The bidirectional polar and unidirectional lateral flagellar motors of Vibrio alginolyticus are controlled by a single CheY species

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Summary
The bacterial flagellar motor is an elaborate molecular machine that converts ion-motive force into mechanical force (rotation). One of its remarkable features is its swift switching of the rotational direction or speed upon binding of the response regulator phosphorylated CheY, which causes the changes in swimming that achieve chemotaxis. Vibrio alginolyticus has dual flagellar systems: the Na+-driven polar flagellum (Pof) and the H+-driven lateral flagella (Laf), which are used for swimming in liquid and swimming over surfaces respectively. Here we show that both swimming and surface-swimming of V. alginolyticus involve chemotaxis and are regulated by a single CheY species. Some of the substitutions of CheY residues conserved in various bacteria have different effects on the Pof and Laf motors, implying that CheY interacts with the two motors differently. Furthermore, analyses of tethered cells revealed that their switching modes are different: the Laf motor rotates exclusively counterclockwise and is slowed down by CheY, whereas the Pof motor turns both counterclockwise and clockwise, and CheY controls its rotational direction.

Introduction
The bacterial flagellar motor is a molecular machine that converts ion-motive force into mechanical force into rotation of helical filament extending from the cell body (for reviews, see Macnab, 1996; Yorimitsu and Homma, 2001; Kojima and Blair, 2004). Another remarkable feature of the flagellar motor is that its direction of rotation switches without changing the direction of ion flow (unlike F1Fo-ATP synthase, in which reverse rotation produces reverse ion flow). This switching of the motor causes two alternate modes of swimming, e.g. run/tumble (in peritrichously flagellated bacteria) or forward/backward in polarly flagellated bacteria, and hence generates the random walk behaviour of the cell. The switching frequency is modulated in response to external stimuli, thereby enabling a cell to migrate towards an attractant or away from a repellent via biased random walk. Many bacterial species, including Escherichia coli, switch the rotational sense of the motor, i.e. between counterclockwise (CCW) and clockwise (CW) (Larsen et al., 1974; Macnab, 1996). Other bacteria employ other modes of switching, such as rotation/stop in Rhodobacter sphaeroides (Armitage and Macnab, 1987) and fast/slow in Sinorhizobium meliloti (Götz et al., 1982; Schmitt, 2002). However, the molecular mechanism underlying motor switching is largely unknown.

Some marine Vibrio spp. (V. alginolyticus and V. parahaemolyticus) have two types of flagella, the polar flagellum (Pof) and the lateral flagella (Laf) (Blake et al., 1980). The small, soluble protein CheY in its active phosphorylated form is the response regulator that binds to the flagellar motor to promote switching. However, the genome of V. parahaemolyticus (Makino et al., 2003; see below for detail) contains only one cheY gene. (The genome sequence of V. alginolyticus 12G01 is available online, but the sequence has not been completed. The che genes of V. alginolyticus are very similar to those of V. parahaemolyticus in terms of sequence and gene order on the chromosome. Considering this situation, we will refer to the genome sequence of V. parahaemolyticus throughout the text.) The two flagellar systems of marine Vibrio play distinct roles in their life cycles: in liquid, a rod-shaped swimmer cell is propelled by a single polar flagellum, which is produced constitutively, whereas on a surface the cell elongates and generates numerous lateral flagella as it differentiates into a swarmer cell that can migrate over the surface (McCarter and Silverman, 1990).

Consistent with their distinct physiological roles, the two types of flagella have many distinct properties. (i) The polar flagellum assembles at a cell pole, and its filament is...
sheathed with an extension of the outer membrane (Allen and Baumann, 1971), whereas the lateral flagella are peritrichous and are not sheathed. (ii) The two types of flagella are encoded by separate (non-overlapping) sets of genes (Makino et al., 2003), and no single mutation that abolishes the Pof and Laf formation has been isolated (McCarter et al., 1988). (iii) The energy sources of the Pof and Laf motors are the Na\(^+\)- and H\(^+\)-motive forces respectively (Atsumi et al., 1992; Kawagishi et al., 1995). (iv) In a high-viscosity environment, the lateral flagella work better than the polar flagellum, although swimming propelled by the polar flagellum is much faster in a low-viscosity environment (Atsumi et al., 1996). (v) The polar flagellum serves as a sensor for the viscosity (via the flagellar rotation speed) to regulate \(\text{laf}\) gene expression (McCarter et al., 1988; Kawagishi et al., 1996).

Signal transduction in chemotaxis has been best studied in \(E.\ coli\) (for reviews, see Sourjik, 2004; Wadhams and Armitage, 2004; Parkinson et al., 2005; Baker et al., 2006). Central to this pathway is a two-component regulatory system that consists of the histidine kinase CheA and its substrate CheY. CheA is activated when coupled to an unliganded chemoreceptor [also known as methyl-accepting chemotaxis protein (MCP)]. CheA phosphorylates itself and transfers the phosphoryl group to CheY. Binding of phospho-CheY to the switch complex of the flagellar motor induces its CW rotation, resulting in an abrupt change in its swimming direction called a tumble.

Chemotaxis-defective (\(\text{che}\)) mutants of \(V.\ parahaemolyticus\) have been isolated. Detailed characterization of the mutants strongly argues that both Pof and Laf, which have their own motors, are controlled by a common chemosensory signalling system (Sar et al., 1990; Kim and McCarter, 2000; McCarter, 2004). Indeed, the genome sequence of \(V.\ parahaemolyticus\) (Makino et al., 2003) has identified that it has one set of \(\text{che}\) genes and two sets of flagellar genes (McCarter, 2004). Thus, the flagellar and chemotaxis systems of \(V.\ parahaemolyticus\) shows a sharp contrast to those of \(Vibrio cholerae\): the latter has a single flagellar system (Pof) but has three sets of \(\text{che}\) genes (Gosink et al., 2002), only one of which is directly involved in chemotaxis (Gosink et al., 2002; Hyakutake et al., 2005).

It is therefore likely that a single CheY species can control two distinct flagellar motors in \(V.\ alginolyticus\), which is closely related to \(V.\ parahaemolyticus\). However, the Laf and Pof homologues of the \(E.\ coli\) motor switch component FlIM, which binds phospho-CheY, are quite dissimilar in \(V.\ alginolyticus\) (see Discussion for details). If a single CheY species indeed controls the Laf and Pof systems, \(V.\ alginolyticus\) becomes a nice system for a comparative study of the two systems for control of flagellar rotation.

It should be noted, however, that swimming (Pof motility) and swarming (Laf motility) are quite different. In particular, surface motility of \(E.\ coli\), which also differentiates into an elongated and hyperflagellated swarmer cell on surfaces (Harshey and Matsuyama, 1994), does not require chemotaxis, although the chemotactic signalling system is essential (Burkart et al., 1998). Therefore, it must be conclusively determined whether surface-swarming of \(V.\ alginolyticus\) involves chemotaxis and whether the lateral flagellar motors are under the control of CheY when cells are swarming on surfaces.

In this study, we cloned and characterized the \(\text{cheY}\) gene of \(V.\ alginolyticus\) and demonstrated that one CheY protein regulates both Pof and Laf motility. We then showed unambiguously that surface-swarming of \(V.\ alginolyticus\) involves chemotaxis. Furthermore, we found that some of the mutations targeting CheY residues conserved among various bacteria had different effects on the Pof and Laf systems, implying that CheY interacts with the two motors differently. We conclude that CheY, presumably in its phosphorylated form, slows rotation when it binds to the Laf motor, whereas it induces CW rotation when it binds to the Pof motor.

## Results

**The \(\text{cheY}\) gene of \(V.\ alginolyticus\)**

The \(V.\ alginolyticus\) \(\text{cheY}\) gene was cloned and sequenced. The deduced amino acid sequence of CheY homologues from various species were aligned using CLUSTAL W version 1.83 (Fig. 1). In \(Vibrio\) species, the CheY amino acid sequence shows high homology (more than 94% identity and 96% similarity, in any combination). The identity and similarity between the \(V.\ alginolyticus\) and \(E.\ coli\) CheY proteins were 66% and 84% respectively. The site of phosphorylation (Asp-57) and the other residues critical for phosphorylation (Asp-12, Asp-13 and Lys-109) of \(E.\ coli\) CheY (Bourret et al., 1990) were conserved in all of the \(Vibrio\) CheY proteins. Alignment with CheY of other species predicted that Asp-60 of \(V.\ alginolyticus\) CheY is the site of phosphorylation. Other key residues conserved among various response regulators (Volz, 1993) and FlIM-interacting residues identified by genetic and structural studies (Roman et al., 1992; Shukla et al., 1998; Lee et al., 2001) were also generally conserved (for details, see Fig. 1). The cloned \(\text{cheY}\) gene of \(V.\ alginolyticus\) also complemented the \(\text{cheY}\) defect of \(E.\ coli\) (Fig. S1 in Supplementary material).

**Deletion of \(\text{cheY}\) abolishes swimming and surface-swarming by \(V.\ alginolyticus\)**

We deleted the \(\text{cheY}\) gene in the wild-type strain 138-2 (Pof\(^+\) Laf\(^+\)) and characterized the motility of the resulting strain (138-2Δ\(\text{cheY}\)). The 138-2Δ\(\text{cheY}\) cells did not spread...
either in VPG-0.3% agar or on VPG-1.3% agar (Fig. 2), indicating that the loss of the cheY gene disrupts both Pof-based and Laf-based motility (hereafter, spreading on hard agar and in soft agar will be referred to as surface-swarming and swimming respectively. Swimming in liquid will be referred to as free-swimming). As uncontrolled expression of CheY failed to complement the swarming defects of the DcheY strain, cheY or its FLAG-tagged derivative was cloned into pSU41 and the resulting plasmids were introduced into the DcheY strain with a lacI-containing plasmid pMMB206. Cells expressing CheY or CheY-FLAG spread both in VPG-0.3% agar and on VPG-1.3% agar in the presence of proper concentrations of IPTG (Fig. 2). These findings are consistent with the reported behaviour of V. parahaemolyticus cheY mutants (Sar et al., 1990; Kim and McCarter, 2000; McCarter, 2004) and directly demonstrate that a single CheY can regulate Pof and Laf.

Overexpression of CheY interferes with both Pof and Laf

When CheY is overexpressed, E. coli or V. cholerae cells tumble or reorient incessantly (Hyakutake et al., 2005).

We examined whether overexpression of CheY has similar effects in V. alginolyticus. Both swimming in VPG-0.3% agar and surface-swarming on VPG-1.3% agar of 138-2 (Pof+ Laf+) cells were inhibited by overexpression of CheY from pVacheY in the absence of LacI (i.e. without pMMB206) (Fig. 3A).

We then directly observed Pof-only or Laf-only cells under a microscope. First, we observed free-swimming of 138-2 cells that were grown in liquid media and hence express the polar flagellum only. 138-2 cells harbouring the vector plasmid (pSU41) showed longer runs and shorter ‘reverse’ runs, with the reorientation frequency at around 1.5 s−1 (Fig. 3B, top left), whereas 138-2 cells overexpressing CheY showed more frequent reorientation (~3.0 s−1) (Fig. 3B, top right). Similar results were obtained for the Pof − Laf− mutant strain YM4 (data not shown).

We used the Pof− Laf+ mutant strain YM19 as Laf-only cells, as lateral flagella are constitutively expressed in strains defective in the formation of polar flagella (McCarter et al., 1988; Kawagishi et al., 1996). YM19 cells harbouring the vector plasmid swam smoothly (Fig. 3B, bottom left). When CheY was overexpressed, the motile fraction became zero: no swimming or tumbling cell was observed (Fig. 3B, bottom right). This was not due to the lack of Laf, as immunoblotting to detect Laf flagellin revealed that Laf-only cells carrying pVacheY expressed the same level of Laf flagellin as do cells carrying the vector plasmid (data not shown). We also observed surface-swarming of Laf-only cells under a digital microscope and found that cells overexpressing CheY hardly move (data not shown). These results suggest that the modes of CheY control on the Laf and Pof motors are different.

Surface-swarming of V. alginolyticus requires chemotaxis

Although the effects of CheY on surface-swarming and free-swimming Laf-only cells are similar, the actual

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movements observed under a digital microscope are quite different. Capillary assays with V. parahaemolyticus and V. alginolyticus already revealed that Laf-only cells are chemotactic in liquid (Sar et al., 1990; Homma et al., 1996), but it has not been established whether they are also chemotactic on surfaces. We therefore examined the effects of saturating concentrations of the attractant L-serine on spreading on hard-agar surfaces as well as in soft agar (Fig. 4). Very high concentrations of serine should saturate the relevant chemoreceptor, thereby inhibiting chemotaxis. As shown in Fig. 4B, chemotactic rings in VPG-0.3% agar became smaller for YM4 (Pof+ Laf+), YM19 (Pof+ Laf+) and wild-type (Pof+ Laf+) strains with increasing concentrations (0–50 mM) of L-serine. The addition of 1 mM L-serine enhanced surface-swarming of the wild-type and Pof– Laf+ strains on VPG-1.3% agar, but higher concentrations (10 mM or higher) inhibited swarming (Fig. 4C). These higher concentrations of serine did not affect cell growth (data not shown). In contrast, L-aspartate failed to inhibit or enhance either chemotactic-ring formation in 0.3% agar or surface-swarming on 1.3% agar (Fig. 4D and E). When serine and aspartate were spotted on the opposite sides of a streak of YM19 cells on VPG-1.3% agar, cells swarmed faster towards serine than towards aspartate (Fig. 4F). Similar results were obtained with synthetic medium agar. For the wild-type strain, the addition of 1 and 5 mM L-serine enhanced swimming and surface-swarming, respectively, but higher concentrations of serine inhibited them (Fig. 4G and H). These results indicate that Laf motility of V. alginolyticus can be modulated by external stimuli and that surface-swarming involves chemotaxis.

Substitution of Ala for Asp-60, affects both types of swarming

We examined the role of the putative phosphorylation site, Asp-60, in the control of the Pof and Laf motors by making the D60A substitution. The D60A mutant cheY gene failed to complement both swimming and surface-swarming defects of the 138-2ΔcheY strain (Pof+ Laf+) (data not shown). The mutant protein (CheY-D60A) was overproduced in 138-2 (Pof+ Laf+) cells carrying pMMB206 (carrying the lacI gene) and swimming in VPG-0.3% agar and swarming on VPG-1.3% agar of the resulting strains were examined (Fig. 5). In the presence of 100 μM or more IPTG, cells expressing CheY-D60A swam and swarmed faster than cells expressing wild-type CheY, indicating that the mutant protein interferes less with both the Pof and Laf motors.

We also examined the role of CheZ, which facilitates dephosphorylation of phospho-CheY. A cheZ-defective mutant of 138-2 lost both Pof-based swimming and Laf-based surface-swarming (data not shown). This result suggests that phosphorylation at the same site of CheY is involved in the control of both types of flagellar motors.

Replacement of conserved CheY residues can have different effects on the Pof and Laf motors

We introduced the following substitutions, which are predicted to affect the interaction with FliM, into the cheY gene: A93V, I98V, Y109L and V111M (Fig. 1). These correspond to the A90V, I95V, Y106L and V108M replacements in E. coli CheY. In E. coli, A90V and V108M decrease the affinity of CheY to CheA and FliM (Shukla
et al., 1998), whereas I95V increases the affinity to the N-terminal residues of FilM (Schuster et al., 2000). These mutations therefore would cause CCW- and CW-biased motor rotation respectively. Y106L confers a null phenotype without affecting phosphorylation or binding to FilM (Zhu et al., 1996). We examined whether these mutant cheY genes could complement the swimming and swarming defects of 138-2ΔcheY cells. Immunoblotting demonstrated that the expression levels of the mutant CheY proteins were essentially the same as that of wild-type CheY at any concentration of IPTG tested (Fig. 6A).

The colony diameters of 138-2ΔcheY cells expressing wild-type or mutant CheY protein were measured in VPG-0.3% agar and on VPG-1.3% agar and plotted against the concentrations of IPTG (Fig. 6B). Cells expressing wild-type CheY swam and swarmed best when induced by 10 μM and 25–50 μM, respectively, IPTG. The V111M protein required higher concentrations of IPTG for both swimming and swarming than wild-type CheY, indicating

![Control of dual flagellar motors](https://example.com/fig4)

**Fig. 4.** Both chemotactic-ring formation in swim agar and surface-swarming require chemotaxis.
A–E. Effects of increasing concentrations of serine and aspartate (0, 1, 5, 10 and 50 mM, from left to right) on spreading by swimming (VPG-0.3% agar) (serine, B; aspartate, D) and surface-swarming (VPG-1.3% agar) (serine, C; aspartate, E) of *V. alginolyticus* strains 138-2 (Pof<sup>+</sup>Laf<sup>+</sup>), YM4 (Pof<sup>+</sup>Laf<sup>-</sup>) and YM19 (Pof<sup>-</sup>Laf<sup>+</sup>) (all arranged as shown in A). F. Surface-swarming of *V. alginolyticus* towards serine. An overnight swarm culture of YM19 cells was streaked on VPG-1.3% agar, and aliquots (10 μl each) of 0.5 M serine and aspartate were spotted at the positions indicated by a star and a circle respectively.

G and H. Effects of increasing concentrations of L-serine (0, 0.01, 0.1, 1, 5, 10 and 100 mM, from left to right) on spreading by swimming (synthetic medium-0.3% agar) (G) and surface-swarming (synthetic medium-1.3% agar) (H) of strain 138-2.

Plates were incubated at 30°C for 5 (B and D), 9 (C), 18 (E), 20 (F), 12 (G) or 36 (H) h.

![Control of dual flagellar motors](https://example.com/fig5)

**Fig. 5.** Inhibition of chemotactic-ring formation in swim agar and surface-swarming by the overexpression of CheY or its mutant derivative CheY-D60A. Overnight cultures of 138-2 (Pof<sup>+</sup>Laf<sup>+</sup>) cells carrying the vector or the plasmid encoding CheY or CheY-D60A were spotted onto VPG-0.3% agar (for swimming) and VPG-1.3% agar (for surface-swarming) supplemented with 2.5 mg l<sup>−1</sup> chloramphenicol and 100 μg ml<sup>−1</sup> kanamycin with 0–1000 μM IPTG (as indicated). Plates were then incubated at 30°C for 5 h (0.3% agar) or 15 h (1.3% agar).
that it has lower affinities for both Pof and Laf motors. Cells expressing the A93V protein showed a similar swimming pattern than those expressing the V111M protein. However, the IPTG concentration (25 μM) required for maximal complementation of surface-swarming by the A93V protein was essentially the same as that required with wild-type CheY, although the swarm diameters were larger than with wild-type CheY. The Y109L protein conferred swimming and swarming phenotypes similar to, but stronger than, those associated with V111M; even higher concentrations of IPTG were required for optimal chemotactic ring formation in VPG-0.3% agar, and the rings were smaller. These complementation profiles are quite different from that shown by the corresponding Y106L CheY of *E. coli*, which has a null phenotype (Zhu et al., 1996).

In contrast to the other three mutant proteins, the I98V protein required lower concentrations of IPTG to complement the swimming defect, and higher levels of expression inhibited formation of chemotactic rings, a profile consistent with the phenotype associated with the *E. coli* I95V protein. However, I98V CheY behaved just like wild-type CheY for surface-swarming. Thus, some substitutions at conserved CheY residues had different effects on the Pof and Laf motors, which suggests that CheY may interact with the two motors in different ways.

The Laf motor rotates only CCW and may be slowed down by phospho-CheY

We observed rotation of Laf motors by preparing tethered YM19 (Pof–Laf−) cells. Although good tethered cells were difficult to obtain, all of the stably rotating tethered YM19 cells rotated exclusively CCW, unlike tethered *E. coli* cells, which rotate in both CCW and CW directions (Fig. 7). The CCW rotation of the Laf motor is supposed to render Laf to form a bundle considering our preliminary microscopic observation based on the method by Shimada et al. (1975) that Laf forms a left-handed helical bundle in liquid (Y. Maekawa, I. Kawagishi and Y. Imae, unpublished). When CheY was overexpressed, no rotating tethered cells were observed. Thus, control of the Laf motor does not involve switching between rotational senses.

To examine the role of CheY in the control of the Laf motor, we deleted the cheY gene in the Laf-only mutant strain (YM19). YM19ΔcheY cells showed a characteristic free-swimming pattern (Table 1): both the motile fraction and the mean swimming speed were higher than those of the parental strain, raising the possibility that CheY interferes with motility itself. 138-2ΔcheY (Pof−Laf−) cells grown in liquid medium swarm without apparent reorienta-
tion but their swimming speed is similar to those of 138-2 cells (data not shown). Rotation of tethered YM19 and YM19ΔcheY cells was recorded, and the video images were analysed by using the software Image J (http://rsb.info.nih.gov/ij/). Rotation angles of a tethered cell were integrated with CCW and CW directions as positive and negative values, respectively, and plotted against frame numbers. As a control, we analysed *E. coli* RP437 cells (Che+). The time-course of the integrated rotation angle of a tethered RP437 cell gave a zigzag line (not shown). So did that of tethered *V. alginolyticus* YM4 (Pof+ Laf–) cells, although we found very few tethered cells for this strain (not shown). The rotational speeds of RP437 and YM4 cells showed both positive and negative values (Fig. 8A and B), clearly demonstrating that the *V. alginolyticus* Pof motor, like the *E. coli* flagellar motor, alternates between CCW and CW rotation. In general, cells tethered with polar flagella are not suited for detailed analyses, but tethered Pof-only ΔcheY cells were found to rotate exclusively CCW (data not shown) as shown in optical nano-metric measurements of the Pof rotation of polar-only Che– cells (Sowa et al., 2003). Together with the fact that overproduction of the wild-type CheY protein, but not that of the D60A mutant protein, increases the reorientation frequency of 138-2 cells grown in liquid medium (Fig. S2 in Supplementary material), these observations suggest that phospho-CheY induces CW rotation of the Pof motor.

**Table 1.** Motile fractions and mean swimming speeds of YM19 and YM19ΔcheY cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Motile fraction (%)</th>
<th>Mean swimming speed (μm s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>YM19</td>
<td>34.7</td>
<td>15.2 ± 4.1</td>
</tr>
<tr>
<td>YM19ΔcheY</td>
<td>75.0</td>
<td>23.1 ± 6.3</td>
</tr>
</tbody>
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Swimming speed was measured as described previously (Nishiyama et al., 1997).
In contrast, the integrated rotation angles of tethered YM19 (Pof–Laf) cells increased monotonously over time, with some intervals of more gentle slopes (not shown). This feature is more apparent when rotational speeds are plotted (Fig. 8C, indicated by arrow heads). For YM19_cheY cells, however, no obviously slower intervals were seen. When integrated rotational angles of three representative cells of YM19 and YM19_cheY were fitted with straight lines and deviations from the fitted lines were plotted (Fig. 8D), YM19_cheY cells clearly had much smaller speed fluctuations than YM19 cells. These results strongly suggest that the function of CheY relative to Laf is to slow down unidirectional CCW rotation of the motor.

Discussion

Vibrio alginolyticus possesses two distinct flagellar systems: the polar flagellum (Pof), which is well suited for swimming in sea water, and the lateral flagella (Laf), which enable swarming over biotic and non-biotic surfaces (Blake et al., 1980; McCarter and Silverman, 1990). The results presented here show that not only Pof-based and Laf-based swimming but also Laf-based surface-swarming involve chemotaxis. A single CheY protein controls both the Pof and Laf motors. The behaviour of cells in which the putative phosphorylation site of CheY, Asp-60, is replaced with Ala suggests that phosphorylation of CheY is involved in the control of both of the motor systems.

The I98V and Y109L substitutions in V. alginolyticus CheY had differential effects on the Pof and Laf motors, suggesting that the motor interaction surfaces of CheY and/or the conformational changes of CheY required for switching are different between the Pof and Laf motors. Tethered cell analyses revealed that phospho-CheY affects the Pof and Laf motors differently. The Pof motor undergoes switching between the CCW and CW rotation, whereas the Laf motor slows down in response to phospho-CheY. Although each of these responses is known from different species, this is the first report that a single CheY species can exert both types of control in the same organism.

The genome sequences of V. alginolyticus and V. parahaemolyticus, which is closely related to V. alginolyticus, contain an additional gene annotated as cheY, but its product seems unlikely to control flagellar motility, as: (i) the gene maps outside of the che gene cluster and is presumably co-transcribed with a neighbouring gene encoding a non-CheA histidine kinase, and (ii) the overexpression of the orthologue in the related species V. cholerae does not affect flagellar rotation (Hyakutake et al., 2005). V. cholerae and other unrelated bacteria, including R. sphaeroides and S. meliloti, have multiple sets of che genes with a single flagellar system (Ferrandez et al., 2002). V. cholerae has four cheY homologues, including the putative cheY described above, but only one of them (cheY3) directly controls flagellar rotation (Hyakutake et al., 2005). In R. sphaeroides, multiple che genes are thought to cross-talk with each other, but only one cheY gene directly regulates the stop-and-go control of the flagellar motor (Porter and Armitage, 2002; 2004; Porter et al., 2002). S. meliloti has a single CheA protein and two CheY proteins. Only one of them directly controls the flagellar motor (slow-and-fast type). The other serves as a phosphate sink (Sourjik and Schmitt, 1996; 1998; Schmitt, 2002).

In E. coli, phospho-CheY binds to the FilM protein, a component of the cytoplasmic ring (C-ring) of the flagellar motor (Welch et al., 1993). The N-terminal residues of FilM constitute a CheY-docking site (Bren and Eisenbach, 1998; Lee et al., 2001). In V. alginolyticus, mutagenic analyses of cheY were consistent with the notion that phosphorylation at the same site of CheY is involved in the control of both types of flagellar motors. Furthermore, the substitution I98V affected only the Pof motor but not the Laf motor. The corresponding substitution, I95V, in E. coli, decreases binding to FilM and interferes with chemotaxis of swimming cells but does not affect surface-swarming (Mariconda et al., 2006). We aligned the deduced amino acid sequences of the filM genes for Pof and Laf that have been identified in the genome sequence of V. parahaemolyticus (Makino et al., 2003; McCarter, 2004) and V. alginolyticus with those of the filM from other bacterial species (not shown). The polar flagellar FilM (Pof FilM) protein has a well-conserved N-terminal CheY-docking site. In Rhodospirillum centenum, which also has two flagellar systems (Ragatz et al., 1995), both the Pof and Laf FilMs have putative CheY-docking sites (McClain et al., 2002). In contrast, the Laf FilMs of V. parahaemolyticus and V. alginolyticus lack this sequence, and the rest of the proteins show a relatively low similarity to that of their Pof counterparts (not shown). It is therefore likely that the CheY-binding site on the Laf motor of marine Vibrio differs from that on the Pof motor: it could reside in a different part of FilM, or other proteins might be involved in CheY binding.

When placed on a surface, a rod-shaped marine Vibrio swimmer cell with a single polar flagellum undergoes a substantial morphological change to become a highly elongated cell with numerous (>100) lateral flagella. The polar flagellum, however, is retained. How can CheY cope with such a large increase in number of its targets (i.e. the flagellar motors)? The swimmer-to-swarmer differentiation might be accompanied with an increased intracellular level of CheY. Alternatively, CheY might have a higher affinity for the Laf motor, or control of the Laf motor might require binding of fewer CheY molecules. In any case, identification of the CheY-binding sites is a next obvious
Experimental procedures

Bacterial strains, plasmids, growth conditions and media

Bacterial strains and plasmids used in this study are listed in Table S1 in Supplementary material. V. alginolyticus strains used are a polar flagellum-defective mutant, YM19 (Pof−Laf+), a lateral flagellum-defective mutant, YM4 (Pof+Laf−), and their parental strain 138-2 (Pof+Laf+). A 138-2-derivative strain, 138-2ΔcheY, in which the cheY gene is deleted, was constructed as described below. All of the E. coli strains are derivatives of K12. Strain RP437 is wild type for chemotaxis (Parkinson and Houts, 1982). Strain RP4979 (J.S. Parkinson, pers. comm.) is a ΔcheY mutant.

Vibrio alginolyticus cells were cultured at 30°C in VC medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K2HPO4, 3% NaCl, 0.2% glucose), VPG medium [1% polypeptone, 0.4% K2HPO4, 3% NaCl, 0.5% (w/v) glycerol] or synthetic medium [0.3 M NaCl, 10 mM KCl, 2 mM K2HPO4, 0.01 mM FeSO4, 15 mM (NH4)2SO4, 5 mM MgSO4, 1% glycerol, 50 mM Tris-HCl (pH 7.5)]. E. coli cells were cultured at 37°C in LB or TG [1% tryptone, 0.5% NaCl, 0.5% (w/v) glycerol] medium. When necessary, chloramphenicol and kanamycin were added to final concentrations of 2.5 and 100 μg ml−1 for Vibrio, or 25 and 25 μg ml−1 for E. coli respectively.

Cloning and mutagenesis of the cheY gene

Primers used for cloning the cheY gene of V. alginolyticus are listed in Table S2 in Supplementary material. The cheY gene was amplified by PCR using chromosomal DNA of strain 138-2 as the template, cloned into the vector plasmid pCR2.1 (Invitrogen). The resulting plasmid was named pCR2.1-VacheY. The 1.1 kb EcoRI fragment of pCR2.1-VacheY was subcloned into the multicloning site of the vector plasmid pSU41 to yield pVacheY. A KpnI site was introduced at the 3′ end of the coding region. The resulting DNA fragment was digested with EcoRI and KpnI and cloned into the vector plasmid pFLAG-CTC (Sigma) to yield pFLAG-CTC-VacheY, so that the FLAG tag is fused to the C-terminus of the CheY protein. Plasmid pVacheY-FLAG was constructed by cloning the 0.6 kb HindIII−SacI fragment of the PCR product into the multicloning site of pSU41. Mutant cheY genes were constructed by a two-step PCR method with the primers listed in Table S2 essentially as described previously (Umemura et al., 1998).

Nucleotide sequencing

The nucleotide sequence of the cloned gene was determined by the dyeoxy-chain termination method, using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence of the cheY gene of V. alginolyticus has been deposited to the DDBJ database (Accession No. AB246040).

Deletion of the cheY gene

An in-frame deletion in the cheY gene was constructed by overlap-extension PCR (Ho et al., 1989; Urban et al., 1997). The 1.4 kb Sphl−SacI fragment with the in-frame deletion in cheY was constructed as described in the note of Table S2 and subcloned into the suicide vector pKY704 (Xu et al., 1994). The resulting suicide plasmid (pKY704-ΔVacheY) was first introduced into the E. coli strain SM10pir (Miller and Mekalanos, 1988) which expresses α protein to support replication of the plasmid, and then transferred by conjugation into V. alginolyticus strains. The suicide plasmid is expected to integrate into chromosome by homologous recombination at chromosomal sequences flanking cheY. Chloramphenicol-resistant (Cm+) transconjugants were inoculated on VPG-0.3% agar to screen for chemotaxis-defective mutants. Two Che− clones were found among 100 Cm+ transconjugants. Next, these Cm+ Che− cells were cultured in liquid broth without antibiotics, and single colonies were isolated on chloramphenicol-free VC-3.0% agar. From 2000 colonies, we found one Cm+ Che− clone. We confirmed the deletion by examining the sizes of PCR-amplified fragments and the complementation of the strain with pVacheY. This mutant strain was named 138-2ΔcheY.

Analyses of amino acid sequences

Amino acid sequences were aligned using the software of CLUSTAL W version 1.83. Sequence homology and similarity were calculated using the BLAST 2 sequence program (Tatusova and Madden, 1999).

Swim and swarm agar assays

VPG-0.3% and 1.3% agar were used for V. alginolyticus swim and swarm assays respectively. TG-0.3% agar was used for E. coli swim assays. When necessary, chloramphenicol, kanamycin, 10–500 μM IPTG, and/or 1–500 mM serine or aspartate (pH 7.0) were added. An aliquot (1 μl) of overnight culture was spotted onto a plate, which was then incubated at 30°C for 5–20 h.

Observation of swimming behaviour

Free-swimming cells were observed essentially as described previously (Homma et al., 1996; Nishiyama et al., 1997). V. alginolyticus cells were resuspended in VPG medium, and cells were observed under a dark-field microscope and recorded on videotape. Swimming speed was measured as described previously (Nishiyama et al., 1997).

Observation of tethered cells

Tethered E. coli and V. alginolyticus cells (grown at 30°C) were prepared according to the protocol of Silverman and Simon (1974), with minor modifications, and were observed under a dark-field microscope at room temperature and recorded on videotape.
Immunoblotting was carried out essentially as described previously (Okumura et al., 1998). An overnight culture of V. alginolyticus in VC medium was diluted 1:30 into fresh VPG medium supplemented, if necessary, with IPTG. Cells were grown with vigorous shaking, harvested at mid-logarithmic phase and washed with Vibrio buffer [25 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM MgCl_2]. The samples were subjected to SDS-PAGE (13% polyacrylamide) followed by immunoblotting with anti-FLAG M2 antibody (Sigma).

Acknowledgements

We thank Drs Yoshiyuki Sowa (Oxford University) and Seiji Kojima (Nagoya University) for discussion and encouragement and Dr Michael Manson (Texas A&M University) for critically reading the manuscript. This work was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (to T.Y., M.H. and I.K.).

References

Control of dual flagellar motors


Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** Complementation of the defect of the cheY knockout *E. coli* strain by the CheY homolog of *V. alginolyticus*.

**Fig. S2.** Reorientation frequencies of wild-type (138-2) cell with or without overexpression of wild-type a mutant (D60A) CheY protein.

**Table S1.** Bacterial strains and plasmids.

**Table S2.** Primers used in this study.

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