Bacillus subtilis CheC and FliY are members of a Novel Class of CheY-P Hydrolyzing Proteins in the Chemotactic Signal Transduction Cascade

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Abbreviation: CCW, counterclockwise; P_i, inorganic phosphate
SUMMARY

Rapid restoration of prestimulus levels of the chemotactic response regulator, CheY-P, is important for preparing bacteria and archaea to sensitively respond to new stimuli. In extending previous work, we describe a new family of CheY-P phosphatases, the CYX family, that is widespread among the bacteria and archaea, in addition to the previously known CheZ of the γ- and β-proteobacteria (e.g., *Escherichia coli*) and alternate CheY serving as a “phosphate sink” among the α-proteobacteria (e.g., *Sinorhizobium meliloti*). In particular, we identify CheC, previously known to be involved in adaptation to stimuli in *Bacillus subtilis*, as a CheY-P phosphatase. Using an *in vitro* assay previously used to characterize the switch protein FliY as a CheY-P phosphatase, we have shown that increasing amounts of CheC accelerate the hydrolyzation of CheY-P. *In vivo*, a double mutant lacking cheC and the CheY-P binding region of *fliY* is almost completely smooth swimming, implying very high levels of CheY-P. CheC appears to be primarily involved in restoring normal CheY-P levels following addition of attractant, whereas FliY seems to act on CheY-P constitutively. The activity of CheC is relatively weak when compared to FliY, but we have shown that the chemotaxis protein CheD enhances the activity of CheC. We suggest a model of how FliY, CheC and CheD work together to regulate CheY-P levels in the bacterium.
INTRODUCTION

Chemotaxis is the process by which bacteria travel to higher concentrations of attractant or lower concentrations of repellent. Peritrichous bacteria like *Bacillus subtilis* rotate their flagella counterclockwise (CCW) to swim smoothly and clockwise to tumble, a random motion without forward progress so that the bacteria can be reoriented for the next smooth swim. Binding of attractant at the *B. subtilis* receptors, the methyl-accepting chemotaxis proteins (MCPs) leads to activation of the associated CheA kinase (1,2), with rapid production of CheY-P. This activation is facilitated by the coupling proteins, CheW and CheV (3). CheY-P interacts with FliM to cause an increased tendency for CCW flagellar rotation (for reviews see (4-6)). Interestingly, in *Escherichia coli*, a positive stimulus decreases CheA activity and therefore CheY-P concentrations in the cell. Additionally the default rotation of the flagella is CCW in *E. coli*, and CheY-P binds FliM to induce clockwise rotation (reviewed in (4,5)). However the final output, to increase CCW rotation and therefore the duration of smooth swimming behavior upon sensing positive stimuli, remains conserved between both organisms.

Following the activation of CheA in *B. subtilis*, the bacteria have to be able to adapt to the presence of stimulus in order to direct their direction of movement up the gradient to ever-higher concentrations of attractant. Adaptation is achieved by a methylation system that reversibly methylates the receptors on conserved glutamate residues (reviewed in (7)), and by the phosphorylation of CheV (8). Besides reducing receptor activity, it is also important to reduce the levels of CheY-P in order to respond to changing levels of receptor bound with attractant. In *E. coli* and other γ— and β—
proteobacteria, CheZ catalyzes dephosphorylation of CheY-P (9). However most chemotactic bacteria do not encode for a CheZ homolog (6). Recently FliY, a flagellar switch protein of *B. subtilis*, was described to have a similar activity (10). While homologs for this protein can be found in most chemotactic Gram positive bacteria and some spirochetes, most bacteria do not encode for a FliY. However, CheC, a homolog of the N-terminal portion of FliY exists in many organism, including *B. subtilis* and in the archaea (11)(Fig. 1A). In particular two regions of 31 amino acids, which we termed CYX1 and CYX2 (for CheC, FliY and CheX), show high similarity in these homologs (Fig. 1B). CheC was identified as a chemotaxis protein since a cheC mutant shows clear defects in chemotactic ability (12). However a biochemical role has remained elusive. Here we were able to show that CheC shares FliY’s ability to increase the rate of CheY-P hydrolysis in vitro. Additionally, the chemotaxis protein CheD, which has been previously identified to bind CheC (13), can augment CheC activity. Studies on cheC and fliY<sub>Δ6-15</sub> single mutants (used since a fliY null mutant is not flagellated (10,14)) and a cheC fliY<sub>Δ6-15</sub> double mutant showed evidence for the importance of this phosphatase in *vivo*. To get insight into why *B. subtilis* encodes for two proteins with an apparently similar function, the specific activity and per cell copy number for both proteins was determined. We propose a model how these two proteins function *in vivo*. 
EXPERIMENTAL PROCEDURES

Chemicals, Columns, Enzymes, and Growth Media—All chemicals were reagent grade. All protein purification columns, as well as PreScission Protease and \([\gamma-^{32}P]\)-ATP were purchased from Amersham. Media was obtained from Difco. Luria-Bertani (LB) medium is 1% Tryptone, 0.5 % yeast extract, and 1 % NaCl. TBAB is tryptose blood agar base.

Plasmid and Strain Construction—The strains and plasmids used are listed in Table I. All \(B.\ subtilis\) strains constructed derived from the \(Che^+\) strain OI1085. Strains deleted for \(fliY\) and \(cheC\) were derived by gene conversion. Briefly, genomic DNA from OI2852 (\(fliY::\text{cat}\)) was transformed into OI3135, selecting for Cm\(^R\). The resulting strain was transformed with pHS108 selecting for Kan\(^R\) and screening for loss of Cm\(^R\), indicating gene conversion. Colonies were cured for the plasmid indicated by loss of Kan\(^R\) through repeated growth in LB medium in the absence of antibiotics. This resulted in strain OI4141. Strain OI4141 was transformed with plasmids pHS111 and pHS110, to produce OI4139 and OI4140, respectively. Plasmids pTM18 (pGEX-6P-2-CheC) and pTM25 (pGEX-6P-2-CheD) were made by amplifying \(cheC\) or \(cheD\) by PCR, introducing a 5’ BamHI and a 3’ EcoRI site, and excluding the ATG start codon for \(cheC\) or \(cheD\). The PCR products were cloned into the respective sites of pGEX-6P-2.

Protein Overexpression and Purification—CheY, CheA and FliY were overexpressed and purified as previously described (10). FliY\(_{\Delta6-15}\) was purified as described for FliY; however, a phenyl-sepharose-column (high substituent) was used instead of an octyl-
sepharose-column. CheC and CheD were overexpressed and purified as GST-fusion proteins, and the GST-tag was cleaved and removed, as described for CheA (10).

Dephosphorylation Assay—The dephosphorylation assay was essentially performed as described (10). Briefly, 25 µM CheA was phosphorylated by incubation with [γ-32P]-ATP (33 µM, 12.5 µCi/µl) for 30 min in a total volume of 120 µl TKMD (50 mM TRIS pH 8.0, 5 mM MgCl₂, 50 mM KCl, 0.2 mM dithiothreitol, 10 % glycerol). Remaining [γ-32P]-ATP was removed by desalting, using a BioRad Micro-Bio-Spin column. The resulting CheA-32P was added to premixed CheY-CheC (and CheD) solutions, resulting in a final concentration of 20 µM CheY, 10 µM CheA-P, varying concentrations of CheC (and CheD) as indicated, and 5 mM cold ATP in TKMD. Aliquots were taken at the indicated times by pipetting 10 µl of each reaction into 10 µl 2 x SDS buffer containing 100 mM EDTA, and separated on a 12 % SDS-PAGE. The gel was exposed to a phosphor imaging screen and developed using a Storm 860 PhosphorImager from Amersham. The experiment was done in duplicate.

Phosphate-Release-Assay—In this assay the evolution of inorganic phosphate (Pᵢ) was measured by the EnzCheck phosphate assay kit (Molecular Probes), essentially as described (15). Release rates for different amounts of CheY-P-hydrolyzing proteins allow for determination of specific release rates and therefore comparison of activity. Briefly, MESG (200 µM), monophosphate imidazol (3 mM, synthesized as described (16)), and CheC, FliY or FliYΔ6-15 at the concentrations indicated were mixed in a final volume of 930 µl of 20 mM Tris, pH 7.5 and 10 mM Mg₂Cl₂ and incubated for 30 min at 25°C. Following this incubation time, mixtures were added to a cuvette. After the
addition of 10 µl (1 unit) nucleoside phosphorylase the absorbance at 360 nm was measured. The absorbance stabilized after 30 s and the phosphate flow through CheY was initiated by addition of 6 µM CheY (resulting in a final volume of 1 ml). P_i release rates were followed for 3 min at a temperature of 25°C. Using a standard curve relating P_i concentration to absorbance at 360 nm, release rates were calculated in µM/min. For this measurements a temperature controlled Shimadzu BioSpec-1601 Spectrophotometer was used.

_Determination of Copy Numbers for FliY and CheC_—Strain OI1085 was grown as previously described for pulse labeled methylation experiments (17). 100 µl of a 2 ml overnight culture was added to 10 ml LB and grown to early stationary phase at 37°C. Cells were washed twice with chemotaxis buffer (10 mM potassium phosphate pH 7.0, 0.1 mM EDTA, 0.05 mM calcium chloride, 0.3 mM ammonium sulfate, 0.05% glycerol, 1.5 mM sodium lactate) and once with protoplast buffer (25 potassium phosphate pH 7.0, 10 mM magnesium chloride, 0.1 mM EDTA, 20% sucrose, 30 mM sodium lactate) supplemented with 250 µg/ml chloramphenicol and diluted to A_{600nm} = 1 in 5 ml protoplast buffer. Serial dilutions of this suspension were plated on TBAB plates to determine cell count. Lysozyme was added to a concentration of 4 mg/ml to the suspension to produce protoplasts. Following incubation at 37°C for 30 min, protoplasts were collected by centrifugation and lysed in 500 µl 1xSDS solublizer boiling for 10 min. 10 µl of lysates were separated on 12 % SDS-PAGE-gels next to purified FliY or CheC at indicated concentrations. Proteins were visualized immunlogically essentially as described (18). However low copy numbers of CheC required anti-CheC antibody to be
preabsorbed as described (19). Antibody dilutions were 1:50000 for anti-FliY-antibody and 1:100 for anti-CheC-antibody. Bands were quantified using ImageQuant software allowing for calculation of copy numbers for CheC and FliY using the resulting standard curves and the cell counts from the serial dilutions.

Tethered Cell Assay—The tethered cell assay was essentially performed as described (20); however \( fliY_{\Delta 6-15} \) expression was induced with 0.1 mM IPTG 3 hours after starting the culture. Each graph shown depicts the average bias of a population of at least 20 cells.

Swarm assay for chemotaxis—The swarm assay was performed as described (21); however semi-solid agar plates contained 0.1 mM IPTG to allow for wild type expression levels of \( fliY \) and \( fliY_{\Delta 6-15} \) in strains OI4140 and OI4139, respectively.

Capillary assay for chemotaxis—The capillary assay was performed as described for \( E. coli \) (22), however modified as described for \( B. subtilis \) (21); \( fliY_{\Delta 6-15} \) expression was induced with 0.1 mM IPTG 2.5 h before harvesting the culture. Each experiment was performed on two different days in duplicate to insure reproducibility.
RESULTS

*CheC Increases the Rate of CheY-P Hydrolysis*—Recently we showed that the flagellar switch protein FliY is capable of increasing the rate of CheY-P hydrolysis (10). Since CheC and FliY share substantial sequence similarities (11), we wanted to test whether these proteins share the same function. In the absence of CheC detectable levels of CheY-P remained after 240 sec, while in the presence of equimolar concentrations of CheY-P and CheC, essentially all CheY-P was hydrolyzed by the 60 sec time point (Fig. 2.). We conclude that CheC and FliY share a common function as CheY-P hydrolyzing proteins.

*FliY is more active than CheC in vitro*—It appears that CheC activity is substantially lower than that of FliY in the dephosphorylation assay described above. To quantify this, a spectrophotometric assay was used that measures the production of inorganic phosphate (Pi) over time. This assay was previously used to compare activities of different *E. coli* CheZ mutants (15). At low concentrations of CheC or FliY relative to CheY-P, the relationship between protein concentration and Pi-release rate is linear (Fig. 3 A and B). Specific rates calculated from the slopes allow for comparison of FliY and CheC activity. The µM specific activities are 49 µM P₁ formed/min/ µM FliY, and 3.1 µM P₁ formed/min/ µM CheC. Therefore CheC’s activity is only about 6 % of that of FliY. Additionally the activity of FliYΔ6-15, a mutant defective in binding CheY-P (10) was determined to be about 20 % of that of FliY, indicating that this mutant is still more active than CheC, at least *in vitro*. 
FliY does not effect CheC’s activity—We also wanted to explore whether the activity of CheC can be affected by the presence of FliY, in a synergistic manner. To test this, a constant amount of FliY (0.02 mM) and varying amount of CheC was present in all hydrolyzing reactions at concentration within the linear range. A change in the specific CheY-P hydrolyzing rate of CheC would indicate either negative or positive cooperativity. However no change was observed, indicating that FliY and CheC apparently hydrolyze CheY-P independently (data not shown).

CheC can be activated by CheD—CheD was identified to have receptor glutamine deamidase activity (23). Additionally it was shown to interact with CheC, and no purpose for this interaction had yet been discovered (13). While CheD had no effect on CheY-P stability by itself, it clearly enhanced CheY-P hydrolysis in the presence of CheC (Fig. 4). Under conditions used, CheC (2 µM) was calculated to be 5.3 fold more active, in the presence than in the absence of 4 µM CheD. We conclude that CheD specifically interacts with CheC to increase its activity.

A cheC fliY_{6-15} double mutant is not capable of performing chemotaxis—In the swarm assay strains are stabbed onto a low agar low nutrient plate. As the cells metabolize nutrient, they create an attractant gradient, which they sense and migrate towards, and thus form a characteristic ring. Since the cheZ mutant in E. coli does not show chemotaxis as measured by this assay (24), we wanted to see how the single mutants of CheC and FliY compare in this assay to a double mutant. Since a fliY strain is not
flagellated, we used the CheY-P binding mutant fliY\(_{\Delta 6-15}\). Both the cheC as well as the fliY\(_{\Delta 6-15}\) strains showed somewhat impaired chemotaxis. The swarm diameter was smaller than that of wild type strain OI1085 (Fig. 5). The double mutant was not capable of forming a ring on a swarm plate and was therefore not chemotactic, as seen for the cheZ mutant in E. coli. We note that reduced ring sizes on swarm plates can reflect problems in signal transduction or suboptimal flagellar rotational biases. The fliY and cheC fliY strains were almost fully complemented to wild type and cheC swarm diameters, respectively, when expressing fliY\(^+\) located at the amyE locus (Fig. 5).

The traditional quantitative assay of chemotaxis is the capillary assay. By this assay the single mutants were tenfold reduced in chemotaxis toward asparagine but the cheC fliY\(_{\Delta 6-15}\) double mutant showed no taxis, consistent with the results from swarm plates (Fig. 6).

The cheC fliY\(_{\Delta 6-15}\) strain shows a 100\% CCW rotational bias in the tethered cell assay—To further explore the nature of the chemotactic defect in the double mutant cheC fliY\(_{\Delta 6-15}\), this strain was subjected to the tethered cell assay, which measures flagellar rotational bias of individual cells. The bias is averaged for a population of cells and is presumed to reflect CheY-P levels in those cells. The double mutant had a very high CCW rotational bias with very few cells ever spinning clockwise (Fig 7.). The bias was even higher than that of a fliY\(_{\Delta 6-15}\) single mutant (10), shown here for comparison. A cheC mutant does not show an increased bias before a stimulus is added; however, it is impaired in adaptation to the addition of stimulus as previously described (11).
Copy numbers for CheC and FliY—To place the activity of both CheC and FliY into perspective, the copy number for each protein was determined (Fig. 8.). The copy number for FliY determined as described in experimental procedures was 535 ± 53 copies per cell, consistent with FliN copy number published for E. coli which is about 100 per flagella, with a cell averaging 4-5 flagella (25). The copy number for CheC was determined to be around 20 ± 7. This is by far the lowest amount of any chemotaxis protein in any bacteria ever reported (Hazelbauer, G.L., personal communication). While copy numbers of proteins might be affected by high turnover, we believe that this is not the case for CheC, as the protein is stable when over-expressed and purified.
DISCUSSION

Identifying a mechanistic function of CheC has remained elusive for many years. Here, we were able to show that it increases the rate of CheY-P hydrolysis. This is the second protein reported to contribute to CheY-P hydrolysis in *B. subtilis*, in addition to FliY, with which it shares sequence homology (10).

Most chemotactic bacteria and all known chemotactic archaea lack CheZ (6), the protein that performs this function in *E. coli* and other γ- and β-proteobacteria (9). A second mechanism of CheY-P removal has been suggested for *Sinorhizobium meliloti* and other α-proteobacteria (26,27). These organisms encode for at least two alternative CheY proteins. While both CheY’s can get phosphorylated, only one can interact with the flagellar switch. The other one acts as a phosphate sink, as the primary CheY can transfer its phosphoryl group back to CheA and thence to the other CheY, where hydrolysis occurs. The identification of FliY’s and CheC’s role describes yet a third mechanism of CheY-P removal in chemotactic bacteria (Fig. 9).

This third mechanism of CheY-P removal appears to be the most widely spread mechanism of the three described. CheC and FliY homologs (which include CheX, a chemotaxis protein with homology to CheC and FliY and therefore likely with a similar function (11,28)) can be found in the archaea, thermatoga, spirochetes, Gram positive bacteria, and even some proteobacteria like *Myxococcus xanthus* and *Vibrio cholera*. Interestingly *V. cholera* has a CheZ as well, making it the only known organisms with both types of CheY-P hydrolyzing proteins. Therefore *V. cholera* might be an evolutionary link utilizing both, the more conserved and therefore presumably more
ancestral system of CheY-P hydrolysis, and the more recent proteobacterial CheY-P hydrolyzing system.

Following the observation of CheC’s activity, an important goal of this study was to ascertain the importance of this function in vivo and to gain insight into why B. subtilis expresses two proteins with an analogous function. A fliY_{\Delta 6-15} mutant and as previously published, a cheC mutant showed reduced taxis both in swarm and capillary assays (11,12). The double mutant, though, was completely defective in chemotaxis, as observed by either assay. This result may mean that CheC and FliY function are partially redundant. This is not the only example in B. subtilis chemotaxis for two proteins to share partially redundant function. CheV and CheW have been shown to both couple CheA to the receptors, a role promoted in E. coli solely by CheW. Deletion of either of these coupling proteins has only a modest effect on chemotaxis but deletion of both abolishes it completely (3).

To further explore the effect on chemotaxis of the reduced CheY-P hydrolyzing activity, the double mutant was subjected to the tethered cell assay. Rotational data for both cheC and fliY_{\Delta 6-15} single mutants has been previously reported (10,11). While the fliY_{\Delta 6-15} mutant showed a phenotype of very high CCW bias indicating an increased level of CheY-P, the cheC mutant promoted a less severe phenotype, with a prestimulus bias similar to wild type (strain OI1085) (29). However following the addition of attractant, cells were impaired in adaptation as evident by a high CCW rotational bias. The double mutant showed the highest CCW rotational bias ever observed for a B. subtilis chemotactic mutant, with very few cells ever rotating clockwise, a result consistent with presence of very high levels of CheY-P.
Comparing the activity of FliY and CheC *in vitro* revealed that CheC is only about 6% as active as FliY, consistent with the more severe phenotype for the *fliY*Δ6-15 mutant. However we were able to show that the chemotaxis protein CheD, previously identified to interact with CheC (13), is capable of increasing CheC’s activity by about 5.3 fold *in vitro*. This effect might be even larger *in vivo*, where CheC and CheD are in the environment of receptor complexes. We hypothesize that FliY is constitutively removing CheY-P around the flagellar switch in order to hold CheY-P at the optimum level. CheC functions mainly just after addition of an attractant, in order to cope with increased levels of CheY-P. This activity is regulated by CheD, which activates CheC. Interestingly CheZ in *E. coli* co-localizes to the receptor complex via CheA_short (30,31), a truncated version of CheA expressed only in enteric organisms (32). Additionally CheA_short is capable of increasing CheZ’s activity *in vitro* (33). Therefore, the CheC-CheD complex might have a similar role as does the CheA_short-CheZ complex in *E. coli*, while FliY is, in a sense, analogous to the cytoplasmic CheZ. A model summarizing the three modes of CheY-P dephosphorylation can be seen in Fig. 9. The possibility that CheC and FliY work in a cooperative manner was rendered unlikely by the observation that a constant amount of FliY does not alter CheC’s specific activity. Additionally, one might speculate that CheC could act as a phosphatase on one of the other two response regulators in the system, CheB or CheV. However tethered cell phenotypes of *cheB* and *cheV* phosphorylation point mutants are similar to a *cheC* mutant in the time course of their response to attractant (8,34). If CheC were dephosphorylating one of these response regulators, then phosphorylation levels should be too high in the absence of CheC. It is
unlikely that the time course of response of attractant would be that of the *cheB* or *cheV* mutants.

Lastly we were able to establish the per cell copy numbers for both FliY and CheC. FliY numbers were around 500 per cell, a very reasonable number considering that its *E. coli* homologue, FliN (which is missing the CheC-homologous domain) has been reported to be expressed at a level of about 100 per flagella (25) with the average cell having four to five flagella. CheC numbers were very low, around 20 copies per cell. This clearly suggests that CheC functions as an enzyme rather than a structural or inhibitory component in the chemotaxis pathway.

As mentioned, CheZ does not share sequence similarity with its functional homologs FliY and CheC. These proteins might still act in a similar manner. Based on the X-ray refraction structures for CheZ in complex with CheY, it is believed that a glutamine residue 147 of CheZ actively contributes in the release of phosphate from CheY-P, by positioning and activating a water molecule in the CheY-P active site (35). While there are no conserved glutamine residues found between CheC and FliY, other highly conserved residues possibly capable of a similar function include an aspartate, a serine, two glutamates and two aspargines (Fig.1B). Ultimately only an X-ray refraction structure will reveal whether the mode of action is similar.
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Prevalence and architecture of CheC and FliY. (A) Shown is a phylogenetic tree of selected chemotactic organisms. Organisms that encode for FliY and/or CheC and/or the homologue CheX are shaded gray. This tree was generated from 16S-RNA sequences using the programs CLUSTALW and DRAWTREE. (B) The architecture of CheC and FliY is shown with two conserved regions termed CYX1 and CYX2 highlighted in gray. An alignment of these regions is shown for selected CheC’s and FliY’s. Residues conserved 100% among both regions are shaded in gray. T.m. is *Thermatoga maritima*; C.a. is *Clostridium acetobutylicum*; B.s. is *B. subtilis*; D.h. is *Desulfitobacterium hafniense*; A.f. is *Archaeoglobus fulgidus*.

Fig. 2. CheY-P hydrolysis assay. Shown are time points tracking dephosphorylation of CheY-P. Lane 1 and 18 contained 10 µM CheA-P before the addition of 20 µM CheY. After addition of CheY, 15, 60, 120 and 240 s time points were taken in the presence of 0 µM (lanes 2-5), 0.5 µM (lanes 6-9), 2 µM (lanes 10-13) and 10 µM CheC (lanes 14-17). See Experimental Procedures.

Fig. 3. Phosphate release assay for FliY, CheC, and FliY_{Δ6-15}. (A) Phosphate release rates in relation to concentrations of CheC (white squares), FliY (dark diamonds), and FliY_{Δ6-15} (white triangles). (B) Enlargement of the linear area framed in (A) which was used to calculate specific P_{i} release rates.
Fig. 4. CheD activates CheC. This assay was performed as in Fig. 2. The last timepoint was taken after 180 s. CheC and CheD concentrations were as indicated.

Fig. 5. Swarming ability of cheC, fliYΔ6-15, and cheC fliYΔ6-15 strains in comparison with OI1085 (wild type). (A) Representative swarm plate for the indicated strains. (B) The average swarming diameter expressed in percent wild type is shown for wild type (OI1085), cheC (OI3135), fliYΔ6-15 (OI4106), fliY+ (OI3942), cheC fliYΔ6-15 (OI4139) and cheC fliY+ (OI4140). The expression fliY+ is used for ΔfliY amyE::fliYWT. Error bars represent the standard deviation from the mean calculated from 20 swarms.

Fig. 6. Capillary assays for cheC, fliYΔ6-15, and cheC fliYΔ6-15 strains in comparison with OI1085 (wild type). Symbols: Black diamonds: OI1085; white triangles: OI3135; black squares: OI4106; white circles: OI4139. (Note the logarithmic scale.)

Fig. 7. Tethered cell assay of chemotaxis mutants. Strains as follows: Wild type (OI1085) (thick dotted line), cheC (OI3135) (thin solid line) and fliYΔ6-15 (OI4106) (thin dotted line), and cheC fliYΔ6-15 (OI4139) (thick solid line). Each graph represents the average counterclockwise rotational bias for a population of at least 20 cells. Downward and upward arrows indicate addition and removal of 0.5 mM of the attractant asparagine.

Fig. 8. Determination of copy numbers for FliY and CheC. (A) Shown are representative blots containing purified FliY at 0.0625, 0.125, 0.25, 0.5, and 1 pmol
(lanes 3-7, respectively), as well as protein extract from $4.2 \times 10^8$ cells of OI1085 (lane 1) and OI3941 (lane 2). (B) Shown are membranes containing purified CheC at 1, 2.5, 5, 10, and 25 fmol (lanes 3-7, respectively), as well as protein extract from $4.2 \times 10^8$ cells of OI1085 (lane 1) and OI3135 (lane 2). Proteins were visualized immunologically.

Fig. 9. Model for the 3 modes of CheY-P hydrolysis. Proteins that are responsible for reducing CheY-P stability are shaded in gray. In *E. coli* and other γ- and β-proteobacteria CheZ hydrolyzes CheY-P (left) and part of it is localized to the receptor complexes via CheA<sub>short</sub> (CheAs) which increases CheZ activity (left). In *B. subtilis* FliY hydrolyzes CheY-P constitutively at the flagellar switch, while CheC gets activated by CheD to hydrolyze CheY-P following the addition of stimulus (middle). In *Sinorhizobium meliloti* and other α-proteobacteria CheA can phosphorylate two alternative CheYs, of which only one (CheY2) interacts with the flagellar switch while the other one (CheY1) can act as a phosphate-sink. CheY2-P levels can get reduced by transferring their phosphoryl-group back to CheA and consequently to CheY1, which quickly dephosphorylates (right).
Table I: Strains and Plasmids used in this study

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<th>Plasmids</th>
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<td>pGEX-6P-2::cheY1 [wild type]</td>
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<td>pGEX-6P-2::cheC2 [wild type]</td>
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<td>pGEX-6P-2::cheD2 [wild type]</td>
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<td>pHS115</td>
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<td>pHS108</td>
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<tr>
<td>pHS110</td>
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</table>
Figure 1

A

Gram-positive bacteria
V. cholerae
Proteobacteria
M. xanthus
Cyanobacteria
Spirochetes
Thermatogae
Archaea

B

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Consensus D---E-N-------------------R
Figure 2

Time in sec.: 15 60 120 240 15 60 120 240 15 60 120 240 15 60 120 240
0 µM CheC 0.5 µM CheC 2 µM CheC 10 µM CheC

CheA →

CheY →
Figure 3

[Graph with two panels, A and B, showing enzyme activity vs. enzyme concentration]
Figure 4

Time in sec.: 15 60 120 180 15 60 120 180 15 60 120 180 15 60 120 180
2 μM CheC 2 μM CheC 0 μM CheC 0 μM CheC
4 μM CheD 0 μM CheD 4 μM CheD 0 μM CheD
Figure 5

A

B

% Wild Type Swarm Diameter

0% 20% 40% 60% 80% 100%

OI1085 (wt/+) OI3135 (cwc) OI4106 (Δcwc) OI4142 (Δcwc Δas) OI4139 (cwc Δcwc Δas) OI4140 (cwc Δas)

(Null/Null) (Null) (Null) (Null) (Null) (Null) (Null)
Figure 6
Figure 7
Figure 8

A

FliY

B

CheC