiny colonies on LB plates. This mutant also showed a smaller swim-colony diameter and was completely defective in swarming (Fig. S2A). The growth defect is sufficient to explain the poor swim/swarm phenotypes of this mutant. Faster-growing variants, all of which were non-motile, accumulated readily, indicating that the growth defect of *E. coli flhE* strain is also related to the flagellar system. The *E. coli flhE* mutant behaved similarly to the *S. enterica flhE* mutant on green plates and in agarose-gel assays for cell lysis, except that these phenotypes were independent of added glucose (Fig. S2 B, C). As in *S. enterica*, the phenotypes depended on filament assembly. For example, in *fliK* and *flgL* mutant backgrounds, the *flhE* mutant failed to show growth defects (Fig. 9A; only *fliK* mutant shown) or release of genomic DNA (data not shown). GFPmut3b reporter assays were more reproducible in *E. coli* (Fig. 9B), although here too we experienced day-to-day variations in the absolute pH values. However, the difference in pH between the strains measured on the same day is statistically significant (p-value < 0.005 for pH difference between wild-type and *flhE* mutant). As with *S. enterica*, the *flhE* mutation lowered the cytoplasmic pH in *E. coli*. The lower pH was associated with the *flhE* mutation in both the *fliK* and *flgL* mutant backgrounds. These data indicate that the cytoplasm is acidified prior to the switch to late secretion (*fliK flhE*), is still seen after the switch (*flgL*

flhE), and gets worse upon filament addition (*flhE*). Because the former two mutants neither lyse nor show growth defects (Fig. 9A), acidification, in and of itself, is not responsible for lysis. How filament assembly contributes to lysis is not clear.

Discussion

This study links FlhE to flagellar T3S function in *S. enterica* and *E. coli*. Based on flagella-specific growth defects and acidification of the cytoplasm in the absence of FlhE, our data suggest a role for FlhE in regulating proton flow through the T3S system.

Acidification of the cytoplasm vs cell lysis

In the absence of FlhE, a lowering of cytoplasmic pH is indicated by two different assays: a Tsr-dependent repellent response to low pH (Fig. 7) and changes in the fluorescence intensity of a GFP reporter (Figs. 8 and 9). We interpret these data to indicate a proton leak in the absence of FlhE. The leak must be through the flagellar T3S channels, because it depends on the flagellar system (Fig. 9). We note that proton leaks have been reported through the flagellar stator proteins in several *motB* mutants (Hosking *et al.*, 2006). The *flhE* mutant phenotype we report is independent of Mot proteins (Fig. 5).

Overall, the *flhE* phenotypes were similar in *S. enterica* and *E. coli*. For example, *flhE* mutants in both bacteria were impaired for swarming (Fig. 2 and S2), were green on pH indicator plates (Figs. 3 and S2), showed filament-dependent cell lysis (Figs. 5, 6, 9, S1 and S2), and had lower cytoplasmic pH (Figs. 8, 9). However, there were important differences. For example, these phenotypes were glucose-dependent in *S. enterica* (Fig. 4), but were not glucose-dependent in *E. coli* (Fig. S2). The *E. coli flhE* mutant grew poorly in LB media in contrast to the *S. enterica* mutant (Figs. 4, 9). Thus, *E. coli* is more sensitive to the loss of *flhE*. This could be due to subtle differences in the T3S systems of the two bacteria, or to better tolerance of acid stress in *Salmonella*.

GFP reporter assays in *E. coli* indicate that the proton leak is present before the switch to late secretion and gets worse after the switch and upon filament addition (Fig. 9). However, cells do not lyse until filaments are added in both *S. enterica* (Figs. 5, 6 and S1) and *E. coli* (Figs. 9 and S2). Thus, cell-lysis is a filament-dependent, post-acidification event. We do not fully understand why this is so. We cannot attribute filament-induced lysis to the viscous load known to be exerted on rotating external filaments, because neither rotation nor an external location of the filaments is required (Figs. 5 and 6). That the lysis phenotype is heightened when flagella grow in the periplasm, where they are not anchored firmly in the peptidoglycan layer (Fig. 6), leads us to propose that, when the internal pH is low, the added stress of long filaments pulling on the inner membrane weakens the membrane further, and exacerbates the proton leak to cause solute efflux and lysis.

Non-viability/cell death of only 10% of cells in the *S. enterica flhE* mutant (Fig. 3) may reflect the fraction of cells that have the critical number of flagella needed to trigger lysis. We suggest that the *flhE* mutation affects swarming more than swimming because the fraction of cells with more flagella tends to lead the swarming pack and, being disproportionally affected, interferes with movement of cells that follow; in addition, the swarming environment is more aerobic than when swimming under the agar (Wang et al., 2004), and produces more lysis (data not shown).

Our data provide an explanation for the observation that absence of FlhE induces the late secretion switch in a PL-ring mutant background (Hirano *et al*., 2009). The flagellar secretion-specificity switch is coupled to HBB completion (Chilcott & Hughes, 2000, Chevance & Hughes, 2008), but it has been reported to occur prematurely, i.e. prior to HBB

completion, in null mutations in *flhE* (Aldridge *et al*., 2006, Chevance *et al*., 2007, Hirano *et al*., 2009). Two events must take place for the flagellar T3S system to change secretion specificity: interaction of $FliK_C$ with $FlhB_C$ and cleavage of $FlhB_C$. Although FlhB can autocleave spontaneously *in vitro* (Ferris *et al*., 2005), FliK interaction is thought to stimulate the cleavage *in vivo*. We propose that acidification of the cytoplasm that accompanies absence of FlhE, switches secretion specificity by changing the conformation of FlhB and/or proteins like FlhA/Flk that have been proposed to protect FlhB until its timely interaction with $FliK_C$ (Hirano *et al*., 2009).

FlhE function

Acidification of the cytoplasm in the absence of FlhE may imply a 'proton plug' or 'lid' function for FlhE. Such a function has been proposed for a periplasmic segment of the stator protein MotB, which together with MotA, forms a transmembrane proton channel (Hosking *et al*., 2006). The plug region was identified as a 20 amino acid segment of MotB, which, when deleted or mutated, caused a massive influx of protons, acidifying the cytoplasm without significantly depleting the proton motive force. The growth inhibition associated with the plug mutation was surmised to be a result of the acidification itself or some following consequence, such as potassium or water efflux from the cells. Under those conditions, cell lysis also depends on filament assembly (Fig. S3).

An alternative function for FlhE is that of a chaperone. Dedicated cytoplasmic chaperones are common in T3S systems, where they help maintain their substrates in a secretion-competent state, delivering them to secretion machinery in manner designed to facilitate travel through the T3S apparatus in an unfolded or partially folded manner (Galan & Wolf-Watz, 2006, Chevance & Hughes, 2008, Minamino & Namba, 2008). Periplasmic chaperones have been identified in assembly of the structural elements of flagella (Nambu & Kutsukake, 2000), needle complexes (Schuch & Maurelli, 2001) and pili (Sauer *et al*., 2000), where they are thought to bind, stabilize, and cap interactive surfaces of subunits until they are assembled. The MS ring scaffold has to accommodate six different T3S transport proteins, all correctly juxtaposed to regulate timely export. It is possible that FlhE assists in optimizing their packing arrangement, ensuring a tight seal.

A plug/lid role for FlhE fits in with the observation that the phenotype in *Salmonella* is manifest when the external pH is low. Such a role would tie in nicely with acquisition of the *flhE* gene mainly by the Enterics, as these bacteria experience low pH during transit through the stomach. However, in *E. coli*, the *flhE* mutant phenotype does not depend on lowering of the external pH. Furthermore, the presence of *flhE* in some non-enterics such as *Azotobacter*, *Chromobacter*, and *Ralstonia*, in which *flhE* is not part of the *flhBA* operon and displays only a 28–38% homology to the *flhE* gene in the enterics (Stafford & Hughes, 2007), also suggests that a role for FlhE as a chaperone is plausible. Although not much is known about the nature and assembly of flagellar secretion apparatus or of the proton channels that drive secretion (Minamino & Namba, 2008, Paul et al., 2008), it is attractive to think that FlhE might play a role in promoting optimal interactions among components of the T3S apparatus.

Experimental Procedures

Bacterial growth conditions, strain construction and reagents

Strains and plasmids are listed in Table 1. Bacteria were grown in 2% L-broth (LB) base unless otherwise stated. Swim plates were made with 0.3% Bacto agar, and swarm plates with 0.6% (*S. enterica*) or 0.5% (*E. coli*) Eiken agar (Eiken Chemical, Tokyo, Japan) supplemented with 0.5% glucose (Wang *et al*., 2004, Wang *et al*., 2005, Mariconda *et al*.,

2006). Both swim and swarm plates were inoculated in the center of the plate with 8 μl of an overnight culture. All experiments were at 37°C for *S. enterica* and 30°C for *E. coli* (Parkinson, 1978). Green plates were prepared with 2% LB supplemented with 0.86% glucose, aniline blue (0.67 g/liter), alizarin yellow (0.065 g/liter), and either 1.5% Bacto agar (hard agar) or 0.5% Eiken agar (swarm agar; glucose concentration was 0.5% in these plates). Photographs of colonies growing on green plates were taken by Canon PowerShot SD1000. Antibiotics used in this study were ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), and tetracycline (12.5 μg/ml).

Deletion of *S. enterica* and *E. coli* genes was achieved by the one-step mutagenesis procedure previously described (Datsenko & Wanner, 2000, Wang *et al*., 2005). All primers used are listed in Table S3. Initial deletions involved selection with a kanamycin (*kan*) or tetracycline (*tetRA*) cassette. In *S. enterica*, the region replaced in *flhE* covers 330 bp, starting from nucleotide (nt) 61 (1 refers to A of the start codon), in *flgL*, 896 bp from nt 51, in *fliD*, 1404 bp from nt 1, in *fliS*, 248 bp from nt 100, and in *flgHI*, 1670 bp from nt 61 in *flgH* to nt 70 in *flgI*. In *E. coli*, for *flhE*, the region replaced covers 330 bp from nt 61, for *flgL* 876 bp from nt 40, and for *fliK* 1028 from nt 61. Verification of deletions was achieved by DNA sequencing. Mutant combinations in *S. enterica* were constructed by P22 transduction. Curing the *kan* cassette was achieved by expression of the FLP recombinase encoded on pCP20 (Datsenko & Wanner, 2000).

Antibodies against *E. coli* FliG were provided by David Blair, *S. enterica* anti-FlgG antibody by May Macnab and *E. coli* anti-TonB antibody by Kathleen Postle. Anti-FljB antibody was purchased from Becton Dickinson, anti-MBP antibody from New England Biolabs, anti-FLAG antibody from Sigma, and Texas-Red conjugated anti-rabbit antibody from Molecular Probes.

Plasmids

Plasmids obtained from other laboratories are indicated in Table 1. Flagellar genes from *S. enterica* were amplified with PCR from the chromosomal DNA of appropriate bacterial strains and ligated into pTrc99a (Amann *et al.*, 1988). Epitope tags such as FLAG were conjugated to the genes by PCR. For GFPmut3b expression, gene sequences were amplified with PCR from the plasmid pMMB1311 and moved to pTrc99a.

Isolation of intact flagella from cells

Bacterial flagella were isolated as previously described (Francis *et al.*, 1994) with the following modifications. A liter of wild-type *S. enterica* cells containing pTrc99-FlhE-FLAG were grown at 37°C with shaking in LB supplemented with 10 μM IPTG and ampicillin. They were collected in late log phase (4 h time point) by centrifugation at 4000 *g* for 20 min, and resuspended with 100 ml of ice-cold sucrose solution (0.5 M sucrose, 0.1 M Tris-HCl, pH 8.0). All solutions added were the same as those in the original protocol. The pH was raised with 1 M NaOH to pH 10–12. The lysate was centrifuged as described, and the pellets were re-suspended in 10 ml of alkaline solution.

SDS-PAGE, flagellin staining and Western blots

All samples were suspended in 6X SDS-PAGE sample buffer before electrophoresis in 12% polyacrylamide gels, followed by transfer to membrane for Western blot analysis. For whole cell extracts, the bacterial suspension was diluted to an OD_{600} = 0.6. Immobilion-P membrane (Millipore) was used for Western blots, performed according to standard protocols (Sambrook *et al.*, 1989) and developed using the Enhanced chemiluminescence (ECL) kit from Amersham.

To detect the secreted flagellin, cells were harvested from on green swarm agar plates with 1XPBS after 6 h and normalized to OD_{600} = 0.3. They were centrifuged at 3000 *g* for 5 min, the supernatant was mixed with 6X SDS-PAGE sample buffer and electrophoresed in 10% polyacrylamide gels. The gels were washed twice with deionized water and stained for 1h with SimpleBlue™ SafeStain (Invitrogen).

Measurement of protein content

Protein concentration was measured using the BioRad Protein Assay according to the manufacturer's instructions. Protein bands obtained from the Western blots were quantitated by BioRad Gel Doc and Quantity One 4.4.0 software.

Live/Dead Staining

The stain kit was purchased from Invitrogen, and cells were stained according to the manufacturer's specifications. The kit includes two nucleic acid stains: green-fluorescent STYO 9 and red-fluorescent propidium iodide (PI). STYO 9 labels both live and dead bacteria alike, whereas PI reduces STYO 9 stain intensity only after crossing damaged cellular membranes. Staining was performed as described (Butler et al., 2010) with the following modifications. Cells were grown on swarm agar plates for 6 h and harvested with 1X PBS and diluted OD_{600} = 0.6. To determine red/green cell numbers, at least 200 cells were counted for each strain analyzed.

Growth curves, cell counts, and agarose gel electrophoresis

The growth profiles of strains were measured as colony-forming units (CFUs). Overnight cultures were diluted 1:100 in fresh LB (50 ml) and 100 μl of the cultures were collected every 1 h for up to 6 h. Serial dilutions were made with 1X PBS and plated to determine CFUs. To measure CFUs on green swarm plates, cells were grown on these plates for 6 h, collected with 5 ml of 1X PBS, normalized to OD_{600} = 0.3, and CFUs were determined after serial dilution.

To detect the chromosomal DNA on green swarm agar, swarm cells were harvested from plates after 6 h and adjusted to similar OD_{600} readings for all strains. The whole cell (WC) suspension was centrifuged at 3000 *g* for 5 min to separate the cell pellet and supernatant. 15 μl of supernatant were mixed with 3 μl of loading dye, electrophoresed on a 0.8% agarose gel with a 1kbp DNA ladder (NEW ENGLAND BioLabs) and visualized by a BioRad Gel Doc. The position and identity of genomic DNA were confirmed independently by monitoring the migration position of isolated genomic DNA under the same conditions, as well as testing the DNA band for chromosomal markers by PCR.

pH measurements of bacterial cultures

S. enterica cultures were grown overnight in LB (pH 7.0), diluted 1:100 in fresh LB broth (100 ml) with and without the addition of 0.5% glucose, and incubated up to 6 h. 5 ml of culture was collected every 30 min and pH of the culture was measured by a UB-10 UltraBasic pH/mV Meter (Denver Instruments).

Transposon mutagenesis to isolate extragenic supressors of the flhE green phenotype

Phage P22 was grown on donor stain (TH341) carrying Tn*10d*Cm (defective Tn*10*, lacking the transposase), and used to generate a random pool of Tn*10d*Cm by infection of a wild-type *S. enterica* strain harboring pNK972 (plasmid expressing the functional transposase gene of Tn*10*). The Cm^R transductants were pooled together, P22 lysates were prepared from the pool, and the Tn*10d*Cm insertions were transduced into the *S. enterica flhE::kan* strain ST005, selecting on green plates supplemented with chloroamphenicol and

kanamycin. Out of 26,827 colonies screened, 915 were yellow. 84% (771/915) of yellow mutants were non-motile. The remaining 144 motile mutants were re-tested by moving the Tn*10d*Cm into a clean *flhE::kan* strain by P22 transduction to confirm the yellow phenotype. Although all the transductants were still motile, only 37 were truly yellow. These yellow motile mutants could be categorized into three classes based on their swimming speed: compared to the parent strain, the motilities of class I –class III mutants were >80%, 40–80%, and <40%, respectively. The Tn*10d*Cm insertion sites of the class I and II mutant subtypes were mapped as follows: Chromosomal DNA from the mutants was purified by Wizard® Genomic DNA purification kit (Promega), partially fragmented by Sau3AI, and then ligated to a BamHI site in the high-copy number plasmid pAmCyan (Clontech) containing Amp^R. Ligation mixtures were electroporated into *E. coli* DH5α. Plasmid DNA from Cm^R/Amp^R transformants was purified using a Qiagen mini-prep kit and sequenced using vector primers from both sides of the insert (Table S3). The data are shown in Table S1; the gene descriptions are from (McClelland *et al.*, 2001).

Microarray experiments

Transcription profiles of *S. enterica* wild-type and *flhE* mutant strains propagated on swarm plates for 3 h were obtained by microarray experiments performed as described (Wang *et al.*, 2004).

Repellent response measured by monitoring trajectories of swimming cells

S. enterica wild-type, *flhE, tsr*, and *tsr flhE* mutant strains containing pBAD33-*flhDC* were used. Overnight cultures were diluted 1:100 in LB and shaken at 37°C until the cultures reached an OD_{600} of 0.6. A 10 μl aliquot (pre-induction) was sampled before arabinose was added (0.2% v/v) to induce *flhDC* expression and a second sample was taken after 30 min. Cells were observed by phase-contrast microscopy and recorded up to 10 min on an external Sony video recording device with Windows Movie Maker software. Three biological replicates were prepared for swim-motility analysis, and 90 to 200 cells were observed under each condition. To measure reversal frequencies, the straight-swimming distance was defined as ten-times the length of a bacterial cell (25 μm). Cells that swam straight without a tumble in this distance were categorized as having a wild-type or ‘normal’ bias. The swimming distance of each cell was measured manually on the computer screen.

Measurement of cytoplasmic pH with GFP reporter plasmid

Cytoplasmic pH was measured using GFPmut3b, a pH-sensitive green fluorescent protein, as described (Wilks & Slonczewski, 2007, Kitko *et al*., 2009). A standard pH curve was generated using a wild-type strain containing pTrc99a-GFPmut3b. An overnight culture grown at 37°C was diluted 1:100 in fresh potassium-modified LB (LBK [pH7.5]), which was buffered with 20 mM homopiperazine-*N*, *N*′-bis-2-(ethanesulfonic acid) (HOMOPIPES) and supplemented with 40 μM IPTG and ampicillin. Cells were grown to OD_{600} = 0.6 and pelleted at 4800 *g* for 4 min. They were resuspended to an OD_{600} of 0.4 in M63 minimal-medium (1.5% casein hydrolysate, 0.8% glycerol, and 50 mM HOMOPIPES). The pH of M63 medium was adjusted with KOH to pH 5.5 to 7.5. To equilibrate cytoplasmic pH with external pH, 30 mM sodium benzoate was added to the cultures.

Measurement of cytoplasmic pH of experimental samples was performed as described above, except that cultures were grown LBK buffered with 20 mM HOMOPIPES pH 5.5, the external pH attained by cultures growing in the presence of glucose (Fig. 4B), and sodium benzoate was omitted. Cultures were kept on ice prior to recording the GFPmut3b excitation spectra.

Fluorescence measurement of pH reporter plasmids

The excitation spectra of GFPmut3b were recorded using a PTI Quanta Master Model C scanning spectrofluorometer. Three ml of cell suspension were placed into a Bio-Rad VersaFluor cuvette with a path length of 10 mm. Excitation was measured from 480 to 510 nm (slit width of 2 nm), using an emission wavelength of 545 nm (slit width of 20 nm) (Wilks & Slonczewski, 2007). Three biological replicates were measured. To determine the standard curve correlating internal pH with fluorescence intensity, GFPmut3b intensities at pH 5.5, 6.0, 6.5, 7.0, and 7.5 were obtained. The intensities at pH5.5 and 7.5 were fitted to a linear equation ($y = mx + b$, where y is the GFPmut3b intensity, x is pH value, m is slope, and b is y-intercept) to obtain the slope ($m = 4.75 \times 10^4$) and y-intercept ($b = -1.9 \times 10^5$). The equation (GFPmut3b signal intensity = $4.75 \times 10^4 \times$ pH - 1.9×10^5) was used to convert the signal intensities (sum of 480 nm to 510 nm) obtained from experimental samples to pH units.

Flagella staining and Microscopy

Flagellar filaments were stained as described (Attmannspacher *et al.*, 2008) and cells were observed as reported previously (Paul *et al.*, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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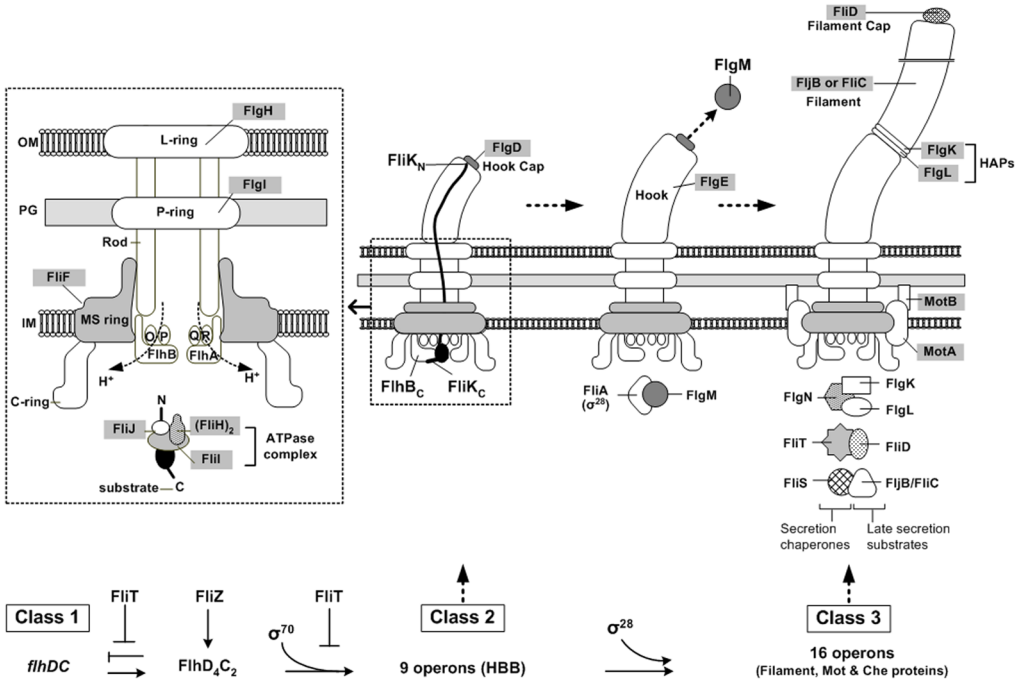

Fig. 1.

Expression and assembly of *S. enterica* flagellar proteins. Only structures/proteins relevant to this study are indicated. See text for details. OM, outer membrane; PG, peptidoglycan; IM, inner membrane; N and C, amino- and carboxy-terminal regions of secreted substrate.

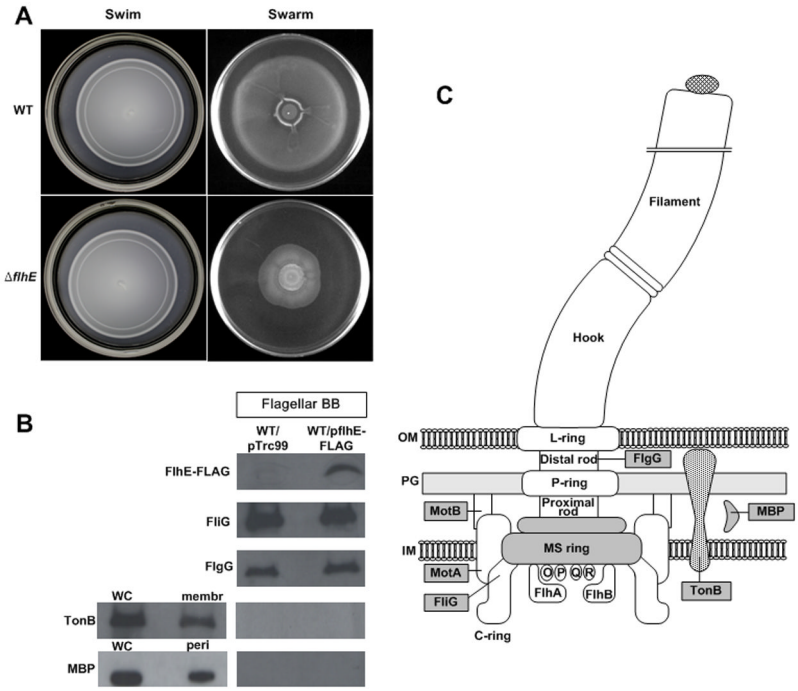

Fig. 2.

Motility phenotype of a *flhE* mutant and localization of FlhE in *S. enterica*. **A.** Swimming and swarming motility of wild-type and *flhE* mutant (ST004) of *S. enterica*. Cultures were inoculated at the center of 0.3 % agar plates for swimming and 0.6 % agar plates for swarming. Plates were incubated at 37°C for 6 h. **B.** Western blots detecting the presence of FlhE-FLAG in isolated flagellar basal bodies. C-ring protein FliG and rod protein FlgG were detected with antibodies specific to these proteins. The bottom two panels show controls that follow the location of the membrane protein TonB and periplasmic maltose-binding protein (MBP) in either whole cells (WC), membranes (membr), the periplasm (peri) or basal body (BB) preparations, using antibodies to these proteins. All lanes contained ~3 μg of protein. **C**. Schematic showing location of the flagellar and membrane proteins shown in B.

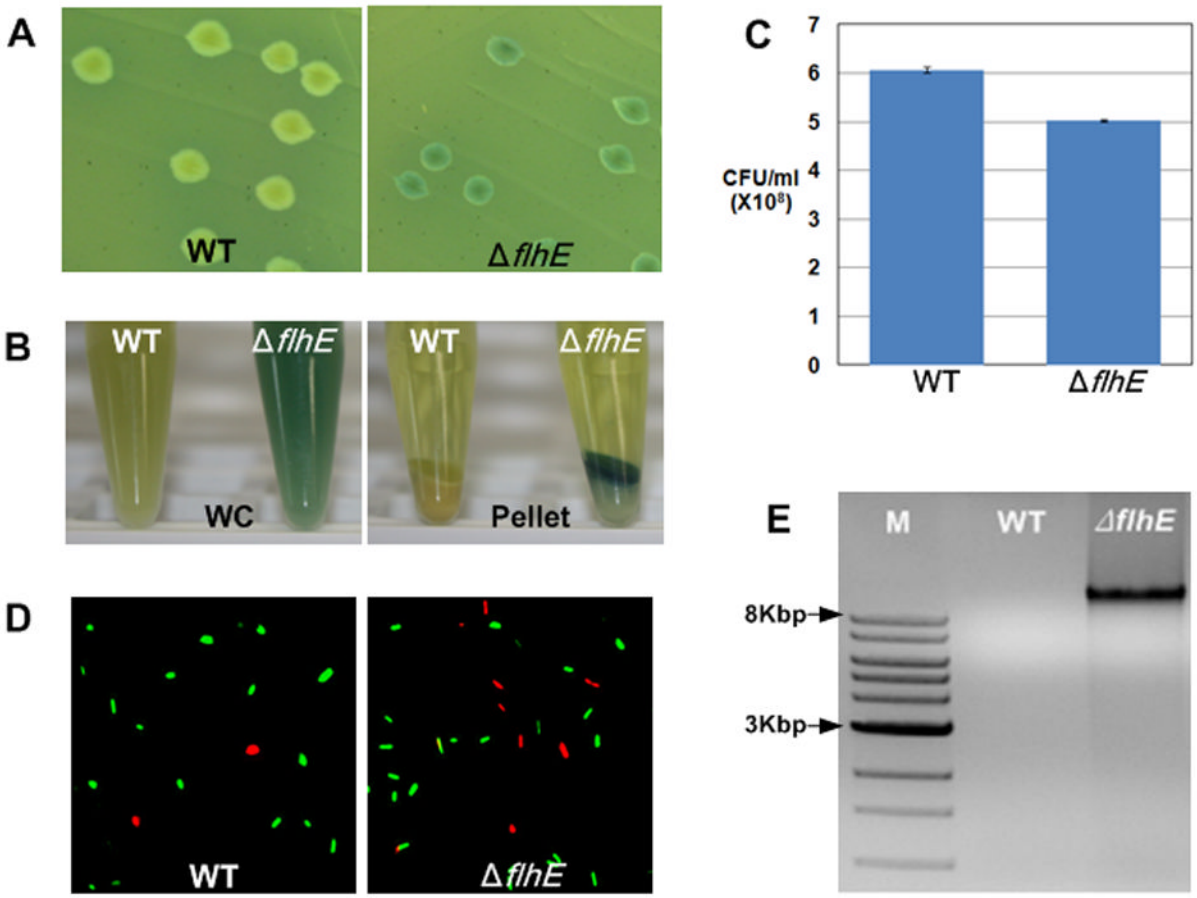

Fig. 3.

S. enterica flhE mutant colonies are green on pH indicator plates and show cell lysis. **A.** Color phenotype of wild-type and *flhE* (ST004) colonies on green plates. **B.** Color of cell suspensions of the strains in A, isolated from green swarm plates after 6 h of growth. WC, whole cell suspension; Pellet, low-speed centrifugation of WC showing a clear supernatant and yellow/green pellets. **C**. Colony forming units (CFU) of strains isolated as in B. **D**. Live-Dead stain used to visualize cell death in strains isolated as in B. **E.** Genomic DNA in the supernatants from B, electrophoresed on 0.8% agarose gels and stained with ethidium bromide. DNA size markers are indicated. See Experimental Procedures for details.

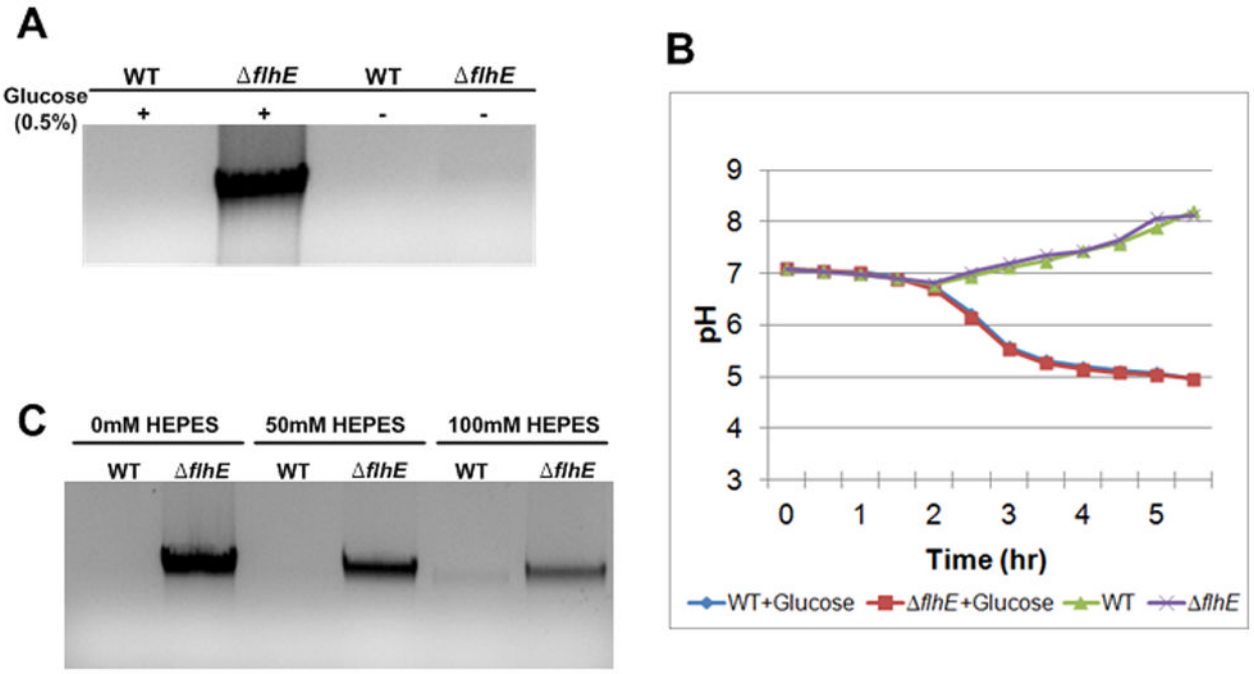

Fig. 4.

Cell lysis in a *S. enterica flhE* mutant depends on added glucose, whose metabolism lowers external pH. **A.** Agarose gel assay for lysis as in Fig. 3. **B.** External pH of indicated strains grown in LB medium with and without the addition of glucose, measured as described in Experimental Procedures. **C.** Effect of increasing HEPES buffer on cell lysis, measured as in A. The buffer was added to green swarm plates, and cells were harvested at 6 h. HEPES buffered well only at concentration over 200 mM. However, at these higher concentrations it was not well-tolerated by wild-type cells, which began to lyse. At 100 mM, the pH of the culture was ~0.5 units higher than the unbuffered culture.

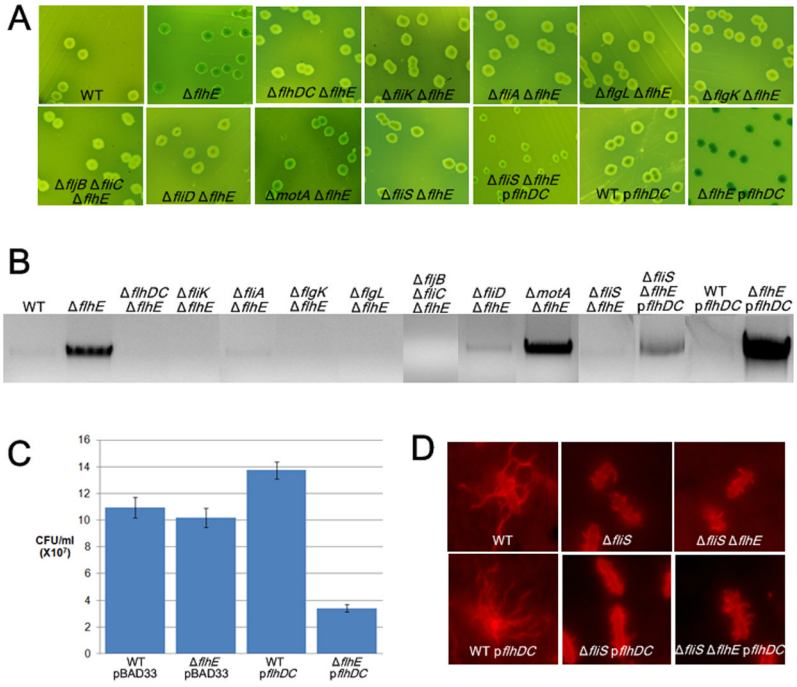

Fig. 5.

Filament assembly, number, and length, but not rotation, are essential for cell lysis in a *S. enterica flhE* mutant. **A.** Color phenotype on green plates of mutants defective in various steps of flagellar assembly. In strains with p*flhDC*, arabinose (0.8%) was substituted for glucose in the medium to induce gene expression. Strains used from left to right (not including those with plasmids): WT 14028, ST004, ST496, ST121, ST214, ST210, ST137, ST212, ST146, ST157, ST442. **B.** Agarose gel electrophoresis of *flhE* mutants shown in A, propagated on green swarm plates, in which arabinose was substituted for glucose in strains with p*flhDC*. **C.** Cell viability of wild-type and *flhE* mutant strains complemented with p*flhDC;* 0.2% arabinose added as inducer. **D**. Flagellar staining of indicated strains with Texas Red coupled anti-IG antibody to FljB antibody.

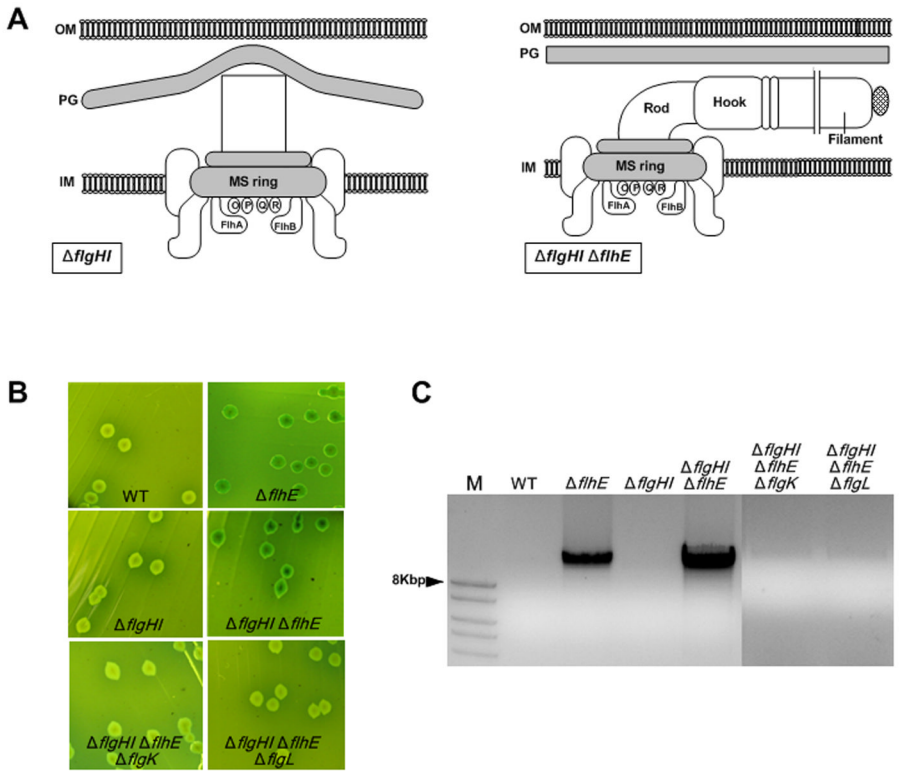

Fig. 6.

Cell lysis of *flhE* mutants in a *S. enterica* PL-ring mutant background. **A.** Cartoon showing impairment of rod growth in a *flgHI* mutant (lacking P and L rings); deletion of *flhE* relieves this defect, allowing filaments to assemble in the periplasm (Hirano *et al*., 2009). **B.** Color phenotype of wild-type and *flgHI* mutants defective in *flhE* and/or *flgK*, *flgL* on green plates. Strains used from left to right: 14028, ST004, ST453, ST454, ST478, ST482. **C.** Agarose gel electrophoresis of mutants shown in B.

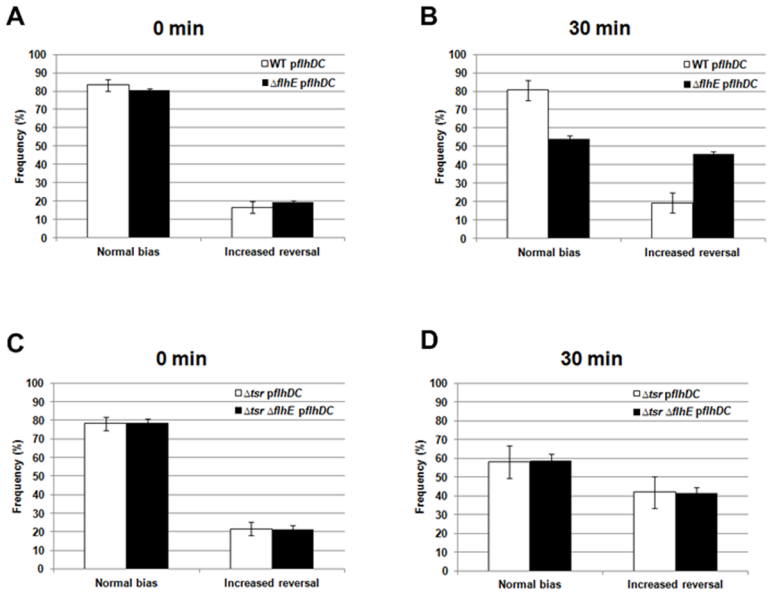

Fig. 7.

Swimming behavior of *flhE* mutants with and without chemoreceptor Tsr. *S. enterica* wild-type, Δ*flhE* (ST004), *tsr* (RH2858), and *tsr flhE* (ST557) strains containing either the vector pBAD33 alone, or p*flhDC*, were induced for *flhDC* expression with addition of arabinose, and their swimming trajectories were observed and recorded at 0 min and 30 min after induction, as described in Experimental Procedures. 'Normal bias' refers to cells that swam straight for a distance of 25 μm without a tumble, which is the wild-type bias of cells in an isotropic medium.

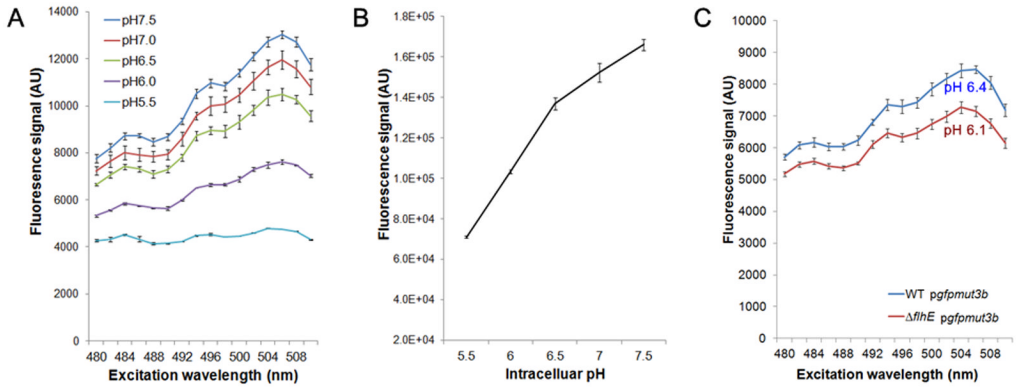

Fig. 8.

Measurement of cytoplasmic pH in *S. enterica* by Green Fluorescent Protein Fluorimetry. **A.** Excitation spectra of cytoplasmic GFPmut3b as a function of pH. Wild-type *S. enterica* cells containing the GFP plasmid were suspended in M63 minimal-media adjusted to pH values varying from 5.5–7.5. Internal pH was equilibrated with external pH by addition of 30 mM sodium benzoate. **B**. Standard curve of cytoplasmic pH as a function of fluorescence signal (sum of 480nm to 510 nm). See Experimental Procedures for details. The error bars represent standard deviation of the mean (n = 3). **C** Wild-type and *flhE* (ST004) *S. enterica* strains were resuspended in media adjusted to pH 5.5, and GFP excitation spectra were recorded without addition of benzoate. Fluorescence measurements were converted to pH units using the standard curve (B) as described in Experimental Procedures. AU, arbitrary units.

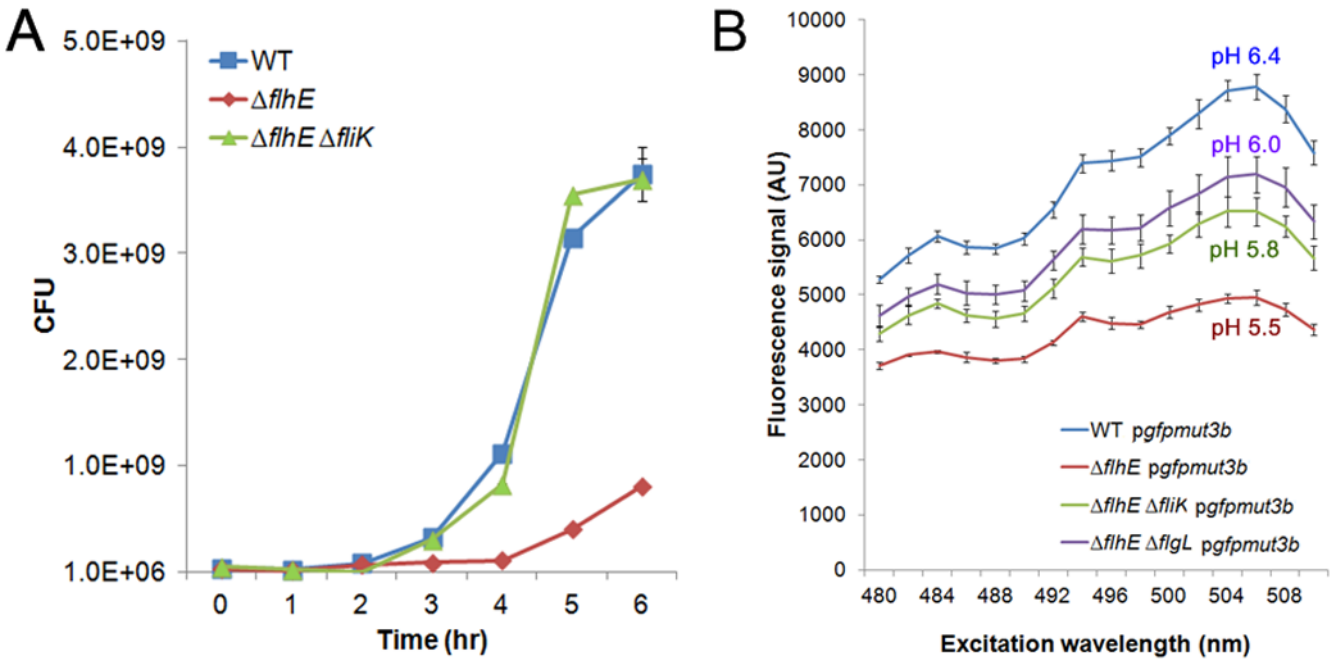

Fig. 9.

Measurement of growth rates and cytoplasmic pH of *E. coli flhE* mutants. **A**. Growth curves in LB broth of the indicated *E. coli* strains, expressed as colony forming units (CFUs). Strains used: wild-type RP437, ST839, ST907. **B**. GFPmut3b excitation spectra in wild-type *E. coli* and its *flhE* mutant derivatives, measured and converted to pH units as described in Fig. 8C. Strains as in A, with the addition of ST964.

Table 1

Strains and plasmids used.

Strain	Genotype[a]	Source (ref.)
Salmonella		
14028	Wild-type ATCC strain	Mariconda *et al.* (2006)
ST005	*flhE::kan*	This study
ST004	Δ*flhE*	This study
ST084	*flhE::FLAG*	This study
QW215	*flhDC::tetRA*	Wang *et al.* (2007)
ST496	*flhDC::tetRA flhE::kan*	This study
RH2153	SJW/*fliA::kan*	Joe Mireles
ST649	*fliA::kan*	This study
ST214	*fliA::kan* Δ*flhE*	This study
RH2217	Δ*fljB* Δ*fliC*	Adam Toguchi
ST212	Δ*fljB* Δ*fliC flhE::kan*	This study
ST155	Δ*fliD*	This study
ST146	Δ*fliD flhE::kan*	This study
TH2955	LT2/*fliK77*::Tn*10*	Kelly Hughes
ST120	*fliK77*::Tn*10*	This study
ST121	*fliK77*::Tn*10* Δ*flhE*	This study
ST209	Δ*flgL*	This study
ST210	Δ*flgL* Δ*flhE*	This study
TH2929	LT2/*flgK563*::Tn*10*	Kelly Hughes
ST136	*flgK563*::Tn*10*	This study
ST137	*flgK563*::Tn*10* Δ*flhE*	This study
ST450	*flgHI::kan*	This study
ST453	Δ*flgHI*	This study
ST454	Δ*flgHI* Δ*flhE*	This study
ST478	*flgHI::kan* Δ*flhE flgK563*::Tn*10*	This study
ST482	*flgHI::kan* Δ*flhE* Δ*flgL*	This study
QW178	Δ*motA*	Wang *et al.* (2005)

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Strain	Genotype[a]	Source (ref.)
ST157	Δ*motA flhE::kan*	This study
QW180	Δ*motB*	Wang *et al*. (2005)
ST580	Δ*motB fliA::kan*	This study
ST441	*fliS::kan*	This study
ST442	*fliS::kan* Δ*flhE*	This study
RH2858	Δ*tsr*	Susana Mariconda
ST557	Δ*tsr flhE::kan*	This study
RH2202	Δ*hin* (flagellar phase variation locked for fljB expression)	Adam Toguchi
RH2204	Δ*hin* (flagellar phase variation locked for fliC expression)	Adam Toguchi
ST149	Δ*hin* (RH2202) *flhE::kan*	This study
ST150	Δ*hin* (RH2204) *flhE::kan*	This study
TH341	F::Tn*10d*Cm	Kelly Hughes
E. coli		
RP437	Wild-type	Parkinson (1978)
ST839	*flhE::kan*	This study
ST893	*fliK::kan*	This study
ST903	Δ*fliK*	This study
ST907	Δ*fliK flhE::kan*	This study
ST963	*flgL::tetRA*	This study
ST964	*flgL::tetRA flhE::kan*	This study
DH5α	Δ(*lacIZYA-argF*) Φ80d*lac* Δ(*lacZ*)M15	New England Biolabs
Phage		
P22	HT12/4*int*103	Mariconda et al. (2006)

Plasmid	Expressed protein	Resistance	Replication Origin	Induction	Source (ref.)
pNK972	Tn*10* transposase in pBR322	Ampicillin	ColE1	Constitutively active	Kelly Hughes
pAmCyan	Expression vector used for Tn*10d*Cm insertion mapping	Ampicillin	pUC	IPTG	CLONTECH
pKD4	Source for Kan cassette	Kanamycin	oriR6K gamma	N.A	Datsenko and Wanner (2000)
pKD46	Lamda Red recombinase	Ampicillin	repA101ts & oriR101	Arabinose	Datsenko and Wanner (2000)
pCP20	FLP recombinase	Ampicillin & Chloramphenicol	repA101ts	Constitutively active	Datsenko and Wanner (2000)
pMMB1311	Source for GFPmut3b	Ampicillin	oriC	Constitutively active	Kitko *et. al.* (2010)
pTrc99a	Expression vector	Ampicillin	pBR	IPTG	Amann *et al*. (1988)

Plasmid	Expressed protein	Resistance	Replication Origin	Induction	Source (ref.)
pBAD33	Expression vector	Chloramphenicol	pACYC	Arabinose	Guzman *et al*. (1995)
pBAD30	Expression vector	Ampicillin	pACYC	Arabinose	Guzman *et al*. (1995)
pJM01	FlhE-FLAG	Ampicillin	pBR	IPTG	This study
pJM37	GFPmut3b	Ampicillin	pBR	IPTG	This study
p*flhDC*	FlhDC	Chloramphenicol	pACYC	Arabinose	Asaka Suzuki
p*motAB*$_{\Delta 51\text{-}60}$	MotA, MotB$_{\Delta 51\text{-}60}$	Ampicillin	pACYC	Arabinose	Hosking *et al*. (2006)

[a]Unless otherwise indicated (e.g. SJW/LT2), all *S. enterica* strains are derivatives of 14028, and all *E. coli* strains constructed in this study are derivatives of RP437. Δ and :: refer to deletion of, or insertion/substitution within, respectively, the indicated gene. Note that deletions created by the Datsenko & Wanner (2000) method leave behind a 'scar' sequence of ~80 base pairs.