

The bacterial flagellar switch complex is getting more complex

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The mechanism of function of the bacterial flagellar switch, which determines the direction of flagellar rotation and is essential for chemotaxis, has remained an enigma for many years. Here we show that the switch complex associates with the membrane-bound respiratory protein fumarate reductase (FRD). We provide evidence that FRD binds to preparations of isolated switch complexes, forms a 1:1 complex with the switch protein FliG, and that this interaction is required for both flagellar assembly and switching the direction of flagellar rotation. We further show that fumarate, known to be a clockwise/switch factor, affects the direction of flagellar rotation through FRD. These results not only uncover a new component important for switching and flagellar assembly, but they also reveal that FRD, an enzyme known to be primarily expressed and functional under anaerobic conditions in *Escherichia coli*, nonetheless, has important, unexpected functions under aerobic conditions.

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Introduction

Bacterial chemotaxis is considered by many as the signalling system for which the level of understanding is most advanced (see, for a review, Eisenbach, 2004). The object of chemotactic signalling in bacteria is to change the direction of flagellar

rotation in response to stimulation, which results in a change in swimming behaviour and direction (Larsen *et al*, 1974). The structural element that is responsible for switching the direction of flagellar rotation is the switch—a ring-like complex (termed the C-ring) at the base of the flagellar motor, which is one of nature's most intriguing molecular machines (see, for reviews, Macnab, 1995; Eisenbach and Caplan, 1998). The switch complex is built from multiple units of the proteins FliG, FliM, and FliN. It is striking how little is known about switch function despite (a) the centrality of switching for chemotaxis, (b) the many known aspects of switch structure, (c) the availability of functional switching mutants, and (d) the extensive effort invested in studying the switch (see, for reviews, Eisenbach and Caplan, 1998; Berg, 2003; Kojima and Blair, 2004). It is known that the default direction of rotation (i.e., the direction in the absence of intracellular signalling) is counterclockwise (see, for a review, Eisenbach, 1996), and that phosphorylation-controlled binding of CheY to the switch protein, FliM, enables the switch to shift to clockwise rotation (see, for a review, Eisenbach, 2004). However, the post-binding processes that occur at the switch are still a mystery.

Over a decade ago, Marwan *et al* (1990) found that fumarate is a factor needed for the archeal *Halobacterium salinarum* to swim back and forth. Subsequently, this dicarboxylate was found to be a clockwise switching factor in *Escherichia coli*. In cytoplasm-free but CheY-containing cell envelopes, it enabled the flagella to switch their direction of rotation (Barak and Eisenbach, 1992; Barak *et al*, 1996). By employing mutants having exceptionally low or high intracellular levels of fumarate due to the absence of one of the enzymes that act on fumarate, that is, succinate dehydrogenase (SDH) or fumarase, Prasad *et al* (1998) and Montrone *et al* (1998) found that, in intact *E. coli* cells, fumarate increases both the fraction of time spent in clockwise rotation and switching frequency. These effects were due, in part, to reduction of the standard free energy difference between the clockwise and counterclockwise states of the switch (Prasad *et al*, 1998). Fumarase, a cytosolic protein, was also shown to be involved in a phosphorylation-independent response to some repellents (Montrone *et al*, 1996).

The effects of fumarate in intact cells of *E. coli* were independent of the presence of CheY in the cell, indicating that fumarate exerted its action on the switch rather than on CheY (Prasad *et al*, 1998). In the present communication, we discovered that the enzyme fumarate reductase (FRD) is the target of fumarate at the switch.

FRD and its closely related homologue SDH are four-subunit membrane-bound enzymes (see Supplementary Figure S1 and Supplementary data), usually thought to be involved in electron transport reactions (see, for review, Cecchini *et al*, 2002). It is well documented that FRD functions in anaerobic respiration of *E. coli* and that SDH is involved in aerobic respiration. We provide evidence for a reversible interaction between FRD (hitherto unknown to be

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associated with motility or flagella) and FliG of the flagellar switch, and we demonstrate that mutants lacking *frd* are defective in flagellar assembly and switching and are not responsive to fumarate.

Results

Fumarate does not bind to any of the known switch proteins

We initiated this work by trying to determine whether fumarate binds to the switch complex. We isolated the intact switch complex of *E. coli* flagella (see Materials and methods and Supplementary Figure s2), incubated it with [¹⁴C]fumarate, and separated it from the medium by centrifugation. We detected no binding of [¹⁴C]fumarate (assayed in the range of 5–50 μM [¹⁴C]fumarate) to the isolated switch complex. We also measured the binding of [¹⁴C]fumarate to each of the three purified switch proteins. We used both equilibrium dialysis and centrifugal ultrafiltration, described in Materials and methods and Supplementary data, to measure binding of [¹⁴C]fumarate in the range 0.5–10 000 μM to each of the three switch proteins (10–200 μM). No binding was detected.

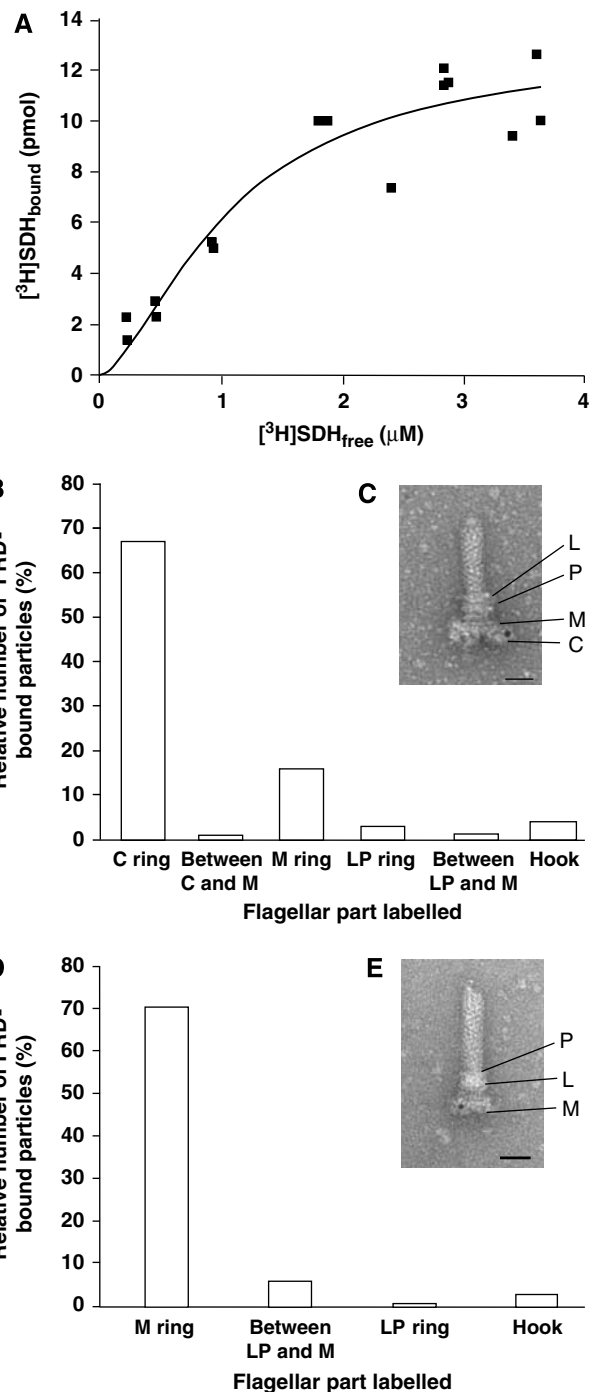
Potential targets of fumarate binding to the flagellar switch

The absence of detectable direct binding to any switch protein suggested that another protein may transmit the fumarate effect to the switch. This protein is expected to be membrane-bound because earlier it was shown that fumarate enhances switching even in envelopes devoid of cytoplasm

(Barak and Eisenbach, 1992; Barak *et al*, 1996). Potential candidates for transmitting the fumarate effect to the switch are SDH and FRD—enzymes similar in amino-acid composition, 3D structure (Supplementary Figure s1), cofactors, and mechanisms of function (Cecchini *et al*, 2002). Both interact with fumarate and are membrane-bound. Thus, we tested the interaction of SDH and FRD with the switch.

Evidence consistent with SDH binding to the switch complex was observed following incubation of [³H]SDH with the isolated intact switch complex and separation by centrifugation (Figure 1A; $K_d = 1.1 \pm 0.3 \mu\text{M}$; mean \pm s.e.m.). To reduce nonspecific SDH binding, the experiment (detailed in Materials and methods) was carried out in the presence of

Figure 1 SDH and FRD binding to the switch complex. (A) Concentration-dependent SDH binding to the isolated switch complex. See Materials and methods for details. The free SDH concentration was calculated by subtracting the concentration of bound SDH from the added SDH concentration. The data shown are normalized and derived from three different switch batches. The concentration of the switch was 0.1 mg protein/ml. The connecting line between the experimental points is a theoretical fit, performed by Origin computing program according to the Hill model ($R^2 = 0.90$). (B) Protein A-gold particles bound to FRD in isolated extended flagellar basal bodies. The preparation of extended basal bodies was preincubated sequentially with FRD, anti-FRD antibody, and Protein A-gold. The number of gold particles counted was 1985. A total of 1265 basal bodies were counted; 715 were labelled at C-ring, M-ring, or between these rings, 87 were nonspecifically labelled in other parts of the basal body, and 463 were not labelled. The average number of gold particles associated with a basal body was 2.5 (the most common being 2 or 3 particles/basal body; range = 1–10 particles/basal body). For the negative control with boiled FRD (not shown in the figure), a total of 382 basal bodies were counted: 3 (0.8%) were labelled at C- or M-ring, 4 (1%) were nonspecifically labelled in other parts of the basal body, and 375 (98%) were not labelled. Similar numbers were observed with BSA substituting for boiled FRD. (C) Image of an isolated extended flagellar basal body, treated as in (B) (bar = 10 nm). (D) Protein A-gold particles bound to FRD in isolated basal bodies lacking the structure of the C-ring. Gold particles counted were 1619. Of the 1679 basal bodies counted, 1156 were labelled at the M-ring, 155 were nonspecifically labelled in other parts of the basal body, and 368 were not labelled. The average number of gold particles associated with such a basal body was 1.3 (the most common being 1 particle/body; range = 1–6 particles/basal body). (E) Image of an isolated basal body lacking the structure of the C-ring, treated as in (B) (bar = 10 nm).



at least 40-fold excess BSA, high salt concentration, and detergent. Insufficient amounts of the switch complex made direct binding measurements with an irrelevant protein impractical.

To examine FRD binding to the switch, we employed immuno-electron microscopy, which requires much less material and does not involve chemical modification of the enzyme. We isolated flagellar hook basal bodies (termed 'extended basal bodies'; Francis *et al*, 1994) and allowed them to bind FRD. (The basal body is the rotor of the flagellar motor, connected to the flagellar filament by means of a hook. It consists of a rod surrounded by coaxial rings: the M ring, a doublet ring embedded in the cytoplasmic membrane; the P ring, embedded in the peptidoglycan layer; and the L ring, embedded in the outer membrane (Figure 1E). Extended basal bodies also contain the C-ring (Figure 1C).) BSA was used as a control both for nonspecific binding/adsorption of the antibodies to the basal bodies and detection of protein A-Gold trapping by the basal body particles in the labelling protocol. Denatured FRD was used as a negative control. After binding, all the samples were incubated sequentially with anti-FRD antibody and protein A-Gold. FRD was localized to the C-ring (i.e., the switch complex) and, to a much lesser extent, to the M-ring (Figure 1B and C). Neither BSA nor denatured FRD was similarly localized. Thus, both SDH and FRD appear to bind to the switch complex.

To substantiate FRD binding to the M-ring, we assessed by immuno-electron microscopy its binding to a preparation of basal bodies that lack the C-ring structure and do not contain the switch proteins FliM and FliN (Francis *et al*, 1994). However, they do contain FliG, as ~80% of the basal bodies are labelled following anti-FliG immunolabelling (Supplementary Figure s3) and as previously demonstrated (Francis *et al*, 1992, 1994). FRD binding to this preparation was evident (Figure 1D and E). The observation that both the C-ring and M-ring bound FRD raised the possibility that the site of FRD and, probably, SDH binding was FliG. This is because FliG is the only switch protein that was present in both basal-body preparations and because it appears to be associated with both the C-ring and M-ring (Oosawa *et al*, 1994; Thomas *et al*, 2001).

To explore this possibility, we employed three independent experimental approaches. (a) Following incubation with His-tagged FliG, biotinylated SDH and FRD were brought down by streptavidin-agarose beads and probed for the presence of FliG by western blotting with anti-His-tagged FliG antibody. His-tagged FliG colocalized with either SDH or FRD (Figure 2A). (b) Real-time surface plasmon resonance analysis showed that both purified SDH (Figure 2B) and FRD (Figure 2C), when individually immobilized onto the sensor surface, bound His-tagged FliG in a concentration-dependent manner. The dissociation rate constants, obtained on the basis of the Langmuir 1:1 bimolecular kinetic model, were similar for SDH and FRD ($2 \times 10^{-3} \text{ s}^{-1}$), as were the association rate constants (3000 and $5000 \text{ M}^{-1} \text{ s}^{-1}$ for SDH and FRD, respectively) and the resulting dissociation constants ($K_d = 0.6 \pm 0.1$ and $0.4 \pm 0.3 \mu\text{M}$; mean \pm s.d. for SDH and FRD, respectively). Earlier tests showed that immobilized SDH bound neither FliM nor FliN (Prasad, 2001). The similarity between the K_d value obtained in this experiment for SDH binding to FliG and the K_d value for SDH binding to the isolated switch complex in Figure 1A strongly supports

the argument that the SDH-switch binding in Figure 1A was specific. (c) Analytical ultracentrifugation was used to assess complex formation between His-tagged FliG and FRD. The shift in the location of the major protein peak from the molar mass of FRD to that of a 1:1 FliG-FRD complex when His-tagged FliG was present (Figure 2D) confirmed the formation of a complex and identified its stoichiometry.

The seminal issues, however, are whether this binding has physiological significance, and whether either or both SDH and FRD are responsible for the action of fumarate on the switch.

FRD but not SDH affects the functions of FliG

FliG is central to the function of the flagellar motor. It is known to be directly involved in motor assembly, torque generation, and switching (see, for a review, Kojima and Blair, 2004). Consequently, defects in this protein may lead to loss of flagella, paralysis, or biased direction of flagellar rotation. Assuming that the interaction of FliG with FRD or SDH is required for its function, absence of FRD or SDH may lead to any one of these phenotypes. Therefore, we prepared three deletion mutants: a Δfrd mutant, deleted for the genes encoding all the subunits of FRD; a Δsdh mutant in which two of the four genes encoding SDH were deleted, resulting in complete absence of SDH (Montrone, 1996; Prasad *et al*, 1998); and a double $\Delta frd \Delta sdh$ mutant.

The Δsdh mutant did not differ from its wild-type parent with respect to motility (data not shown), whereas, strikingly, the Δfrd mutant and the double mutant were barely motile. As shown for the Δfrd mutant (Figure 3A), many cells did not swim at all, others swam more slowly than usual and, in most of these latter cases, the movement was wobbly. This behaviour resulted from a decrease in the number of flagella (Figure 3B and C). The wild-type parent had a median of 5 flagella/cell, but the Δfrd mutant had a median of only 1 flagellum/cell, with many cells having no flagella at all. Similar data (not shown) were obtained for the double mutant. To verify that the observed phenotype was due to the absence of FRD, we complemented the *frd* deletion with a plasmid, producing a single copy of FRD under its native promoter (pEWF1). The plasmid restored, at least partially, the number of flagella (median of 3 flagella/cell; Figure 3B and C) and increased the fraction of motile cells (Figure 3A). As the *frd* deletion did not affect the expression level of FliG, as evident from western blots with anti-FliG antibody (Supplementary Figure s4), the results suggest that FRD is required for normal flagellar assembly.

FRD deletion could potentially reduce the energy level and elevate the fumarate level in the cell, contributing to the observed phenotypes of the Δfrd mutant. No evidence for these scenarios was found. We measured the intracellular ATP concentration and found it to be similar in all the strains used, with a variation of ~10% from the value of $2.5 \pm 0.1 \text{ mM}$ measured for the wild-type strain. (It should be mentioned, however, that although removal of FRD did not have a significant effect on cellular ATP levels, its absence might have other, unexplored effects on cellular metabolism.) The possible contribution of an elevated fumarate level was ruled out earlier by showing that, in cells deleted of the genes encoding fumarase, elevated cellular fumarate has no effect on the motility level (Montrone *et al*, 1998; Prasad *et al*, 1998).

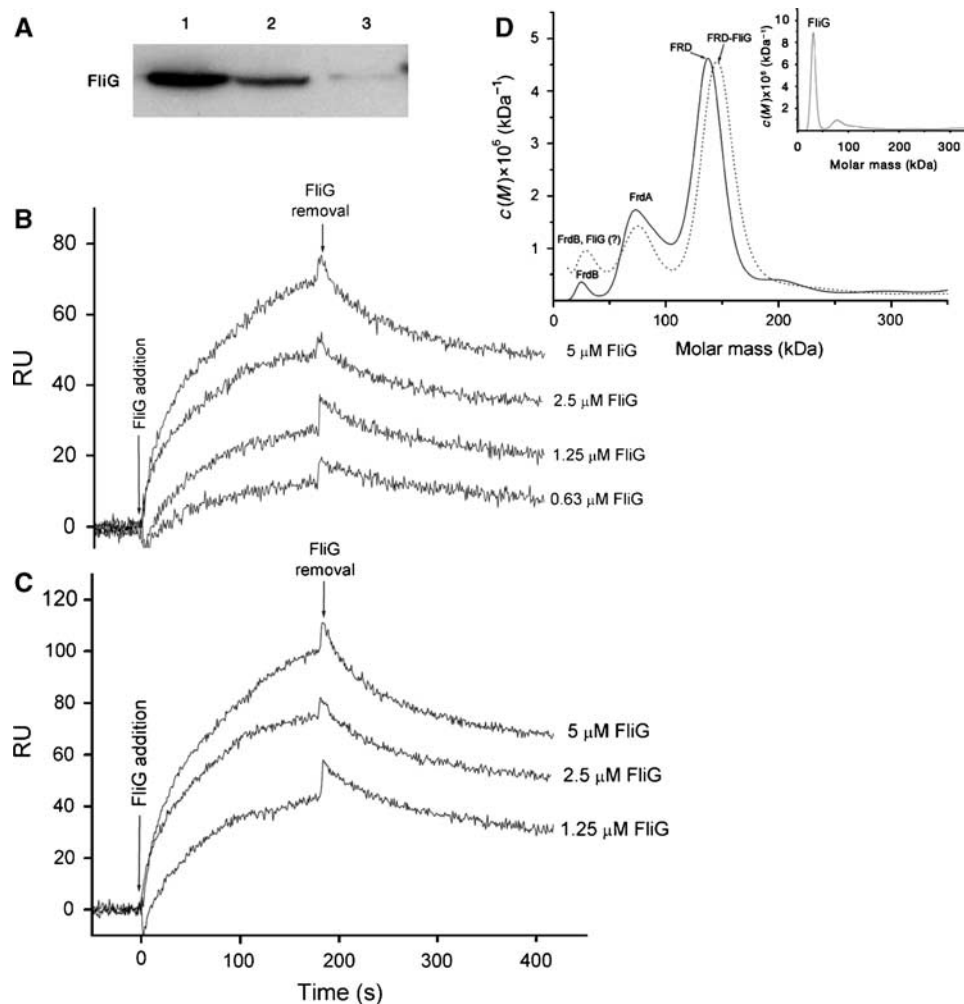


Figure 2 Binding of purified His-tagged FliG to purified FRD or SDH. (A) Pull-down assay. FliG was incubated with biotinylated FRD (lane 1), biotinylated SDH (lane 2), or, as a negative control, with neither FRD nor SDH (lane 3), precipitated with streptavidin–agarose beads, and probed with an anti-His-tagged FliG antibody. (B, C) Real-time interaction of His-tagged FliG with biotinylated SDH (B) or biotinylated FRD (C), measured by surface plasmon resonance. RU, resonance units. The values shown are after subtraction of the value of the empty channel. The kinetic and equilibrium constants, shown in the text, were obtained by a global fit, using the Langmuir 1:1 bimolecular kinetic model ($A + B \rightleftharpoons AB$; fit quality: $\chi^2 = 5$ and 11 for the SDH and FRD curves, respectively). (D) Analytical ultracentrifugation of the FliG–FRD complex. The $c(M)$ distribution is a variant of the distribution of Lamm equation solutions using Sedfit (see Supplementary data). Three samples were run: FRD alone (2 μ M; solid line), His-tagged FliG alone (16 μ M; inset), and both together (2 μ M each; dotted line). The major peak observed in the His-tagged FliG and FRD mixture (dotted line) was at 150 kDa (corresponding to a 1:1 FliG:FRD complex; 158 kDa theoretical value, calculated from the amino-acid composition). The changes of the sedimentation rates of the lesser peaks of FRD were minor in the presence of His-tagged FliG. The major peak of His-tagged FliG (inset) was at 32 kDa (37 kDa theoretical); the low peak at 75 kDa probably reflects a FliG dimer. The peaks of FRD (solid line) correspond, in the order of decreasing abundance, to FRD (134 kDa, consisting of all four subunits FrdA, FrdB, FrdC, and FrdD; 121 kDa theoretical), FrdA (71 kDa; 66 kDa theoretical), and FrdB (23 kDa; 27 kDa theoretical). FrdC and FrdD are not observed in the figure, as expected, due to their extreme hydrophobicity (Cecchini *et al*, 2002), which precludes them from the solution (either sedimenting to the bottom or adhering to the wall of the tube).

To examine the effect of *frd* deletion on flagellar rotation of those cells that had residual flagella, we tethered them through a flagellum to a glass slide and monitored the rotation of the cells (Silverman and Simon, 1974). The tethered Δ *frd* cells rarely switched their direction of rotation, making a reversal once every 2.5 min on average (Table I, line 2) and rotating counterclockwise 99.9% of the time (line 1). The addition of the strong repellent benzoate increased the probability of clockwise rotation in both the Δ *frd* strain and its wild-type parent (line 3 versus line 1). However, in the Δ *frd* strain, the fraction of responsive cells was smaller (line 5), and the level of clockwise rotation achieved after this stimulation was significantly lower than that in the wild type (line 3). In contrast, the switching frequency was higher than

that in the wild type (line 4), possibly reflecting futile attempts to switch to clockwise rotation. These results are consistent with the clockwise-promoting effect of fumarate in the intact cells of *E. coli* (Montrone *et al*, 1998; Prasad *et al*, 1998) and with the possibility, investigated below, that FRD is the protein that transmits the fumarate effect to the switch.

FRD transmits the effect of fumarate to the flagellar switch

To examine this possibility, we employed two approaches. First, we studied the binding of [14 C]fumarate to a preparation of M-rings associated with FliG (Figure 4A and B)—the minimal structure shown to bind FRD (Figure 1D and E)—in the presence and absence of FRD. The presence of FRD

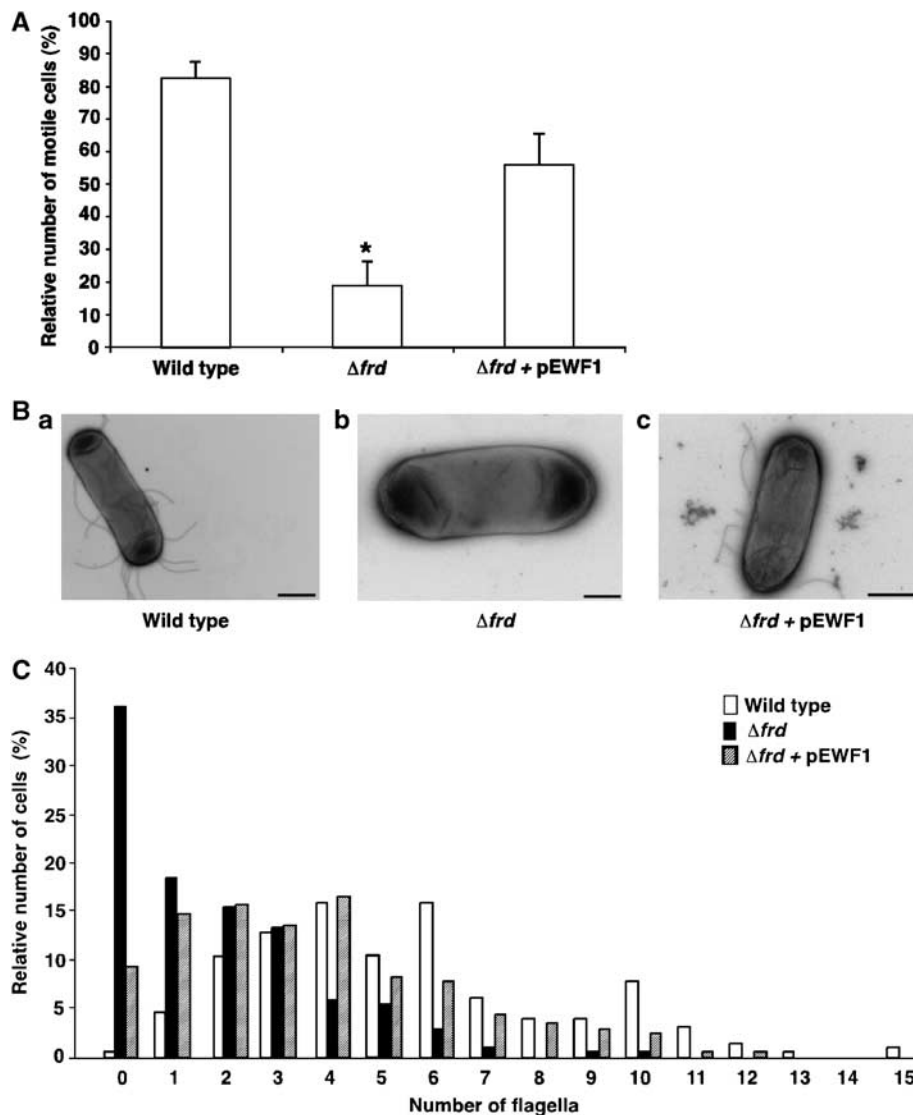


Figure 3 Effects of *frd* and *sdh* deletions on swimming, assembly of flagella, and switching the direction of flagellar rotation. (A) Percentage of motile cells. Swimming cells were video-recorded and the fraction of motile cells was determined blindly. Values shown are the mean \pm s.e.m. of three experiments, 100–200 cells for each strain in each experiment. The asterisk indicates a statistically significant difference from the other columns ($P < 0.01$ and $P < 0.05$ for wild-type and $\Delta frd + pEWF1$ columns, respectively; one-way ANOVA plus Tukey–Kramer tests). (B) Typical micrographs of strains RP437 (a; bar = 1 μ m), RP437 Δfrd (b; 0.5 μ m), and RP437 Δfrd containing pEWF1 (c; 1 μ m). (C) Distribution of the number of flagella per cell. Cells were negatively stained with uranyl acetate and photographed using a transmission electron microscope. The number of flagella of ~ 200 cells of each strain were counted without prior knowledge of the strain being counted. The difference in the number of flagella between the strains was very significant ($P < 0.001$; Kruskal–Wallis test). Open columns, strain RP437; solid columns, RP437 Δfrd ; hatched columns, RP437 Δfrd containing pEWF1.

Table I Comparison between wild-type and Δfrd strains with respect to the parameters of flagellar rotation

Parameter tested ^a	Wild type	Δfrd	<i>P</i> -value for the difference between the strains ^b
<i>Before stimulation</i>			
1. Time fraction spent in clockwise rotation \pm s.e.m. (%)	2.0 \pm 0.9	0.1 \pm 0.1	0.25
2. Switching frequency \pm s.e.m. (min ⁻¹)	2.1 \pm 0.5	0.4 \pm 0.2	<0.009
<i>After stimulation</i>			
3. Time fraction spent in clockwise rotation \pm s.e.m. (%)	70 \pm 4	13 \pm 4	<0.0001
4. Switching frequency \pm s.e.m. (min ⁻¹)	0.7 \pm 0.2	16 \pm 2	<0.0001
5. Fraction of responsive cells (%)	96	75	0.001

^aThe rotation of 46–59 tethered cells was monitored for periods of 40 s before and subsequent to stimulation with the repellent benzoate (50 mM).

^bCalculated by the Mann–Whitney test, except for the fraction of responsive cells, which was calculated by the Fisher’s exact test.

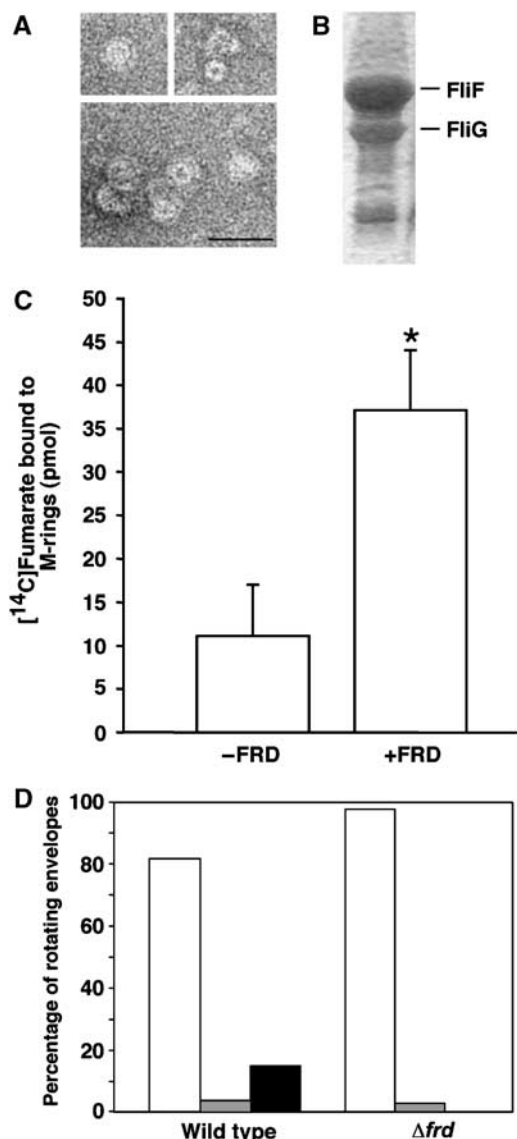


Figure 4 FRD transmits the effect of fumarate to FliG at the switch-motor complex. (A) Electron micrographs of purified M-rings. Bar = 50 nm. (B) SDS gel of the M-rings (composed of the protein FliF), demonstrating the co-occurrence of FliG. (C) Binding of fumarate to the M-ring in the presence and absence of FRD. Fumarate binding was measured with 50 μ M [14 C]fumarate and 20 μ M FRD. The concentration of the M-ring proteins was 10 μ M. The data shown are the means (\pm s.e.m.) of 3–4 repetitions. The asterisk indicates a statistically significant difference ($P < 0.04$, unpaired *t*-test). (D) Effect of *frd* deletion on the rotation of tethered, CheY-containing, cytoplasm-free envelopes in the presence of fumarate at room temperature. The figure shows the distribution of envelopes according to their direction of rotation. Open columns, counterclockwise-rotating cells; hatched columns, clockwise-rotating cells; solid column, switching cells. The numbers of monitored envelopes were 115 and 39 for the wild-type strain and the Δfrd mutant, respectively.

significantly increased the binding of fumarate to this preparation (Figure 4C). Next, we measured the effect of fumarate on flagellar rotation using tethered, cytoplasm-free envelopes containing His-tagged CheY. In intact bacteria, fumarate mainly increases the fraction of time spent in clockwise rotation (Montrone *et al*, 1998; Prasad *et al*, 1998). In envelopes, fumarate enables switching: tethered

envelopes require this dicarboxylate to switch from one direction of rotation to the other (Barak and Eisenbach, 1992; Barak *et al*, 1996). If fumarate binding to the switch complex depends on FRD, envelopes prepared from the Δfrd mutant should be incapable of switching either in the presence or absence of fumarate. The results confirmed this prediction. Although fumarate was present, none of the Δfrd envelopes switched directions, rotating exclusively in one direction, mostly counterclockwise. In contrast, wild-type envelopes showed switching (Figure 4D). These results (Figure 4C and D), taken together with the observation that Δsdh mutant envelopes continue to switch when fumarate is added (Barak *et al*, 1996), suggest that, FRD, but not SDH, transmits the fumarate effect to the switch and that FRD may be a functional component of the switching mechanism of *E. coli*.

Discussion

In this study, we have made three important discoveries, none of which were predictable on the basis of pre-existing knowledge. First, we demonstrated that FRD is important for the function of the switch and appears to form a dynamic complex with it. This conclusion is based on the observations that (a) absence of FRD results in a sharp decrease in the number of flagella per cell and, consequently, in cell motility (Figure 3), without affecting the level of FliG in the cell (Supplementary Figure S4); (b) the ability of existing flagella to switch rotation to the clockwise direction is much reduced in the absence of FRD (Table I); and (c) FRD forms a 1:1 complex with the switch protein FliG (Figures 1 and 2). These observations thus demonstrate that FRD is involved in both flagellar assembly and switching to clockwise rotation.

Second, we identified FRD as the target binding site of fumarate at the switch, enabling fumarate to exert its stimulating effect on clockwise switching (Figure 4).

Third, we have demonstrated that FRD has an important role under aerobic conditions in *E. coli*, unassociated with ATP production. All the results shown were obtained under aerobic conditions, where FRD is minimally expressed ($\sim 4\%$ of the expression level compared to anaerobic conditions; Jones and Gunsalus, 1985) and, according to the current understanding, it has no essential function (Cecchini *et al*, 2002). Furthermore, although FRD, similar to SDH, is known to be involved in the energy-conversion system, it has not been associated until now with flagellar function or with the motility system. Our observations raise significant new questions about the relationship between FRD and the switch.

Complex formation between the switch and FRD

The formation of a complex between FRD/SDH and FliG at the switch was demonstrated by five different lines of evidence (Figures 1 and 2). One of them, surface plasmon resonance analysis, indicated that the complex formed is not permanent, slowly dissociating when FliG is removed (Figure 2B and C). These observations suggest that the complex formed is in a dynamic equilibrium with non-bound FRD. Further studies are needed to determine which subunits of FRD and domain of FliG are involved in complex formation. The similarity between the phenotypes of the Δfrd mutant (Figure 3) and those of *fliG* mutants with tryptophan replacements at residue 163 or 165 in the middle domain of

Table II List of strains and plasmids used in this study

Strain/plasmid	Relevant genotype	Reference and/or source
<i>Strains</i>		
BL21(λDE3)	HsdS <i>gal ompT</i> r _B ⁻ m _B ⁻ [ΔC1857(T _s)indI Sam7 min5 <i>lacUV5-T7</i> gene 1]	Studier <i>et al</i> (1990), Novagen
BL21(λDE3)pLysS	HsdS <i>gal ompT</i> r _B ⁻ m _B ⁻ [ΔC1857(T _s)indI Sam7 min5 <i>lacUV5-T7</i> gene 1]	Studier <i>et al</i> (1990), Novagen
RP437	Wild type for chemotaxis; <i>his thr leu metE thi eda rpsL</i>	Parkinson (1978)
DW35	<i>zjd::Tn10Δ(frdABCD)18sdhC::kan</i>	Westenberg <i>et al</i> (1993)
RP437Δ <i>sdh</i>	Wild type for chemotaxis; <i>his thr leu metE thi eda rpsL</i> ; Δ <i>sdh</i>	Montrone (1996)
RP437Δ <i>frd</i>	Wild type for chemotaxis; <i>his thr leu metE thi eda rpsL</i> ; Δ <i>frd</i>	This study
RP437Δ <i>sdh</i> Δ <i>frd</i>	Wild type for chemotaxis; <i>his thr leu metE thi eda rpsL</i> ; Δ <i>sdh</i> Δ <i>frd</i>	This study
DFB225Δ <i>fliG</i>	RP437 parent containing an <i>fliG</i> in-frame deletion	Received from David Blair (Lloyd <i>et al</i> , 1996)
M15 [pREP4]	LacZ Str ^R Kan ^R	Zamenhof and Villarejo (1972), QIAGEN
<i>Plasmids</i>		
pQE12-CheY-His tag	6 × His–CheY overproducing	Received from A Wolfe
pKOT107	FliG and FliF overproducing	Oosawa <i>et al</i> (1994)
pFAS	SDH overproducing	Maklashina <i>et al</i> (1998)
pKLR2	FliM and FliN overproducing	Oosawa <i>et al</i> (1994)
pEWG1	10 × His–FliG overproducing	This study
pEWG2	10 × His–FliG	This study
pBAD24	Expression vector containing the P _{Bad} promoter; Ap ^r	Guzman <i>et al</i> (1995)
pH3	FRD overproducing	Blaut <i>et al</i> (1989)
pEWF1	FRD single-copy producing	This study

FliG (Brown *et al*, 2007) raises the possibility that the FliG region(s) affected by these tryptophan replacements are involved in the interaction with FRD.

Comparative roles of FRD and SDH

An intriguing question is why the phenotypes are specific for FRD and not for SDH, even though both homologues bind to FliG with similar affinities (Figure 2). Two alternative explanations seem plausible. (a) The similarity of the affinities suggests that either or both of the cytoplasmic localized A and B subunits, known to be highly similar in both enzymes (Supplementary Figure s1), participate in this binding. Thus, they are reasonable candidates for interaction with the switch complex, which resides in the cytoplasm and is tethered to the membrane (Khan *et al*, 1992; Francis *et al*, 1994; Macnab, 1995). This suggests that the subtle differences between SdhAB and FrdAB (e.g., the more exposed C-terminus of FrdB) are sufficient to make only the latter active in switching and flagellar assembly. (b) The part of the molecule that is responsible for the observed activities of FRD may be the membrane domain, which is the region of these proteins with significant differences between FRD and SDH (Supplementary Figure s1).

Functional differences between FRD and SDH also may account for their different effects on switching. FRD is a more versatile enzyme, functional over a much wider range of physiological conditions than SDH (Ackrell *et al*, 1993; Léger *et al*, 2001). For example, FRD may be able to compensate for the changes in the metabolic conditions better than SDH, and it functions with a variety of diverse quinones, whereas SDH appears to function only with higher potential quinones (Maklashina *et al*, 2006). One could speculate that these differences in activity could affect the conformations of FRD and SDH and thus the interaction with FliG at the switch complex.

It is interesting to note that the flagellar master regulon induces the expression of all FRD subunits and represses those of SDH (Prüss *et al*, 2003). This correlation between the expression of flagellar genes and *frd* genes, in contrast to the *sdh* genes, is consistent with the present study showing an association between flagellar number and FRD. It is further supported by the finding of Landini and Zehnder (2002) that *E. coli* grown under anaerobic conditions has increased flagellation.

Flagellum-related activities of FRD and its involvement in electron transport

In *E. coli*, although FRD is considered to be exclusively involved in anaerobic respiration and electron transport, this versatile enzyme can physiologically substitute for SDH in aerobic respiration (Guest, 1981). The question is whether the observations made herein are related to the electron transport properties of FRD or to some hitherto unrecognized protein–protein interactions. It is clear that *E. coli* FRD does not generate a proton-motive force during electron and proton transfer (Cecchini *et al*, 2002; Lancaster, 2002). Therefore, the observations reported in this article are not directly related to enhanced energization. This conclusion is in accordance with the previous observation of Barak *et al* (1996) that the promoting effect of fumarate on switching in cytoplasm-free envelopes of *E. coli* is not related to oxidation–reduction processes because other dicarboxylates, which can bind FRD and SDH but cannot accept electrons, also cause switching although less efficiently. Thus, the electron transfer from menaquinol to fumarate is unlikely to be involved in fumarate's effect on switching. However, this does not exclude the possibility that FRD is just one part of a larger energy-conversion complex associated with the switch–motor complex.

Potential mode by which FRD may affect the switch

The results of this study do not answer the question how fumarate binding to FRD enables switching or how FRD enhances flagellar assembly. It is well established that binding of a dicarboxylate or full reduction of the iron-sulphur clusters results in long-range conformational changes in FRD (Iverson *et al*, 2000; Hudson *et al*, 2005). It is plausible that the binding of fumarate or one of its analogues induces a conformational change in FRD that may modify either the interaction with FliG or the physiological response. These possibilities are clearly speculative and more data are required to resolve this issue.

FRD in other bacterial species

At present, it is not clear whether the observations reported here are restricted to *E. coli* and similar species. It is known from available databases of bacterial genomes that there are many motile bacterial species that do not possess FRD. FRD is considered an evolutionary precursor of SDH (Ackrell *et al*, 1992), and may have evolved under anaerobic conditions. Involvement of FRD with the flagellar switch-motor complex may have arisen to support under anaerobic conditions the high energetic requirements for the synthesis and function of the flagella. Facultative anaerobic bacteria such as *E. coli* often contain both FRD and SDH. In these cases, FRD may maintain its association with the flagella. In contrast, in strictly aerobic bacteria lacking FRD, perhaps SDH or another membrane protein may substitute for FRD. Such possibilities remain to be explored.

Materials and methods

Chemicals

2,6-Dichloroindophenol, phenazine methosulphate, succinate (sodium salt), fumarate (sodium salt), polyoxyethylene(9)dodecylether, and [¹⁴C]fumarate were obtained from Sigma (St Louis, MO, USA); Ni-NTA matrices were from QIAGEN (Hilden, Germany); sodium boro[³H]hydride, [¹⁴C]leucine, and [³H]leucine were from Amersham (Buckinghamshire, UK). All the compounds were of the highest purity grade available. (See Supplementary data for the complete list.)

Bacterial strains

The strains and plasmids used in this study are listed in Table II. Strains RP437Δ*frd* and RP437Δ*sdh*Δ*frd* were constructed by P₁ transduction as described (Silhavy *et al*, 1984). A P₁ phage lysate prepared on DW35 (Δ*frd*Δ*ABCD*, *zjd*::Tn10) was used to infect RP437 and RP437Δ*sdh*. Positive colonies were selected for tetracycline resistance and for their inability to use fumarate for growth on fumarate/glycerol minimal medium under anaerobic conditions as described (Westenberg *et al*, 1993).

Constructs

For the construction of the plasmid pEWG1 (a His-tagged FliG expression vector), the gene *fliG* was amplified from the *E. coli* chromosome by PCR, with the primer sequences 5'-GGAATCCA TATGAGTAACCTGACAGGCACC-3' and 5'-CGGGATCCTCAGACAGG TATCCTCG-3'. The amplified PCR fragments were cloned into the pET19b vector (Novagen, Darmstadt, Germany) with *Nde*I/*Bam*HI sites. The construct was confirmed by DNA sequencing. To verify that the His-tagged FliG is functional, we constructed the plasmid pEWG2, which expresses His-tagged FliG under the arabinose promoter. Strain DFB225 deleted of *fliG* was transformed with the pEWG2 plasmid (a His-tagged FliG expression vector), described below, and the functionality of the His-tagged FliG was confirmed by the formation of expanding rings on tryptone broth semi-solid agar plates. The plasmid pEWG2 was constructed in two ligation steps. First, the His-tagged *fliG* fragment was excised from pEWG1 by digestion with *Nco*I and *Bam*HI, followed by ligation with

pBAD24 predigested with *Nco*I and *Sma*I. Next, the *Bam*HI site was filled and ligated with the *Sma*I blunt end to form pEWG2.

To construct the plasmid pEWF1 (FRD expression vector), plasmid pH3 was digested with *Hind*III to release the complete *frd* coding sequence including its natural promoter and ligated into a similarly digested pZS24-lac/ara-MCS-1 plasmid (containing the pSC-101 origin of replication). The functional status of FRD expressed from pEWF1 was verified by the ability of the RP437Δ*frd* mutant, transformed with this plasmid, to grow under anaerobic conditions in a minimal medium of glycerol/fumarate.

Preparations

Switch complex and M-ring. To prepare the switch complex, we expressed both the plasmids pKOT107 (overproducing FliG and FliF of *Salmonella typhimurium*) and pKLR2 (overproducing FliM and FliN of *S. typhimurium*) in *E. coli* strain BL21(λDE3). To prepare the M-ring, we expressed only pKOT107. The overproduced switch complex (having the form of a ring) and M-ring were purified by differential centrifugation as described by Sagi *et al* (2003). The preparation of isolated switch complexes was tested for functionality by measuring, as described below, phosphorylation-dependent binding of [¹⁴C]CheY to them (Sagi *et al*, 2003).

Flagellar hook basal bodies. Extended basal bodies for electron microscopic studies were isolated as described by Francis *et al* (1994) from *S. typhimurium* cells of strain SJW134 (a wild-type strain except for the lack of expressed flagellin gene; Thomas *et al*, 2001) grown to late-log phase. The cells were cooled on ice, pelleted, lysed, and the outer membrane was dissociated with KOH, pH 11. To prepare specimens of basal bodies lacking the C-ring, extended basal bodies were spun down and the pellet was incubated in glycine (50 mM, pH 3.5) for 1 h at 0°C. The sample was again pelleted and resuspended in Tris-HCl (10 mM, pH 8), EDTA (5 mM), and Triton X-100 (0.1% v/v). The samples were kept at 0°C during all the steps.

Flagellated, CheY-containing, cytoplasm-free envelopes. Flagellated cell envelopes (Eisenbach and Adler, 1981) were prepared by penicillin treatment followed by osmotic lysis as described (Ravid and Eisenbach, 1984). The lysis medium contained purified, His-tagged CheY (36 μM, for inclusion in the formed envelopes; Ravid *et al*, 1986), Tris-HCl (50 mM, pH 7.9), Tetren (0.1 mM), and DL-lactate (2 mM).

Antibodies. Three polyclonal anti-FliG antibodies were used: anti-His-tagged FliG antibody for the pull-down experiments (Figure 2A); anti-FliG antibody received from K Oosawa for the immunolabelling of basal bodies (Supplementary Figure s3); and anti-FliG antibody obtained from M Macnab for western blots of bacterial extracts (Supplementary Figure s4). The polyclonal antibodies against His-tagged FliG (a band cut from gel) and those against FRD (purified native protein) were raised in rabbits as described (Harlow and Lane, 1988). To purify the antibodies, we used Immunolink beads (Pierce, Rockford, IL, USA). A lysate of a Δ*fliG* or Δ*frd* strain was used to remove all reactive material according to the manufacturer's protocol. The non-retained antibody was checked for purity and specificity by western blotting. The resulting antibodies were highly specific (Supplementary Figure s5).

Protein purification

His-tagged FliG and His-tagged CheY. *E. coli* 10 × His-FliG (for use in binding assays with SDH and FRD) was expressed from the plasmid pEWG1, which had been transformed into *E. coli* strain BL21(λDE3)pLysS (Table II). *E. coli* 6 × His-CheY, demonstrated earlier to be functional (Sagi *et al*, 2003), was produced from the plasmid pQE12, pre-transformed into *E. coli* strain M15 (Table II). Both proteins were purified by Ni-NTA affinity chromatography (QIAGEN) according to the manufacturer's instructions. Proteins were concentrated by ultrafiltration (Ultracel Amicon) and dialysed against the buffer used in the assay. The purities of the isolated proteins were high (Supplementary Figure s2).

SDH and FRD. *E. coli* SDH and FRD were purified to a high degree (Supplementary Figure s2) as described by Tornroth *et al* (2002) and Luna-Chavez *et al* (2000), respectively. Purified SDH and FRD had

specific activities of 17 and 20 $\mu\text{mol}/\text{min}/\text{mg}$ protein, as determined by the succinate:phenazine methosulphate/2,6-dichloroindophenol reaction at 37°C (Ackrell *et al*, 1978). The isolation procedure retains quinone and lipids in isolated SDH and FRD (Luna-Chavez *et al*, 2000; Yankovskaya *et al*, 2003) and yields a 1:1:1 stoichiometry of subunits in both enzymes (Supplementary Figure s2).

Switch proteins. His-tagged FliM was purified to >95% purity from *E. coli* strain BL21(λ DE3)pLysS, carrying the plasmid pEWM1 that overexpresses *S. typhimurium*'s His-tagged FliM, as described by Bren and Eisenbach (1998). *S. typhimurium* FliG (for use in binding assays with [^{14}C]fumarate) and FliN were overexpressed in *E. coli* strain BL21(λ DE3)pLysS and partially purified as described by Welch *et al* (1993).

Protein modifications

Radiolabelling of His-tagged CheY. Labelling of 6 \times His-CheY was performed *in vivo* by metabolic incorporation of [^{14}C]leucine during growth, as described by Sagi *et al* (2003).

Radiolabelling of SDH. SDH was radiolabelled by methylating its free amino groups with formaldehyde and NaB[^3H] $_4$, as described previously (Blat and Eisenbach, 1996). This radiolabelling approach had the disadvantage that it reduced the enzymatic activity of SDH by about half.

Biotinylation of SDH and FRD. The proteins in KP $_i$ (20 mM, pH 7.5) were added to 5- to 10-fold molar excess of biotin-3-sulpho-*N*-hydroxy succinimide ester (sodium salt), incubated overnight at 4°C, and dialysed against the binding assay buffer. Biotinylation was verified with streptavidin-agarose beads (Pierce) according to the manufacturer's protocol.

Binding of [^{14}C]CheY, [^3H]SDH, and [^{14}C]fumarate to the switch complex and to the M-ring

To assess binding of radiolabelled compounds to the switch complex or to the M-rings, samples were incubated at room temperature (25°C) with the radiolabelled protein or radiolabelled fumarate for 5 min (with the switch complex) or 1 h (with M-rings). For [^{14}C]CheY or [^{14}C]fumarate binding, 1.6 mg/ml switch complex was used. For [^3H]SDH binding, 0.1 mg/ml switch complex was used. The switch complex was incubated with [^{14}C]CheY (3.6 μM , 13–17 d.p.m./pmol), [^3H]SDH (0.25–4 μM , 2600–7400 d.p.m./pmol), or [^{14}C]fumarate (10–500 μM , 14 d.p.m./pmol) in a solution of Tris-HCl (50 mM, pH 7.9), MgSO $_4$ (5 mM), and BSA (10 mg/ml or, in the case of fumarate binding, 1 mg/ml). In addition, the solution contained [^3H]leucine or [^{14}C]leucine (600 d.p.m./pmol), as appropriate, to correct for the entrapped volume as described by Sagi *et al* (2003). For [^3H]SDH binding, the solution also contained NaCl (150 mM) and Thesit (0.05% w/v). For phosphorylation conditions in the case of [^{14}C]CheY binding, AcP (25 mM) was added to the reaction mixture. Following the incubation, the switch complex was centrifuged at 200 000 g for 10 min at 4°C. The pellet was resuspended in 100 μl of Tris-HCl (50 mM, pH 7.9). Aliquots of the reaction mixture before centrifugation and aliquots of the supernatant and pellet subsequent to centrifugation were counted for ^{14}C and ^3H with a liquid scintillation analyser.

Binding of [^{14}C]fumarate to isolated switch proteins

The binding of [^{14}C]fumarate to the switch proteins (distinct from its binding to the switch complex, described above) was carried out by equilibrium dialysis (a classical approach for studying the binding of a small molecule to a protein) and centrifugal ultrafiltration (a technique employed even for low-affinity binding) as detailed in Supplementary data.

Binding of FRD or SDH to FliG

Pull-down assay. Biotinylated FRD or SDH (4 μM) was mixed with His-tagged FliG (8 μM) in solution A containing KP $_i$ (20 mM, pH 7.5), NaCl (150 mM), and Thesit (0.05%), followed by overnight incubation with shaking at 4°C. Subsequently, the reaction mixture was incubated with streptavidin-agarose beads and BSA (1.6 mg/ml) for 1 h at 4°C, washed with solution A, boiled for 5 min with SDS sample buffer, applied to 12% SDS-PAGE, and the gels probed with anti-His-tagged FliG antibody.

Measurement of binding by surface plasmon resonance. Measurement of FliG-FRD/SDH binding by surface plasmon resonance was carried out with a ProteOn™ XPR36 Protein Interaction Array System (Bio-Rad). Biotinylated FRD and SDH were individually immobilized onto five NeutrAvidin-coated channels of a Proteon NLC (Bio-Rad) sensor chip, leaving one channel blank as reference. Immobilization was achieved by injecting biotinylated FRD (180 μl) in a solution of KP $_i$ (50 mM, pH 7.4), MgSO $_4$ (5 mM), NaCl (150 mM), and the surfactant Tween 20 (0.005%) at a rate of 30 $\mu\text{l}/\text{min}$. About 750–900 RU of biotinylated FRD and SDH was typically immobilized under these conditions. His-tagged FliG was passed continuously over the immobilized proteins in each channel at a rate of 100 $\mu\text{l}/\text{min}$. Regeneration of the surface was carried out with short pulses of NaOH (10 mM) for 30 s at a flow rate of 100 $\mu\text{l}/\text{min}$. As a negative reference, we used interferon $\alpha 2$. The dissociation and rate constants were calculated using the ProteOn Manager v.2 (Bio-Rad).

Sedimentation velocity measurement. Sedimentation velocity measurements were performed in an analytical ultracentrifuge as detailed in Supplementary data.

Electron microscopy assays

FRD binding to basal bodies. Basal bodies were incubated at 0°C for ~12–14 h with activated FRD (1 mg/ml). Anti-FRD antibody was added and the solution was incubated at 0°C for 15 min. Protein A-gold was added to the complex and incubated for 10 min at 0°C. The samples were centrifuged in a Beckman TLA rotor at 50k r.p.m. for 5 min. The pellet was washed 3 \times , applied to carbon-coated grids, and stained with 2% uranyl acetate. The samples were imaged with an FEI Morgani 268 electron microscope equipped with an AMT CCD camera, 1k \times 1k (FEI, The Netherlands) or Philips CM12 TEM (Phillips Electronic Instruments Co., Mahwah, NJ).

Negative staining for counting flagella. Cells were grown on tryptone broth to mid-logarithmic phase and washed with KP $_i$ (pH 7.4, 20 mM). The cell suspension (4 μl) was deposited on a nitrocellulose-coated electron microscopy grid. The sample was blotted with a filter paper and washed twice with droplets of 1% uranyl acetate.

Determination of the direction of flagellar rotation by tethering assay

The direction of flagellar rotation of intact cells and cell envelopes was determined by the tethering assay (Silverman and Simon, 1974), detailed in Supplementary data.

Measurement of intracellular ATP

Bacteria were grown on tryptone broth to a mid-exponential phase, frozen in aliquots of 1×10^7 cells, and diluted 1:6 in nucleotide-releasing buffer for bacterial cells (Lumac bv, The Netherlands). The ATP level in the cells was measured by the luciferin-luciferase method (Stanley and Williams, 1969), using a Lumac/3M Biocounter, model 2010A. Each measurement was performed in duplicate or triplicate. The calculations were based on an internal volume of 1.2×10^{-15} l/cell and 2.2 $\mu\text{l}/\text{mg}$ protein (Welch *et al*, 1995).

Statistical analysis

All statistical analyses were carried out by InStat software (GraphPad Software, San Diego, CA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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