Bacillus subtilis CheC and FliY Are Members of a Novel Class of CheY-P-hydrolyzing Proteins in the Chemotactic Signal Transduction Cascade

Received for publication, October 20, 2003, and in revised form, January 12, 2004
Published, JBC Papers in Press, January 27, 2004, DOI 10.1074/jbc.M311497200

Hendrik Szurmant†, Travis J. Muff‡, and George W. Ordal§

From the Department of Biochemistry, Colleges of Medicine and Liberal Arts and Sciences, University of Illinois, Urbana, Illinois 61801

Rapid restoration of prestimulus levels of the chemotactic response regulator, CheY-P, is important for preparing bacteria and archaea to respond sensitively to new stimuli. In an extension of previous work (Szurmant, H., Bunn, M. W., Cannistraro, V. J., and Ordal, G. W. (2003) J. Biol. Chem. 278, 48611–48616), we describe a new family of CheY-P phosphatases, the CYX family, that is widespread among the bacteria and archaea. These proteins provide another pathway, in addition to the one involving CheZ of the γ- and β-proteobacteria (e.g. Escherichia coli) or the alternative CheY that serves as a “phosphate sink” among the α-proteobacteria (e.g. Sinorhizobium meliloti), for dephosphorylating CheY-P. In particular, we identify CheC, known previously to be involved in adaptation to stimuli in Bacillus subtilis, as a CheY-P phosphatase. Using an in vitro assay used previously to demonstrate that the switch protein FliY is a CheY-P phosphatase, we have shown that increasing amounts of CheC accelerate the hydrolysis of CheY-P. In vivo, a double mutant lacking cheC and the region of fliY that encodes the CheY-P binding domain is almost completely smooth swimming, implying that these cells contain very high levels of CheY-P. CheC appears to be primarily involved in restoring normal CheY-P levels following the addition of attractant, whereas FliY seems to act on CheY-P constitutively. The activity of CheC is relatively low compared to that of FliY, but we have shown that the chemotaxis protein CheD enhances the activity of CheC 5-fold. We suggest a model for how FliY, CheC, and CheD work together to regulate CheY-P levels in the bacterium.

Chemotaxis is the process by which bacteria travel to higher concentrations of attractant or lower concentrations of repellent. Perirhically flagellated bacteria like Bacillus subtilis rotate their flagella counterclockwise (CCW)1 to swim smoothly and rapidly production of CheY-P. This activation is facilitated by the coupling proteins CheW and CheV (3). CheY-P interacts with FliM to increase the probability of CCW flagellar rotation (for reviews see Refs. 4–6). Interestingly, in Escherichia coli an attractant stimulus decreases CheA activity and therefore lowers the CheY-P concentration 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 Refs. 4, 5). However, the final output, which is to increase CCW rotation and therefore lengthen the duration of smooth swimming upon sensing attractant stimuli, remains conserved between the two organisms.

Following the activation of CheA in B. subtilis, the bacteria have to be able to adapt to the presence of a stimulus in order to orient their direction of movement up the gradient to ever higher concentrations of attractant. Adaptation is achieved by a system that reversibly methylates the receptors at conserved glutamate residues (reviewed in Ref. 7) and by the phosphorylation of CheV (8). Besides reducing receptor activity, it is also important as part of adaptation to reduce the levels of CheY-P. In E. coli and other γ- and β-proteobacteria, CheZ catalyzes dephosphorylation of CheY-P (9). However, most chemotactic bacteria do not encode a CheZ homolog (6). Recently, FliY, a flagellar switch protein of B. subtilis, was found to have a similar activity (10). Although homologs of this protein can be found in most chemotactic Gram-positive bacteria and some spirochetes, most bacteria do not encode FliY. However, CheC, a homolog of the N-terminal portion of FliY, exists in many organisms, including B. subtilis and the archaea (11) (Fig. 1A). In particular, two regions of 31 amino acids, which we termed CYX1 and CYX2 (for CheC, FliY, and CheX), are very similar in these homologs (Fig. 1B).

CheC was identified as a chemotaxis protein because a cheC mutant shows clear defects in chemotactic ability (12). However, a biochemical role for CheC has remained elusive. Here, we show that CheC shares the ability of FliY to increase the rate of CheY-P hydrolysis in vitro. Additionally, the chemotaxis protein CheD, which was previously discovered to bind CheC (13), can augment CheC activity. Studies on cheC and fliY6–15 single mutants (used because a fliY null mutant is not flagellated; Refs. 10 and 14) and a cheC fliY6–15 double mutant provided evidence of the importance of this phosphatase in vivo. To gain insight into why B. subtilis encodes two proteins with apparently redundant functions, the specific activity and per-cell copy number for both proteins was determined. Based on our findings, we propose a model for the function of these two proteins in vivo.

EXPERIMENTAL PROCEDURES

Chemicals, Columns, Enzymes, and Growth Media—All chemicals were reagent grade. All protein purification columns, as well as Pre-
of antibiotics. This procedure resulted in strain O14141. Strain O14141 was transformed with plasmids pHS111 and pHS110 to produce strain O14139 and O14140, respectively. Plasmids pTM25 (pGEX-6P-2-CheC) and pTM25 (pGEX-6P-2-CheD) were made by amplifying cheC or cheD by PCR, introducing 5’ BamHI and 3’ EcoRI sites and replacing the ATG start codon for cheC or cheD. The PCR products were cloned into the respective restriction sites of pGEX-6P-2.

Protein Overexpression and Purification—CheY, CheA, and FliY were overexpressed and purified as described previously (10). FliY\textsubscript{AT,AT}, purifying as described for FliY; however, a phenyl-Sepharose column (high substitute) was used instead of an octyl-Sepharose column. CheC and CheD were overexpressed and purified as glutathione S-transferase (GST) fusion proteins, and the GST tag was cleaved and removed as described for CheA (10).

Dephosphorylation Assay—The dephosphorylation assay was performed essentially as described (10). Briefly, 25 μM CheA was phosphorylated by incubation with {\gamma}^{32P}-ATP (33 μM, 12.5 μCi/μl) for 30 min in a total volume of 120 μl of TKMD (50 mM Tris, pH 8, 5 mM MgCl\textsubscript{2}, 50 mM KCl, 0.2 mM dithiothreitol, and 10% glycerol). The remaining {\gamma}^{32P}-ATP was removed by desalting, using a BioRad Micro-BioSpin column. The resulting CheA\textsubscript{32P} was added to premixed CheY-CheC (and sometimes CheD) solutions, resulting in a final concentration of 20 μM CheY, 10 μM CheA-P, various concentrations of CheC (and CheD), and 5 mM cold ATP in TKMD. Aliquots were taken at the indicated times by pipetting 10 μl of each reaction into 10 μl of 2 × SDS PAGE buffer and separated by SDS-PAGE (12% acrylamide). The gel was exposed to a phosphor-imaging screen and developed using a Storm 8600 phosphorImager from Amersham Biosciences. Each assay was run in duplicate.

Phosphate Release Assay—In this assay, the evolution of inorganic phosphate (P\textsubscript{i}) 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 direct comparison of activities. Briefly, 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG; 200 μM), monophosphate imidazole (3 mM, synthesized as described in Ref. 16), and Che, CheY, or FliY\textsubscript{AT,AT} at the concentrations indicated were mixed in a final volume of 930 μl of Tris, pH 7.5, and 10 mM MgCl\textsubscript{2} and incubated for 30 min at 25 °C. Following this incubation, mixtures were added to a cuvette. After the addition of 10 μl (1 unit) of nucleoside phosphorylase, the absorbance at 360 nm was measured. The absorbance stabilized after 30 s, and the phosphate flow through CheY was initiated by the addition of 6 μM CheY (resulting in a final volume of 1 ml). P\textsubscript{i} release rates were followed for 3 min at 25 °C. Using a standard curve that relates P\textsubscript{i} concentration to absorbance at 360 nm, release rates were calculated in micromolars per minute. For these measurements, a temperature-controlled Shimadzu BioSpec-1601 spectrophotometer was used.

Determination of Copy Numbers for FliY and CheC—Strain O11085 was grown as described previously for pulse-labeled methylation experiments (17). A 100-μl aliquot of a 2-ml overnight culture was added to 10 ml of LB and grown to early stationary phase at 37 °C. Cells were washed twice with chemotaxis buffer (10 mM potassium phosphate pH 7, 0.1 mM EDTA, 0.05 mM calcium chloride, 0.5 mM ammonium sulfate, 0.05% glycerol, and 1.5 mM sodium lactate) and once with protoplast buffer (25 potassium phosphate pH 7, 10 mM magnesium chloride, 0.1 mM EDTA, 20% sucrose, and 30 mM sodium lactate) supplemented with 25 μg/ml chloramphenicol and diluted to an\textsubscript{A\textsubscript{578}} = 1 in 5 ml of protoplast buffer. Serial dilutions of this suspension were plated on tryptose blood agar base plates to determine cell counts. 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 washed twice by centrifugation and lysed in a 500-μl 1 × SDS solubilizer by boiling for 10 min. Then, 10 μl of lysates were separated by SDS-PAGE (12%) in parallel with purified FliY or CheC at various known concentrations. Proteins were visualized immunologically, essentially as described (18). However, the low copy numbers of CheC required anti-CheC antibody to be preadsorbed as described (19). Antibody diluted 1:10000 for the anti-CheY-antibody and 1:100 for the anti-CheA-antibody. Bands were quantified using ImageQuant software that allows calculated copy numbers for CheC and FliY from the standard curves with purified protein and the cell counts from the serial dilutions.

Tethered Cell Assay—The tethered cell assay was performed essentially as described (20). However, FliY\textsubscript{AT,AT} expression was induced with 0.1 mM IPTG 3 h after inoculating the culture. The response of each strain was based on 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

---

**Phylogenetic distribution and architecture of CheC and FliY. A, a phylogenetic tree of selected chemotactic organisms.** Organisms that encode FliY and/or CheC and/or their homolog CheX are shaded gray. This tree was generated from 16 S 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. An alignment of these regions is shown for Thermatoga maritima, C. a., Clos- thermatogae, D. h. is Thermatoga maritima; C. a. is Clostridium acetobutylicum; B. s. is B. subtilis; D. h. is Desulfotobacterium hafniense; and A. f. is Archaeoglobus fulgidus.
to achieve wild-type expression levels of FliY and FliY<sub>Δσ<sub>−15</sub></sub> in strains OI4140 and OI4139, respectively.

**Capillary Assay for Chemotaxis**—The capillary assay was performed as described for E. coli (22) but modified as described for B. subtilis (21); the expression of FliY<sub>Δσ<sub>−15</sub></sub> 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 ensure 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). Because 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 s, whereas in the presence of equimolar concentrations of CheY-P and CheC, essentially all of CheY-P was hydrolyzed by the 60-s 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 difference, a spectrophotometric assay was used to measure the production of inorganic phosphate, Pi, over time. This assay was used previously to compare activities of different E. coli CheY-P-hydrolyzing proteins. Recently, we showed that the flagellar switch protein FliY is capable of increasing the rate of CheY-P hydrolysis (10). Because 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 s, whereas in the presence of equimolar concentrations of CheY-P and CheC, essentially all of CheY-P was hydrolyzed by the 60-s time point (Fig. 2). We conclude that CheC and FliY share a common function as CheY-P-hydrolyzing proteins.

**FliY Does Not Affect CheC Activity**—We also wanted to explore whether the activity of CheC can be affected in a syner-

---

**FIG. 2. CHEY-P HYDROLYSIS ASSAY** Shown are time points tracking dephosphorylation of CheY-P. Lanes 1 and 18 contained 10 μM CheA-P before the addition of 20 μM CheY. After the addition of CheY, 15, 60, 120 and 240 s time points were taken in the presence of 0 (lanes 2–5), 0.5 (lanes 6–9), 2 (lanes 10–13), and 10 μM CheC (lanes 14–17). See “Experimental Procedures” for details.

---

**TABLE I** Strains and plasmids used in this study

| Strains | Relevant phenotype or comment | Reference or source |
|---------|-------------------------------|---------------------|
| B. subtilis | | |
| OI1085 | Che<sup>+</sup>, trpF7 hisH2 metC133 | 7 |
| OI2852 | fliY<sup>+</sup>:cat<sup>+</sup> | 14 |
| OI3135 | ΔcheC1 | 12 |
| OI3942 | ΔfliY2 amyE5720::fliY3 (wild type fliY) | 10 |
| OI4106 | ΔfliY2 amyE5720::fliY1 (fliY<sub>Δσ<sub>−15</sub></sub>) | 10 |
| OI4139 | ΔfliY2ΔcheC1 amyE5720::fliY4 | This work |
| OI4140 | ΔfliY3cheC1 amyE5720::fliY3 | This work |
| OI4141 | ΔfliY2ΔcheC1 | This work |

E. coli

| Strains | Relevant phenotype or comment | Reference or source |
|---------|-------------------------------|---------------------|
| BL21 | Protease-deficient expression host | Amersham* |
| TG1 | Cloning host | Amersham* |
| GJ1158 | Host for pT7 derived plasmids; induction by 0.3 mM NaCl | 36 |

**Plasmids**

| Plasmids | Comment | Reference or source |
|----------|---------|---------------------|
| PGEX-6P-2 | GST tag expression plasmid | Amersham* |
| PHS101 | pGEX-6P-2::cheA1 (wild type) | 10 |
| PHS102 | pGEX-6P-2::cheY1 (wild type) | 10 |
| PTM18 | pGEX-6P-2::cheC2 (wild type) | This work |
| PTM30 | pGEX-6P-2::cheD (wild type) | This work |
| PTT-6 | Expression plasmid | 37 |
| PDB32 | pTT-6::fliY3 (wild type) | 14 |
| Plasmid SK<sup>*</sup> | Cloning (and expression) plasmid | Stratagene |
| pHS115 | Plasmid SK::fliY4 | 10 |
| pEB112 | B. subtilis-E. coli shuttle vector | 38 |
| pHS110 | pEB112::“fliM::fliY::fliQ<sup>+</sup>” | 10 |
| pDR67 | AmyE integration plasmid with IPTG inducible pSpac promoter | 39 |
| pH111 | PDR67::fliY4 | 10 |
| pH110 | PDR67::fliY3 | 10 |

<sup>*</sup> Amersham Biosciences.

---

A cheC fliY<sub>Δσ<sub>−15</sub></sub> Double Mutant Is Not Capable of Performing Chemotaxis—In the swarm assay, strains are stabbed onto a
low agar, low nutrient, semi-solid medium. As the cells metabolize the nutrient, they create an attractant gradient, which they sense and migrate toward and thus form a characteristic ring. Because the cheZ null mutant of _E. coli_ does not show chemotaxis as measured by this assay (24), we wanted to see how the single _cheC_ and _fliY_ mutants compare to the double mutant in this assay. Because a _fliY_ strain is not flagellated, we used the CheY-P binding mutant _fliY_<sup>Δ6-15</sup>. Both the _cheC_ and _fliY_<sup>Δ6-15</sup> strains showed somewhat impaired chemotaxis (Fig. 5). The double mutant was incapable of forming a ring on a swarm plate, as seen for the _cheZ_ null mutant of _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_<sup>Δ6-15</sup> strains were almost completely complemented to wild-type and _cheC_ swarm diameters, respectively, when they expressed _fliY<sup>+</sup>_ located at the _amyE_ locus (Fig. 5).

The traditional quantitative assay of chemotaxis is the capillary assay. In this assay, the single mutants were 10-fold reduced in chemotaxis toward asparagine, and the _cheC_ _fliY_<sup>Δ6-15</sup> double mutant showed no chemotactic response, consistent with the results from swarm plates (Fig. 6).

**FIG. 6.** Capillary assays with _cheC_, _fliY_<sup>Δ6-15</sup>, and _cheC_ _fliY_<sup>Δ6-15</sup> mutants in comparison with _OI1085_ (wild type). The symbols and their meanings are as follows: black diamonds, _OI1085_; white triangles, _OI3135_; black squares, _OI4106_; and white circles, _OI4139_. Note the logarithmic scale.

measures flagellar rotational bias of individual cells. The average bias for a population of cells is presumed to reflect mean CheY-P levels in those cells. The double mutant had a very high CCW rotational bias, and very few cells ever spun clockwise (Fig. 7). The average bias was even higher than that of a _fliY_<sup>Δ6-15</sup> single mutant (10), which is shown (Fig. 7) for comparison. The _cheC_ mutant did not show an increased bias before an attractant was added; however, it was impaired in adaptation, as described previously (11).

**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. The other is FliY, with which CheC shares sequence homology (10).

Most chemotactic bacteria and all known chemotactic archaea lack CheZ (6), the protein that hydrolyzes CheY-P in E. coli and other \( \gamma \) and \( \beta \)-proteobacteria (9). A second mechanism of CheY-P removal has been demonstrated in Sinorhizobium meliloti and other \( \alpha \)-proteobacteria (26, 27). These organisms encode at least two alternative CheY proteins. Although both CheY proteins can be phosphorylated, only one interacts with the flagellar switch. The other one acts as a phosphate sink, and the signaling CheY can transfer its phosphoryl group back to CheA and, thence, to the other CheY, which undergoes spontaneous dephosphorylation. The identification of the role of FliY and CheC describes yet a third mechanism for CheY-P removal in chemotactic bacteria (Fig. 9).

This third mechanism of CheY-P removal appears to be the most widespread of the three described. CheC and FliY homologs (which include CheX, a chemotaxis protein with homology to CheC and FliY and, therefore, likely to have a similar function; Refs. 11 and 28) can be found in archaea, thermotoga, spirochetes, Gram-positive bacteria, and even some proteobacteria like Myxococcus xanthus and Vibrio cholera. V. cholera has a CheZ as well, making it the only organism known to have both types of CheY-P-hydrolyzing proteins. Therefore, V. cholera might represent an evolutionary link that utilizes both the more conserved and, therefore, presumably more ancestral system of CheY-P hydrolysis and the more restricted CheZ pathway for CheY-P-hydrolysis found only in \( \beta \) and \( \gamma \)-proteobacteria.

An important goal of this study was to ascertain the importance of \( \text{cheC} \) function in vivo and gain insight into why B. subtilis expresses two proteins with an analogous function. A \( \text{fliY}_{6-15} \) mutant and a \( \text{cheC} \) mutant both showed reduced taxis in swarm and capillary assays (11, 12). The double mutant was completely defective for chemotaxis in either assay. This result may mean that the CheC and FliY activities are partially redundant. This is not the only example in B. subtilis chemotaxis for two proteins to show a partially redundant function. CheV and CheW both couple CheA to the receptors, a role

---

**Fig. 7. Tethered cell assay with chemotaxis mutants.** Strains were as follows: wild type (OI1085) (thick dotted line); \( \text{cheC} \) (OI3135) (thin solid line); \( \text{fliY}_{6-15} \) (OI4106) (thin dotted line); and \( \text{cheC fliY}_{6-15} \) (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, representative blots containing purified FliY at 0.0625, 0.125, 0.25, 0.5, and 1 pmol (lanes 3–7, respectively), as well as a protein extract from 4.2 \( \times 10^8 \) cells of strains OI1085 (lane 1) and OI3941 (lane 2). B, representative blots containing purified CheC at 1, 2.5, 5, 10, and 25 fmol (lanes 3–7, respectively), as well as a protein extract from 4.2 \( \times 10^8 \) cells of strains OI1085 (lane 1) and OI3135 (lane 2). Proteins were visualized by immunoblotting with antibodies directed against the respective proteins.

**Fig. 9. Model for the three modes of CheY-P hydrolysis.** The proteins responsible for hydrolyzing CheY-P are shaded gray. In E. coli and other \( \gamma \) and \( \beta \)-proteobacteria, CheZ hydrolyzes CheY-P (left), and some CheZ localizes to the receptor complexes via CheA\(_{\text{short}}\) (truncated CheA proteins), which increases CheZ activity (left). In B. subtilis, FliY hydrolyzes CheY-P constitutively at the flagellar switch, whereas CheC is activated by CheD to hydrolyze CheY-P following the addition of an attractant (middle). In S. meliloti and other \( \alpha \)-proteobacteria, CheA can phosphorylate two alternative CheY proteins, of which only one (CheY2) interacts with the flagellar switch, whereas the other one (CheY1) acts as a phosphate sink (right). CheY2-P levels can be reduced when their phosphoryl group is transferred back to CheA and, subsequently, to CheY1, which quickly dephosphorylates.
carried out solely by CheW in *E. coli*. Deletion of either *cheV* or *cheW* has only a modest effect on chemotaxis, but deletion of both genes abolishes it completely (3).

To explore the effect on chemotaxis of reduced CheY-P-hydrolyzing activity further, the *cheC fliY*Δ-15 double mutant was subjected to the tethered cell assay. Rotational data for the *cheC* and *fliY*Δ-15 single mutants has been reported previously (10, 11). Whereas the *fliY*Δ-15 mutant had a very high CCW bias, indicating an increased level of CheY-P, the *cheC* mutant exhibited a less severe phenotype with a prestimulus bias similar to the wild-type strain OI1085 (29). However, following the addition of attractant, *cheC* cells were impeded in adaptation, as evidenced by a prolonged 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 that is consistent with the presence of very high levels of CheY-P in the cells.

Comparing the activities of *FliY* and *CheC* in *vivo* revealed that CheC is only ~6% as active as *FliY*, a result consistent with the more severe phenotype for the *fliY*Δ-15 mutant. However, the chemotaxis protein CheD, previously identified as interacting with CheC (13), is capable of increasing the activity of CheC by ~5.3-fold in *vivo*. This effect might be even larger in *vivo*, where CheC and CheD interact with receptor complexes. We hypothesize that *FliY* constitutively removes CheY-P around the flagellar switch to maintain CheY-P concentration at the optimum level. CheC may function mainly after the addition of an attractant 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 CheAshort (30, 31), a truncated version of CheA expressed only in enteric organisms (32). Additionally, CheAshort is capable of increasing the activity of CheZ in *vivo* (33). Therefore, the CheC-CheD complex might play a role similar to that of the CheAshort-CheZ complex in *E. coli*, whereas *FliY* is more analogous to the cytoplasmic CheZ.

A model summarizing the three modes of CheY-P dephosphorylation is presented in Fig. 9. The possibility that CheC and *FliY* work in a cooperative manner was rendered unlikely by the observation that *FliY* does not alter the specific activity of CheC. 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, the tethered cell phenotypes of *cheB* and *cheV* phosphorylation point mutants are similar to a *cheC* mutant in the time course of their response to an 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 the attractant response would be the same in the *cheC* mutant as in the *cheB* or *cheV* mutants if CheC functions to dephosphorylate CheB or CheV.

We were able to establish the per cell copy numbers for both *FliY* and *CheC*. *FliY* numbers were ~500 molecules per cell, a very reasonable number considering that its *E. coli* homolog, FlIN, which lacks the CheC-homologous domain, exists in ~100 copies per flagellum (25), with the average cell having 4–5 flagella. CheC numbers were very low, ~20 copies per cell. This low abundance clearly suggests that CheC functions as an enzyme rather than as a structural component or stoichiometric activator or inhibitor 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 diffraction structure for CheZ in complex with CheY, it is believed that glutamine residue 147 of CheZ contributes in the release of phosphate from CheY-P by positioning and activating a water molecule in the CheY-P active site (35). Although no glutamine residues are conserved in CheC and *FliY*, other highly conserved residues that are possibly capable of exercising a similar function include an aspartate, a serine, two glutamates and two arginines (Fig. LB). Ultimately, only an x-ray crystal structure will reveal whether the catalytic mechanism of the families of the Y-P phosphatases are similar.

REFERENCES

1. Fuhrer, D. K., and Ordal, G. W. (1993) *J. Bacteriol.* 175, 7443–7448
2. Bischoff, D. S., and Ordal, G. W. (1992) *Mol. Microbiol.* 6, 23–28
3. Rosario, M. M., Fredrick, K. L., Ordal, G. W., and Helmann, J. D. (1994) *J. Bacteriol.* 176, 2736–2743
4. Armitage, J. P. (1999) *Adv. Microb. Physiol.* 41, 229–289
5. Stock, J. B., and Surette, M. G. (1996) in *Escherichia coli and Salmonella, Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss, R. III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Zhulin, I. B., and Ordal, G. W., ed) pp. 632-633
6. Ferguson, S. R., and Stamm, L. V. (1999) *J. Bacteriol.* 181, 232–237
7. Porter, S. L., and Armitage, J. P. (2002) *J. Bacteriol.* 184, 87–91
8. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Bacteriol.* 181, 1092–1109
9. Hess, J. F., Oosawa, K., Kaplan, N., and Simon, M. I. (1988) *Cell* 53, 79–87
10. Ullah, A. H., and Ordal, G. W. (1981) *J. Bacteriol.* 145, 958–965
11. Boesch, K. C., Silversmith, R. E., and Bourret, R. B. (2000) *J. Mol. Biol.* 298, 515–528
12. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Bacteriol.* 181, 232–237
13. Rosario, M. M., Fredrick, K. L., Ordal, G. W., and Helmann, J. D. (1994) *J. Bacteriol.* 176, 2736–2743
14. Greene, S. R., and Stamm, L. V. (1999) *Mol. Microbiol.* 32, 59–68
15. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Bacteriol.* 181, 232–237
16. Rathbun, T., and Vesper, T. (1956) *Arch. Biochem. Biophys.* 65, 319–339
17. Tsuboi, A. H., and Ordal, G. W. (1998) *J. Bacteriol.* 180, 3816–3823
18. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Biol. Chem.* 274, 27536–27542
19. Ullah, A. H., and Ordal, G. W. (1981) *J. Bacteriol.* 145, 958–965
20. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Bacteriol.* 181, 232–237
21. Porter, S. L., and Armitage, J. P. (2002) *J. Mol. Microbiol.* 32, 59–68
22. Kirby, J. R., Saulmon, M. M., Kristich, C. J., and Ordal, G. W. (1999) *J. Bacteriol.* 181, 232–237
23. Greene, S. R., and Stamm, L. V. (1999) *Mol. Microbiol.* 32, 59–68
24. Porter, S. L., and Armitage, J. P. (2002) *J. Mol. Microbiol.* 32, 59–68