Control of speed modulation (chemokinesis) in the unidirectional rotary motor of *Sinorhizobium meliloti*

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Summary

Swimming cells of *Sinorhizobium meliloti* are driven by flagella that rotate only clockwise. They can modulate rotary speed (achieve chemokinesis) and reorient the swimming path by slowing flagellar rotation. The flagellar motor is energized by proton motive force, and torque is generated by electrostatic interactions at the rotor/stator (FliG/MotA-MotB) interface. Like the *Escherichia coli* flagellar motor that switches between counterclockwise and clockwise rotation, the *S. meliloti* rotary motor depends on electrostatic interactions between conserved charged residues, namely, Arg294 and Glu302 (FliG) and Arg90, Glu98 and Glu150 (MotA). Unlike in *E. coli*, however, Glu150 is essential for torque generation, whereas residues Arg90 and Glu98 are crucial for the chemotaxis-controlled variation of rotary speed. Substitutions of either Arg90 or Glu98 by charge-neutralizing residues or even by their smaller, charge-maintaining isologues, lysine and aspartate, resulted in top-speed flagellar rotation and decreased potential to slow down in response to tactic signalling (chemokinesis-defective mutants). The data infer a novel mechanism of flagellar speed control by electrostatic forces acting at the rotor/stator interface. These features have been integrated into a working model of the speed-modulating rotary motor.

Introduction

Motile bacteria swim towards attractant stimuli in their environment by rotating helical flagella. The rotary motor at the flagellar base is embedded in the cell envelope, and is energized by a proton (or in some species, sodium ion) gradient across the cytoplasmic membrane (Manson et al., 1977; Hirota et al., 1981). Electron microscopy and biochemical-genetic studies have established the overall organization of a flagellum as drawn in Fig. 1A (Francis et al., 1994; Macnab, 1996; Berg, 2003). The helical filament joins the basal body via a flexible hook and a straight rod that is held by two rings in the cell envelope and firmly connected to the MS ring. The motors of many flagellated bacteria reverse (‘switch’) the sense of rotation, whereas the motors of others – like the soil bacterium *Sinorhizobium meliloti* described in this report – rotate unidirectionally and can modulate their rotary speed (Götz et al., 1982; Schmitt, 2002). The motor–switch complex or C ring contains two proteins, FilM and FilN, and a third protein, FliG, connects it to the MS ring. The MS ring plus the proteins involved in torque generation and switching of direction constitute the rotor.

The stator complexes surrounding the MS ring are composed of two transmembrane proteins, MotA and MotB, that interact in a (MotA); (MotB); stoichiometry to function as proton-conducting channels (Sato and Homma, 2000; Braun and Blair, 2001; Schmitt, 2003). Each MotA contributes four transmembrane helices and each MotB contributes one to the proton channel (Fig. 1B). The C-terminal periplasmic domain of MotB is probably anchored in the cell wall peptidoglycan (Macnab, 1996; Berg, 2003). The energy-transducing elements consist of the MS ring, with the 26 ± ~2 copies of FliG attached to its cytoplasmic face constituting the rotor, and the 8–10 MotA–MotB proton-conducting complexes constituting the stator (Blair and Berg, 1988; Khan et al., 1988; Thomas et al., 1999).

Cells of *Escherichia coli* and *Salmonella* swim by clockwise (cw) and counterclockwise (ccw) rotation of their left-handed helical flagella; frequent switches between the two modes of rotation enable them to change direction (for review, see Macnab, 1996). Unlike the enterobacteria, cells of the soil bacterium *S. meliloti* swim by unidirectional cw rotation of their right-handed helical flagella and respond to tactic stimuli by modulating the flagellar rotary speed thus facilitating changes of swimming direction (Götz and Schmitt, 1987). Full-speed cw rotation causes the four to six right-handed helical flagella to form a bundle that pushes the cell forward into a straight run. A new direction is assumed when the rotary speeds of individual flagella decline at different rates, which drives the bundles...
loop of the stator protein MotA (named MotA-L) that is diagrammed in Fig. 1B (Lloyd and Blair, 1997; Zhou et al., 1998; Lloyd et al., 1999). A quite similar pattern of conserved charged residues is present in the FliG-C and MotA-L domains of the S. meliloti flagellar motor. However, in the present study of about 40 single, double and triple replacements of these residues it became evident that the effect of certain charged positions on swarming and free-swimming speed (i.e. rotary speed) clearly differs between S. meliloti and E. coli. Unlike in E. coli, we identified reciprocal pairs of substitutions in MotA-L that strongly impaired or abolished chemokinesis, suggesting that rotary speed modulation is controlled at the rotor/stator interface. On the basis of these data we propose a model featuring two different rotor–stator alignments, one of which promotes fast the other slow flagellar rotation.

Results

Experimental background

Mutational analyses in E. coli have demonstrated the importance of charged residues in both FliG-C and MotA-L for motor function (Lloyd and Blair, 1997; Zhou et al., 1998). Accordingly, in FliG-C, the residues Arg281, Asp288 and Asp289 are most important (in that order), whereas Lys264 and Arg297 play a secondary role. In the E. coli MotA-L the charged residues Glu98 and Arg90 are essential and Glu150 is secondary for torque generation. Sequence alignments of the corresponding portions of the E. coli and S. meliloti FliG and MotA polypeptide chains, respectively, revealed that the MotA residues, Arg90, Glu98 and Glu150, are perfectly conserved between the two species (Fig. 2A). In contrast, only two of the important FliG residues, Arg281Ec and Asp289Ec, are conserved as Arg294Sm and Asp302Sm, respectively, whereas the third, Asp288Ec, was exchanged to Ser301Sm. Among FliG residues of secondary importance in E. coli, the positive charge of Lys264Ec was conserved as Arg277Sm, and Arg297Ec, was replaced by the neutral Asn311Sm. To facilitate comparison, these congruencies and disparities are diagrammed as edge-on views in Fig. 2B (left, middle). In view of the conservation of these charged FliG-C positions among diverse species equipped with a bidirectional rotary motor (such as E. coli, Vibrio alginolyticus, Borrelia burgdorferi and Thermotoga maritima) and, conversely, the two-charge deviations seen in species with unidirectional, speed-modulated flagellar rotation (including S. meliloti, Agrobacterium tumefaciens, Mesorhizobium loti and Rhizobium lupini H13-3), we initially inferred that these differences in charge distribution at the rotor–stator interface might be the basis for the different modes of flagellar rotation. However, when we exchanged the two deviant positions to S301D and N311R in the S. meliloti
FliG-C domain to mimic the *E. coli* charge pattern, the resulting *S. meliloti* strain (Fig. 2B, right) behaved like wild type with 98% swarming and 97% free-swimming proficiency and normal chemokinetics. This was also true, when in addition Glu300 was replaced by Arg to imitate Arg287 Ec (Fig. 2A; not shown in Fig. 2B). Therefore, these charge differences do not account for the different types of flagellar rotation, but merely reflect the 500 million year evolutionary divergence of *γ*-subgroup (*E. coli*) and *α*-subgroup (*S. meliloti*) proteobacteria (Ochman and Wilson, 1987; Olsen et al., 1994). Hence, we had to look for other substitutions of conserved residues in the FliG-C and MotA-L domains that would affect the *S. meliloti* pattern of swarming and swimming behaviour.

**Targeted mutagenesis of motA and fliG and motility tests**

Amino acid exchanges in FliG-C and MotA-L were introduced into the coding sequences by polymerase chain reaction (PCR) mutagenesis. The mutant sequences were used to transform *E. coli* S17-1 and were then conjugally transferred to wild-type *S. meliloti* R11/001 (Simon et al., 1986). Stable mutants resulting from allelic exchange by homologous recombination were isolated by selection against the suicidal plasmid-borne *sacB* marker (Selschka et al., 1993). By using this more laborious way of allelic exchange rather than using complementation of null alleles by plasmid-borne alleles, all introduced mutant alleles were expressed under the native promoter. This strategy avoided potential complications caused by abnormal stoichiometries of the mutant MotA and FliG proteins that might have biased the interpretation of results. Moreover, by using quantitative Western blot analysis of typical FliG mutants, we have certified that amino acid replacement did not destabilize the mutant FliG proteins (Fig. 3).

Two methods served for assessing the motile behaviour of mutant strains in relation to wild-type. First, the swarm-
ing diameters obtained on Bromfield soft-agar plates were compared with the wild-type standard. On such plates, strains with normal motility swarm outward rapidly from the point of inoculation, whereas those with impaired motility swarm more slowly or not at all. These tests were fast and inexpensive and provided a basis for comparison with swarming rates obtained in *E. coli* (Lloyd and Blair, 1997; Zhou *et al*., 1998). The swarming rates of the important mutant strains relative to wild type are listed in Table 1. Given the interdependence of motile and chemotaxis proficiencies that contribute to swarming (as an outwardly directed attractant gradient is formed by the swarming cells that metabolize and eliminate attracting amino acids on the plate), the swarm ring provides an indirect measure of motility. Hence, an additional assay of motility was used that monitors and averages free-swimming speed. We have previously shown that averaging the speeds of 100–300 *S. meliloti* cells by computerized motion analysis yielded reproducible values of mean population speed with clear responses to external stimuli (Sourjik and Schmitt, 1996). This method is especially suited for a system of unidirectional flagellar rotation, where average free-swimming speed is a direct function of flagellar rotary speed, and hence can be used for characterizing flagellar motor mutants. Table 2 lists the free-swimming speeds observed for all relevant strains of *S. meliloti* in both the absence and the presence of a chemoattractant.

**Table 1.** Swarming rates in soft agar of *Sinorhizobium meliloti* motA and fliG single and double mutants measured relative to wild-type (WT) controls.

<table>
<thead>
<tr>
<th>MotA mutation</th>
<th>FliG mutation</th>
<th>WT</th>
<th>R90A</th>
<th>R90E</th>
<th>R90K</th>
<th>E98Q</th>
<th>E98K</th>
<th>E98D</th>
<th>E150Q</th>
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<tr>
<td>WT</td>
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<td>0.17</td>
<td>0.46</td>
<td>0.32</td>
<td>0.00</td>
<td>0.29</td>
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<tr>
<td>R294A</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R294D</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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</tr>
<tr>
<td>R294V</td>
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<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E300R</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td></td>
</tr>
<tr>
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<td>0.99</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td></td>
</tr>
<tr>
<td>D302A</td>
<td>0.86</td>
<td>0.27</td>
<td>0.11</td>
<td>0.25</td>
<td>0.29</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>D302K</td>
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<td>0.09</td>
<td>0.29</td>
<td>0.00</td>
<td>0.00</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R294A/S301D</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>R294A/D302A</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
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<tr>
<td>S301D/N311R</td>
<td>0.98</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
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</tbody>
</table>

Table 2 lists the free-swimming speeds observed for all relevant strains of *S. meliloti* in both the absence and the presence of a chemoattractant.

**Residues important for torque generation**

Flagellar motor function vitally depends on charged residues located at conserved positions in FliG-C (rotor) and MotA-L (stator) (Fig. 2B), clearly suggesting that electrostatic forces are at the heart of energy transduction between the rotor and the stator (Zhou *et al*., 1998; Schmitt, 2003). In trying to understand how the speed-modulated *S. meliloti* motor (as opposed to the switch-type *E. coli* motor) might function, we have first defined residues that are important for torque generation, and then analysed mutations that impaired chemokinesis. In our analysis of torque generation we have studied the phenotypes of defined mutants: (i) to classify relevant amino acid residues with regard to their importance for motor function, (ii) to analyse cases of synergism and (iii) to identify residues of special significance for the operation of the *S. meliloti* motor.

(i) The importance of single residues for motor function was assessed by comparing the effects of charge-neutralizing mutations with (more severe) charge-reversing mutations (Tables 1 and 2): if neutralization of a single charged residue led to paralysis, it indicated that the charged residue is essential for torque generation. In *S. meliloti* (but not in *E. coli*) this applies to the MotA residue Glu150, where substitution of a neutral glutamine residue (E150Q) resulted in a dominant loss of motor function (Fig. 4A). In contrast, although charge-reversing mutations of Arg294 (R294D in FliG) and Glu98 (E98K in MotA) paralysed the motor, charge-neutralizing substitutions for these residues (R294A and E98Q) merely resulted in gradual loss of swarming proficiency (Table 1). [It should be noted that the FliG ‘null’ muta-

tion R294V represents a special case; that is also seen in E. coli (Zhou et al., 1998). The effect of this replacement is apparently not a simple neutralization of charge, but probably also involves a 'spreading side-chain' effect when valine replaces Arg294, which may cause rotor–stator jamming.

(ii) Residues of secondary importance in torque generation have been revealed by the synergism observed in FliG/MotA double mutants as well as in double mutants of each protein. Synergisms have been indicated by boldface type in Tables 1 and 2. The synergisms exhibited by the intermolecular double mutant R294A(FliG)/R90A(MotA) (which was paralysed) – and the intramolecular FliG double mutant, R294A/D302A, identified Arg90(MotA) and Asp302(FliG) as charged residues that make secondary contributions to function. Finally, synergisms that involve the 'cryptic' FliG residues Glu300 (corresponding to E. coli Arg287) and Ser301 (corresponding to E. coli Asp288; Fig. 2) revealed the functional significance of those residues. To be more specific, two double mutants, namely, S301D(FliG)/R90E(MotA) and E300R(FliG)/R90E(MotA), exhibit such synergism (Tables 1 and 2). Taken together, these data suggest the following order of importance for torque generation in the S. meliloti rotary motor, FliG: Arg294–Asp302–[Ser301–Glu300] and MotA: Glu150–Glu98–Arg90. This hierarchy has been represented by different intensities of shading in the schematic diagrams (Figs 2, 4, 5 and 6).

(iii) Two mutation patterns that differentiate the electrostatic interactions operating at the S. meliloti rotor–stator interface from those operating in E. coli are presented in Fig. 4. The paralysing effect of a single neutralizing mutation, E150Q (MotA), is evidenced by the inability of this mutant to either swarm or swim (Fig. 4A). Unlike in E. coli (Zhou et al., 1998), the S. meliloti E150Q (MotA) reflects a unique case in which the electrostatic interaction of a single charged MotA residue critically affects motor function. As suggested in the diagram of Fig. 4A, the absence of the normal torque-generating interaction between

<table>
<thead>
<tr>
<th>FliG mutation</th>
<th>MotA mutation</th>
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<tbody>
<tr>
<td>WT</td>
<td>R90A/R90E/R90K/E98Q/E98K/E98D/E150Q</td>
</tr>
<tr>
<td>WT</td>
<td>38.5</td>
</tr>
<tr>
<td>R294A</td>
<td>28.5</td>
</tr>
<tr>
<td>R294D</td>
<td>0.0</td>
</tr>
<tr>
<td>R294V</td>
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</tr>
<tr>
<td>E300R</td>
<td>36.0</td>
</tr>
<tr>
<td>S301D</td>
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</tr>
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<td>D302A</td>
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<tr>
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<td>R294A/D302A</td>
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<td>D302A/S301D</td>
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</tr>
<tr>
<td>N311R</td>
<td>39.9</td>
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</table>

<table>
<thead>
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<th>MotA double mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
</tr>
<tr>
<td>WT</td>
</tr>
<tr>
<td>R294A</td>
</tr>
</tbody>
</table>

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Table 2. Free-swimming speeds (μm s⁻¹) of S. meliloti motA and fliG single and double mutants without and with stimulation by proline.α, β

α. Instantaneous velocity (absolute speed) averaged for every swimming track. Mean values for 1000 individual tracks were determined from each sample by computerized motion analysis and were averaged from at least five independent cell populations with standard deviations from the mean ranging between 3% and 7%.

β. The second line in each row gives the swimming speed in the presence of 10⁻² M proline (chemokinesis). An asterisk (*) marks primary chemokinesis defects characterized by high free-swimming speed and little speed increase upon tactic stimulation; a cross (+) marks secondary chemokinesis defects, i.e. mutants affected in free-swimming speed (torque generation) and chemokinesis. Synergism is indicated by boldface type.
Control of chemokinesis in *S. meliloti*

Glu150 (MotA) and Arg294 (FliG) cannot be compensated for by interactions of Arg90 and Glu98 on the stator side with Asp302 and Arg294 on the rotor. As will be detailed in the next section, these two MotA residues are primarily responsible for the chemokinetic response. A specific pattern of synergism involving the FliG double mutation R294A/S301D is depicted in Fig. 4B. Whereas the single substitutions, R294A and S301D, exhibited moderate or no effects on swarming, their combination resulted in the complete loss of motor function. These data came as a surprise, as a swarming rate of 0.86 was reported for the corresponding combination in the *E. coli* FliG, R281A-D288 (Zhou *et al.*, 1998). We propose that the loss of the major positive charge, Arg294, on the FliG side (which would normally attract Glu98 and Glu150) opens the field for strong repulsive interactions between Glu98/Glu150 (MotA) and Asp301 (FliG) – a repulsion that precludes any force-generating rotor–stator interaction (Fig. 4B). Given the conserved pattern of charged residues at the *E. coli* and *S. meliloti* stator–rotor interfaces (Fig. 2), the distinctive effects of charge variations imply that different topologies of charge–charge interactions at the rotor–stator interface distinguish these two motor systems.

**Mutations that impair chemokinesis**

Chemokinesis, the response to chemoattractants by an increase in free-swimming speed (reflecting an increase in flagellar rotary speed), is the salient feature that distinguishes the speed-modulating rotary motor of *S. meliloti* from the switch-type motor of *E. coli* (Sourjik and Schmitt, 1996; Schmitt, 2002). To elucidate the molecular basis for this phenomenon, we have looked for *S. meliloti* mutations that impair or abolish chemokinesis without affecting full-speed swimming (torque generation). Such mutations should result in smooth-swimming mutants that have lost their chemotaxis proficiency, and that therefore form swarm rings much smaller than those of wild-type controls. So we have examined all mutants with reduced swarming rates (Table 1) for their swimming proficiency (Table 2), and have thereby discovered that certain exchanges at two conserved MotA positions, R90 and E98, yielded the anticipated phenotype.

Figure 5 displays swarm-plate photographs and descriptive diagrams for four strains bearing substitutions at one or both of these MotA positions. Three of these strains had chemokinesis defects and the fourth had a non-swarming, slow-swimming phenotype. The three chemokinesis mutants with swarming rates around 0.3 had free-swimming speeds that were about 10% above the wild-type level of 38.5 m m s\(^{-1}\) (Table 2), but only increased their swimming speed upon tactic stimulation by about 2%, on average, compared with the 7.5% increase in speed exhibited by the wild-type strain. (Fig. 5).

Two strains that had a single charge-neutralizing mutations (R90A or E98Q) that abolished one of the two electrostatic interactions of MotA with FliG (but left the other interaction intact) exhibited a significant increase in free-swimming speed combined with an 80–90% loss of chemokinesis proficiency – which was reflected in their low-swarming rates (Fig. 5A and Table 2). However, when these two charge-neutralizing mutations were combined in an R90A/E98Q double mutant, the cells lost their swarming and chemokinesis proficiencies almost entirely (Fig. 5B, right).

Interestingly, chemokinesis defects were also observed in the R90K and E98D mutants, in which Arg90 or Glu98 had been replaced by an identically charged but smaller residue, Lys or Asp respectively (Table 2). Although each
of these mutants still had two charged MotA residues of the type required to interact with countercharges in FliG, they swam faster than wild type in the absence of stimulation, but their chemokinetic potentials were substantially reduced, resulting in low-swarming rates (Tables 1 and 2). Similar results were observed with the R90K/E98Q and R90A/E98D double mutants in which one of the charged residues had been replaced by a smaller charged analogue and the second had been replaced by an uncharged residue, and in the R90K/E98D double mutant in which both charged residues had been replaced by their smaller charged analogues. All three of these mutant strains swam faster than wild type in the absence of tactic stimulation, but had reduced chemokinetic potentials and reduced swarming rates (Tables 1 and 2; Fig. 5A and B). These data may be summarized and rationalized as follows. Controlled modulation of swimming speed (chemokinesis) depends on the presence in MotA of two conserved, charged residues: Arg90 and Glu98. Their coordinated action is absolutely required for the controlled increase and decrease of free-swimming speed, and this function is lost if either of them is replaced by a neutral residue or by its smaller charged analogue (Lys or Asp respectively). On the other hand, only one of these charged MotA residues — and it can be either the native amino acid or its smaller analogue — is necessary and sufficient for maintaining high-swimming speed. However, loss of both MotA charges results in a very slow, uncoordinated swimming pattern and a complete lack of swimming. A working model of the S. meliloti flagellar motor that accounts for these observations will be discussed.

Discussion

Motile bacteria alternate between straight ‘runs’ and brief ‘tumbles’ that change the direction of the swimming path. It is the way flagellar rotation is modified to cause tumbling that differentiates the E. coli-type and the S. meliloti-type rotary motors. Whereas the left-handed helical flagella of E. coli switch from ccw to cw rotation to initiate a tumble, the S. meliloti right-handed helical flagella always rotate cw, but they individually decrease their rotary speeds to initiate tumbling (Scharf, 2002; Schmitt, 2002). We have shown in this study that chemokinesis depends on a normal stator–rotor interface (Fig. 1) and that controlling rotary speed depends on the integrity of two highly conserved charged MotA residues: Arg90 and Glu98 (Fig. 5, Table 2).

How can we envisage the action of such charged residues in the flagellar motor? In a series of elegant experiments in E. coli, Blair and colleagues have demonstrated that motor function absolutely depends on certain charged residues present in the MotA-L (stator) and FliG-C (rotor) domains that face each other (Lloyd and Blair, 1997; Zhou et al., 1998; Lloyd et al., 1999). Based on these and our own results, we have recently proposed (Schmitt, 2003) a concept of motor function predicting that proton flow generates short, reversible helix rotations of the MotA–MotB channel complex (the stator) that are
transmitted by Coulomb forces to the FliG segments at the rotor surface (Fig. 6A). The present data are being interpreted in this context.

The rotation pattern of the *S. meliloti* flagellar motor requires components for generating torque and for decreasing rotary speed. We found that a singular dominant player in torque generation is the MotA residue Glu150, because a charge-neutralizing replacement (E150Q) resulted in a complete loss of swarming and swimming proficiency (Fig. 4A). This single torque-eliminating mutation is so far the strongest case for electrostatic force transmission from the stator to the rotor (but see also Zhou et al., 1998). It may seem surprising initially that the obvious charge-antipode of Glu150 in the FliG active site, Arg294, can be neutralized without causing complete paralysis (Fig. 4B, 1). It has been previously suggested that the equivalent phenomenon that is observed in *E. coli* may reflect the involvement of FliG in multiple electrostatic interactions, each of which participates in – but none of which is indispensable for – torque generation (Lloyd and Blair, 1997). Future analyses will show whether this suggestion appears equally valid for *S. meliloti*. For example, screening secondary mutations for ones that are synergistic with the charge-neutralizing R294A mutation should reveal whether positively charged residues near the FliG active site, such as Arg277 or Arg297 (Fig. 2A), function as alternative partners for Glu150 (MotA) in torque generation.

In distinction to the situation in *E. coli* (Zhou et al., 1998), the other two essential charged MotA residues of *S. meliloti*, Arg90 and Glu98, function in rotary speed modulation rather than in torque generation. This effect became obvious because of the surprising observation that charge neutralization of one residue resulted in high free-swimming speeds but tiny swarm rings (Table 2; Fig. 5A). This behaviour was found to be caused by an inability of mutant cells to modulate their swimming speed and, hence, an inability to respond chemotactically. The fact that the doubly charge-neutralized MotA mutant, R90A/E98Q, did not swarm (Fig. 5B-2), but still retained 59% of the wild-type free-swimming speed (Table 2), reinforces the conclusion that MotA Arg90 and Glu98 are at most secondary players in torque generation.

A particularly curious observation is that although either Lys at position 90 or Asp at position 98 will support high free-swimming speed, neither of those two smaller-charged isologues will support proper speed modulation (Table 2). If we can understand why Lys and Asp are incapable of replacing their larger isologues, Arg and Glu, in maintaining the full potential for rotary speed variation, we will be well on our way to understanding the molecular mechanism of speed modulation.

The essential difference between the isologous amino acids is the greater lengths of the side-chains of Arg and Glu relative to Lys and Asp. With the former two residues, the charged atoms are positioned one C–N bond (1.27Å) or one C–C bond (1.54Å), respectively, farther from the MotA polypeptide backbone, so that these charges may be more exposed at the surface of MotA. Given the importance of these two charged stator residues which face the rotor, a straightforward interpretation of the data is that precise charge–charge interaction at a critical distance for Coulomb forces to act is mandatory for matching the two interacting surfaces.

With these restrictions in mind, we propose a working model of stator–rotor interactions that accounts for fast
and slow rotation. The top view in Fig. 6A illustrates our concept of stator–rotor dynamics during a single power stroke, showing two FliG-rotor segments and one MotAB stator unit facing each other (Schmitt, 2003). Torque is generated by electrostatic force transmission between the antipodal charged residues. This model predicts that rotational movements of the stator (driven by the proton flow) are transmitted to the rotor by an alternation between electrostatic engagement (Fig. 6A, I) and disengagement (Fig. 6A, II) that turns the rotor one step around. The scheme shown in Fig. 6B features two static alignments of stator and rotor (each corresponding to Fig. 6A, I). The alignments reflect two extreme positions of the dominant charged MotA residue, Glu150, relative to the charged FliG residues, Arg294 and Asp302. In alignment I (Fig. 6B), the Glu150 (MotA) and Arg294 (FliG) residues are favourably positioned for an electrostatic interaction that is optimal for torque generation and fast rotary speed. In the absence of a chemoattractant, however, a change in FliG conformation (induced by the activated response regulator, CheY2-P) leads to a new stator–rotor alignment II (Fig. 6B) that places Glu150 (MotA) in juxtaposition to Arg294 and Asp302 (FliG), so that an imbalance between attractive and repulsive forces ensues (as in Fig. 4B), resulting in lower torque and slow rotation. Postulating participation of Asp302 in the repulsive interaction (Fig. 6B) appears logical, because it is the only FliG residue in which we have found that a charge inversion, D302K, affects chemokinesis (Table 2).

The central feature of the S. meliloti motor is the role attributed to the MotA residues Arg90 and Glu98, both of which must be intact to achieve chemokinesis (Fig. 5; Table 2). We propose that alignment II (which is required for slow flagellar rotation; Fig. 6B) is stabilized by the electrostatic forces exerted by both Arg90 and Glu98 interacting with their charged FliG antipodes, as illustrated by the dashed lines in Fig. 6B. If one of these stabilizing interactions fails (as in the mutants R90A and E98Q; Fig. 5A), alignment II cannot be maintained, and Glu150 swings back to a position of optimum torque generation and fast rotation, as in Fig. 6A. This interpretation also permits us to rationalize the advantage of Arg90 and Glu98 for deceleration over their smaller isologues: presumably their longer side-chains place their charged groups farther from the stator surface, closer to the antipodal charges on the rotor, and hence capable of exerting stronger Coulomb forces on the rotor.

The importance of an exact alignment of MotA and FliG for flagellar motor function was previously suggested by Garza et al. (1996), who studied dominant mutations in the motB gene of E. coli that were believed to misalign the stator relative to the rotor. A comprehensive analysis of extragenic suppressors (located in the motA and fliG genes) led these authors to conclude that the suppressor mutations ‘restore motility by introducing compensatory realignments in MotA and FliG’. In their model of flagellar motor mechanics and motor function, Braun et al. (1999) proposed that electrostatic interaction at the stator–rotor interface could participate in driving rotor movement. This interpretation is consistent with our prediction that torque is generated by electrostatic force transmission from the stator to the rotor (Fig. 6A). Our second prediction that electrostatic interactions are required for securing and stabilizing a defined alignment of MotA and FliG (Fig. 6B, II) is an added feature accounting for the integral role of Arg90 and Glu98 in achieving chemokinesis.

The major difference in motor operation between the switch-type (E. coli) and the speed-modulating (S. meliloti) rotary systems is clearly revealed by contrasting the swarming rates that result from charge-neutralizing mutations of three MotA residues: R90A: 0.29/0.71; E98Q: 0.32/0.98; and E150Q: 0.00/1.09 (S. meliloti/E. coli swarming rates respectively; data from Table 1 and Zhou et al., 1998). These data reveal clear differences in function and indicate the unique role played by Glu150 in torque generation and the crucial roles played by Arg90 and Glu98 in chemokinesis in S. meliloti, but not in E. coli.

Sequence differences between the orthologous motor proteins of the two species are remarkable – only 36% similarity/23% identity for FliG and only 53% similarity/43% identity for MotA – suggesting that many minor differences in the MotA and FliG sequences are unimportant for wild-type motile behaviour (Fig. 2). However, the key charged residues are highly conserved, and the contrasting consequences of conservative exchanges in these residues in S. meliloti and E. coli motor proteins indicate a different topology of antipodal charges interacting at the rotor–stator interface, presumably as a result of subtle differences in MotA and/or FliG conformation in the two species.

Biochemical, physiological and structural evidence strongly suggests that in E. coli tactic signalling by an activated response regulator binding to FlIM at the cytoplasmic face of the flagellar motor (Fig. 1) induces a conformational change in FliG, causing a switch in the direction of rotation (Scharf et al., 1998; Bren and Eisenbach, 2000; Brown et al., 2002). Given a similar chain of events in S. meliloti, the ensuing change of FliG conformation must be different from that in E. coli to produce a decrease of rotary speed. Indeed, the S. meliloti FlIM N-terminal region (corresponding to the E. coli CheY binding site) and its potential ligand, the activated chemotaxis response regulator CheY2-P (signalling slowdown), have diverged so greatly from their E. coli orthologues that a different signal may be generated and may induce a different conformational change of FliG (Riepl et al., 2004; B. Scharf, K. Pichler and R. Schmitt, unpublished data).

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Molecular genetic analyses are underway to define the site in FliM that interacts with CheY2-P.

**Experimental procedures**

**Bacterial strains and plasmids**

*Escherichia coli* S17-1 (recA1 endA1 thi hsdR RP4-2-Tc::Mu-Tn7 Tp" Sm"; Simon *et al*., 1986) was used for conjugal transfer of the mobilizable plasmid pK18 mob sacB (lacZ mob Km; Schäfer *et al*., 1994) to *S. melloti*. All motA and flIG mutations listed in Tables 1 and 2 were introduced by allelic exchange into homologous gene loci of *S. melloti* RU11/001 (‘wild type’ with regard to swimming behaviour), a spontaneous SmR derivative of the original isolate MVII-1 (Kamberger, 1979; Krupski *et al*., 1985).

**Media and growth conditions**

*Escherichia coli* 17-1 was grown in Luria broth (Luria *et al*., 1960) at 37°C overnight. *S. melloti* strains were grown in TYC (0.5% tryptone peptone, 0.3% yeast extract, 0.13% CaCl₂ · 6 H₂O) at 30°C for 2 days (Platzer *et al*., 1997). Samples for motion analysis were prepared in 10 ml of RB minimal medium (Götz *et al*., 1982), inoculated with 20 µl of stationary phase culture and layered on Bromfield agar plates (Sourjik and Schmitt, 1996). Plates were incubated at 30°C for 15 h to an OD₆₀₀ of 0.1–0.15 (Sourjik and Schmitt, 1996). Plates were incubated at 30°C for 2 days (Platzer *et al*., 1997). Samples for motion analysis were prepared in 10 ml of RB minimal medium (Götz *et al*., 1982), inoculated with 20 µl of stationary phase culture and layered on Bromfield agar plates (Sourjik and Schmitt, 1996). Plates were incubated at 30°C for 15 h to an OD₆₀₀ of 0.1–0.15 (c. 10 ml) to yield highly motile cells. Antibiotics were used at the following final concentrations: *E. coli*, kanamycin at 50 mg l⁻¹; *S. melloti*, neomycin at 120 mg l⁻¹ and streptomycin at 600 mg l⁻¹.

**Construction of motA and flIG codon exchanges**

Amino acid substitution listed in Tables 1 and 2 were generated by in vitro codon exchanges using the overlap extension PCR method described by (Higuchi, 1989). PCR products containing the exchanges were cloned into the mobilizable suicide vector pK18 mob sacB (Schäfer *et al*., 1994), used to transform *E. coli* S17-1, and then conjugally transferred to *S. melloti* by filter matings according to Simon *et al*. (1986). Allelic replacement was achieved by sequential selections on neomycin and 10% sucrose as described previously (Selbitschka *et al*., 1993). Correct allelic replacement was verified by gene-specific primer PCR and sequencing.

**DNA methods**

Plasmid DNA for sequence analysis was purified with NucleoSpin (Macherey Nagel, Düren, Germany). DNA fragments or PCR products for cloning purposes were purified from agarose gels or from solutions by the use of the Qiaex DNA purification kit (Qiagen, Hilden, Germany). DNA fragments for sequence analysis were purified using the GFX kit (Amersham Pharmacia, Freiburg, Germany). Chromosomal DNA templates for PCR were obtained from a single bacterial colony resuspended in 50 µl of water and incubated at 100°C for 15 min. Sequencing was performed with a model 310 automatic sequencer (Applied Biosystems, Weiterstadt, Germany).

**Western blot analysis**

Extracts of wild-type and mutant *S. melloti* (1 ml of cells of OD₆₀₀ = 0.3) were separated on a 10% SDS-polyacrylamide gel, electroblotted to a nitrocellulose membrane (Amersham Biosciences Europe GmbH, Freiburg, Germany), probed with purified anti-FliG polyclonal antibody (raised against recombinant FlIG) at 1:100 dilution and a horseradish peroxidase-linked anti-rabbit immunoglobulin antibody at 1:5000 dilution (Scharf *et al*., 2001). Signals were visualized by using the ECL Western blotting analysis system on Hyperfilm ECL (Amersham Biosciences), and films were scanned using an Epson Perfection 1640SU.

**Swarming plate assay**

Swarming plates containing Bromfield medium and 0.3% BactoAgar were inoculated with 3 µl droplets of an overnight TYC culture and incubated at 30°C for 3 days. Swarm diameters were determined and swarming rates expressed relative to wild-type controls present on the same plates.

**Computerized motion analysis of free-swimming cells**

Motile cell samples at OD₆₀₀ 0.1–0.3 were observed with a Zeiss Standard 14 phase-contrast microscope (400× magnification) at room temperature (22°C). Averaged free-swimming speeds of c. 50 cells were determined by computerized motion analysis using the Hobson Bactracker system (Hobson Tracking System, Sheffield, UK) as previously described (Sourjik and Schmitt, 1996). Speed values were averaged from 5 × 200 tracks each, and the data given in Table 2 represent the mean of five independent experiments.

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**References**


