# Chemotactic Methylation and Behavior in *Bacillus subtilis*: Role of Two Unique Proteins, CheC and CheD†

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ABSTRACT: We characterized mutants in two novel genes of *Bacillus subtilis*, *cheC* and *cheD*. Mutants in CheC had a high smooth swimming bias and exhibited poor adaptation to positive stimuli. Analysis of tethered cells revealed two distinct subpopulations which differ in their prestimulus bias and extent of adaptation. The receptors, the methyl-accepting chemotaxis proteins (MCPs), of this mutant strain were overmethylated, as a result of an increase in CheR activity. We speculate that CheC helps to control tumbling frequency by regulating CheR, perhaps by a feedback mechanism through the MCPs. In contrast, a *cheD* mutant exhibited very tumbly behavior, and many of the MCPs were unmethylated. It seems that some *B. subtilis* MCPs require the presence of CheD for CheR to methylate them, a unique feature of *B. subtilis* chemotaxis. It is hypothesized that CheD is part of a complex that facilitates methylation of some of the MCPs, and dissociation of CheD from this complex affects CheA activity and may help bring about adaptation.

Bacterial chemotaxis is the process by which motile bacteria respond to their environment by migrating toward more favorable conditions and away from adverse surroundings. The study of chemotaxis in *Escherichia coli* has provided a prototype (Bourret et al., 1991), and many of the chemotaxis proteins of *Bacillus subtilis* (CheA, CheY, CheB, CheR, and CheW) exhibit homology to those of *E. coli* (Fuhrer & Ordal, 1991; Bischoff & Ordal, 1991; Kirsch et al., 1993a,b; Hanlon et al., 1992). To some extent, the chemotaxis enzymes of these two species demonstrate functional resemblance. Purified *B. subtilis* CheY can be phosphorylated *in vitro* by *E. coli* CheA, and dephosphorylation of CheY-P is enhanced some by *E. coli* CheZ (Bischoff et al., 1993). Moreover, the methyltransferases from both *B. subtilis* and *E. coli* are able to methylate the heterologous methyl-accepting proteins (MCPs)[1] *in vitro* (Burgess-Cassler & Ordal, 1982). The CheB methylesterase of *B. subtilis* demethylates *E. coli* MCPs *in vitro* (Nettleton & Ordal, 1989) and can substitute for it *in vivo* (Kirsch et al., 1993a).

Characterization of null mutants of the chemotactic genes of these two organisms, however, indicates that the mechanisms are considerably different. For instance, the phenotype of a null mutant in *cheY* or *cheA* in *E. coli* is smooth swimming (Oosawa et al., 1988; Parkinson, 1978) while in *B. subtilis* it is tumbly (Bischoff & Ordal, 1991; Fuhrer & Ordal, 1991). Thus, CheY-P causes tumbling in *E. coli* but smooth swimming in *B. subtilis*. It is thought that unmethylated MCPs activate CheA poorly, producing little CheY-P, since the phenotype of *cheR* mutants in *E. coli* is smooth swimming while being more tumbly in *B. subtilis* (Kirsch et al., 1993b). However, *cheB* or *cheR* null mutants in *B. subtilis* are not completely smooth swimming or tumbly and exhibit partial adaptation to high concentrations of attractant (as opposed to being always tumbly or smooth swimming in *E. coli*), implicating the existence of an adaptation system independent of methylation (Kirsch et al., 1993a).

The process of reversible methylation of the chemoreceptors is considerably different in the two organisms. In *B. subtilis*, addition of an attractant causes an increase in turnover of methyl groups on the receptors (Goldman et al., 1982; Thoelke et al., 1988; Bedale et al., 1988). This is unveiled in a cold chase experiment, where the addition of attractant effects an immediate decrease in receptor labeling, caused by the remethylation of the MCPs with less radioactive methyl groups from S-AdoMet (Thoelke et al., 1988, 1989). There is little net loss of methyl groups from the MCPs as a result of this addition of attractant (Thoelke et al., 1988; Bedale et al., 1988). Removal of attractant in the presence of cold methionine leads to a return of methyl groups with high specific activity to the receptors. The "hot" methyl groups appear to remain fixed in the system, not released as methanol as occurs in *E. coli* (Thoelke et al., 1989). It is believed that the direct source of methanol in *B. subtilis* is a methylated acceptor (Thoelke et al., 1987, 1989, 1990; Bedale et al., 1988), which remains yet unidentified. In *E. coli*, the addition of repellent or removal of attractant enhances methanol formation, and removal of repellent or addition of attractant inhibits methanol formation (Springer et al., 1977; Toews et al., 1979; Kehry et al., 1984; Thoelke et al., 1989). In *B. subtilis*, both addition and removal of attractant enhance methanol formation, with the greatest release of methanol occurring on the third or fourth cycle, not on the first, as in *E. coli* (Thoelke et al., 1989).

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[1] Abbreviations: MCPs, methyl-accepting chemotaxis proteins; kb, kilobase(s); ORF, open reading frame; kDa, kilodaltons; IPTG, isopropyl 1-thio-β-D-galactopyranoside; CCW, counterclockwise; CW, clockwise; KU, Klett units; S-AdoMet, *S*-adenosylmethionine; MT, methyltransferase buffer; FP, French press buffer.

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Table 1: Bacterial Strains Used in This Study

| strain | relevant genotype | ref |
|---|---|---|
| *E. coli* | | |
| TG-1 | cloning host | Amersham |
| RP3098 | Δ(*flb*B-*fla*H), *che* | J. S. Parkinson |
| OI2918 | RP3098(pGP1-2) | T7 expression host; this work |
| OI2986 | OI2918 (pMR113) | this work |
| OI2988 | OI2918 (pT7-6) | this work |
| OI2989 | OI2918 (pT7-7) | this work |
| OI2714 | RP3098 (pAZ283) | Kirsch et al. (1993b) |
| OI3124 | OI2918 (pMR126) | this work |
| *B. subtilis* | | |
| OI1085 | *che*$^+$, *his*H2, metC, trpF7 | Ullah and Ordal (1981) |
| OI2991 | *cheC::cat* | this work |
| OI2934 | *cheD::cat* | this work |
| OI3135 | ×*c6cheC* | this work |
| OI3136 | OI3135 (pMR109) | this work |
| OI3138 | OI3135 (pEB112) | this work |
| OI3139 | OI3135 (pEBAB) | this work |
| OI3140 | OI2934 (pEB112) | this work |
| OI3142 | OI2934 (pWN5) | this work |
| OI3165 | OI3135 *amyE::cheC* | this work |
| OI2680 | OI1085 *cheR::pAZ286* | Kirsch et al. (1993b) |
| OI1100 | *cheR* | Burgess-Cassler et al. (1982) |
| OI3154 | Δ*cheC cheR* | this work |
| OI2923 | *cheR cheD::cat* | this work |

The differences in the two organisms very likely reflect some novel proteins in one or both of them. Two candidates for such proteins might arise from two open reading frames (ORFs) identified as lying between *cheW* and *sigD* at the distal end of the *che/fla* operon of *B. subtilis* (Marquez-Magaña et al., 1994). In this article we show that these ORFs indeed encode proteins having no homologs in the database and that inactivation of the corresponding genes, named *cheC* and *cheD*, causes defects in chemotaxis. Our results indicate that CheC interferes with MCP methylation, perhaps in order to help set the prestimulus tumbling frequency. They also suggest that CheD facilitates CheA activation and is an obligatory requirement for CheR methyltransferase to methylate many of the MCPs.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Plasmids.* Strains and plasmids used in this study are described in Tables 1 and 2, respectively. All plasmids were propagated in *E. coli* strain TG-1.

*Growth Media and Conditions.* TBr (tryptone broth) is 1% tryptone and 0.5% NaCl. LBr is 1% tryptone, and 0.5% NaCl, 0.5% yeast extract. Minimal media is 50 mM potassium phosphate buffer, pH 7.0, 1 mM $MgCl_2$, 1 mM $(NH_4)SO_4$, 0.14 mM $CaCl_2$, 0.01 mM $MnCl_2$, 0.2 mM $MgCl_2$, 50 μg/mL required amino acids (his, met, trp), and 20 mM sorbitol. When appropriate, antibiotic was added at the following concentrations: chloramphenicol, 5 μg/mL; ampicillin, 100 μg/mL; kanamycin, 5 μg/mL (*B. subtilis*), 75 μg/mL (*E. coli*).

*Expression of cheC and cheD.* pMR113 and pMR126 were used to express *cheC* and *cheD*, respectively, in strain OI2918 (an *E. coli che*$^-$ strain), employing the method of Tabor and Richardson (1985). Induction of expression was done at 42 °C in the presence of L-[$^{35}$S]methionine and rifampicin. Proteins were separated on a 15% polyacrylamide gel, and radiolabeled bands were visualized by autoradiography.

*Construction of Null Mutants.* Since *cheC* is translationally coupled with *cheD*, an in-frame deletion of *cheC* (strain OI3135) was constructed to ensure the nonpolarity of the mutation on the expression of *cheD*. About 92% of the gene was eliminated, and this was done by cleaving pMR112 at an *Eco*RI site introduced by site-directed mutagenesis at the N-terminus of *cheC*, and at a unique *Mst*II site at the C-terminus of the *cheC* gene (Figure 1). The 3′-recessed ends created by these enzymes were filled in using DNA polymerase I and deoxynucleoside triphosphates (final concentration of 20 μM). Ligation of these blunt ends resulted in pMR118, carrying the *Pst*I-*Eco*RI distal fragment of the operon (Figure 1) that encodes only 18 amino acids of CheC. The deletion was verified by sequencing. The *Pst*I-*Bgl*II fragment from pMR118 was cloned into the *Pst*I-*Bam*HI site of pEB112 (creating pMR119), and the mutation was crossed onto the chromosome of strain OI2991 (*cheC::cat*) by gene conversion. Chloramphenicol-sensitive transformants were selected, and the conversion of the mutant strain was verified by Southern blot analysis.

A null mutant in *cheD* was created by inserting a *cat* gene from pDB4 into the unique *Sst*I site of *cheD* (Figure 1). The resulting plasmid, pWN2, was linearized and transformed into wild-type *B. subtilis* strain OI1085, selecting for chloramphenicol resistance. Transformants were verified for the desired mutation by Southern blot analysis.

*Complementation of the Null Mutants.* For complementation experiments, the parent plasmid pEB112 or plasmids carrying the appropriate wild-type gene under the control of the IPTG-inducible *tac* promoter (pMR109, pWN5, and pEBAB) were transformed into OI1085 (wild-type), OI3135 (*cheC*), or OI2934 (*cheD*) (Table 1). Linearized pMR130 was transformed into OI3135 for integration into the *amyE* locus, selecting for chloramphenicol resistance and screening for inability to degrade starch. This allows expression of a single copy of the wild-type CheC gene under the control of an IPTG-inducible promoter. Transformants were tested on semisolid agar plates (0.27% agar) containing tryptone medium or minimal medium with 0.1 mM mannitol. IPTG (range 1−10 mM) and antibiotic (kanamycin, 5 μg/mL; chloramphenicol, 5 μg/mL) were added appropriately. Single colonies were stabbed on swarm plates and incubated for 6 h at 37 °C.

*Capillary Assays.* Capillary assays have been described (Ordal & Goldman, 1975). Cells were grown overnight at 30 °C on tryptose blood agar base plates, resuspended ($10^8$ bacteria/mL) in TBr, and diluted 1:50 in minimal media. After 4 h of shaking at 37 °C, the culture was supplemented with 0.05% glycerol and 5 mM sodium lactate and incubated for an additional 15 min. Cells were harvested and resuspended at $A_{600}$ = 0.001 and assayed for chemotaxis. The number of colony forming units that accumulated in an attractant-filled capillary was determined. All 20 common amino acids, mannitol, glucose, and α-methyl glucoside were used as attractants. Each treatment was done in triplicate. Background accumulation (no attractant) was subtracted from each of the treatments.

*Tethered Cell Analysis.* Cells were prepared for growth as described for capillary assays above, except the culture was diluted 1:100 in minimal media. After incubation at 37 °C for 4.5 h, the cells were tethered as previously described (Berg & Tedesco, 1975; Berg & Block, 1984). Each tethered cell was subjected to the addition and removal of chemoeffectors (10 μM azetidine-2-carboxylic acid; 3.15 mM indole) over a period of 8 min and recorded by

Table 2: Plasmids Used in This Study

| plasmid | description | source or ref |
|---|---|---|
| pGP1-2 | contains gene encoding T7 polymerase; $kan^R$ | Tabor and Richardson (1985) |
| pT7-6 | *E. coli* expression plasmid with cloned insert under control of T7 promoter; $amp^R$ | Tabor and Richardson (1985) |
| pT7-7 | *E. coli* expression plasmid with cloned insert under control of T7 promoter and fused with the translation start site of the T7 gene 10 protein; $amp^R$ | Tabor and Richardson (1985) |
| pBluescript | $amp^R$ | Stratagene |
| pEB112 | *B. subtilis*/*E. coli* shuttle vector; contains IPTG-inducible *"tac"* promoter; $kan^R$, $amp^R$ | Leonhardt and Alonso (1988) |
| pDB4 | pUC19 with the *cat* gene from pBR329 cloned into the *Bam*HI site; $amp^R$, $cm^R$ | Bischoff and Ordal (1992) |
| pDR67 | *amyE* integration plasmid; contains IPTG-inducible "*spac*" promoter; $amp^R$ $cm^R$ | Henner (1990) |
| pWN0 | 1.9-kb *Pst*I-*Eco*RI fragment containing *cheW*, *cheC*, and *cheD*, cloned into pUC18; $amp^R$ | this work |
| pWN1 | 0.9-kb *Sal*I-*Eco*RI fragment containing *cheD* subcloned from pWN0 into pUC19; $amp^R$ | this work |
| pWN2 | 0.7-kb *cat* gene from pDB4 cloned into unique *Sst*I site of *cheD*; $amp^R$, $cm^R$ | this work |
| pWN3 | 0.9-kb *Sal*I-*Eco*RI fragment subcloned from pWN0 into pBluescript; $amp^R$ | this work |
| pWN5 | 0.9-kb *Hinc*II-*Pst*I from pWN3 cloned into *Sma*I-*Pst*I site of pEB112; $amp^R$ $kan^R$ | this work |
| pMR100 | 1.9-kb *Pst*I-*Eco*RI fragment from pWN0 cloned into M13mp19 | this work |
| pMR104 | 1.4-kb *Pst*I-*Dra*I fragment from pWN0 subcloned into *Pst*I-*Sma*I of M13mp19; *Eco*RI site at start of *cheC* created via site-directed mutagenesis | this work |
| pMR105 | 0.7-kb *cat* gene cloned into unique *Eco*RI site of pMR104; $amp^R$, $cm^R$ | this work |
| pMR108 | 0.65-kb *Hin*dIII-*Rsa*I fragment containing *cheC* sucloned from pWN0 into *Hin*dII-*Eco*RV site of pBluescript; $amp^R$ | this work |
| pMR109 | 0.65-kb *Pst*I-*Cla*I fragment from pMR108 cloned into *Pst*I-*Sma*I of pEB112; $amp^R$, $kan^R$ | this work |
| pMR112 | pMR100 with *Eco*RI site created via site-directed mutagenesis at start of *cheC* | this work |
| pMR113 | 0.65-kb *Hind*III-*Eco*RI fragment from pMR108 cloned into pT7-6; $amp^R$ | this work |
| pMR 118 | pMR112 with 0.6-kb *Eco*RI-*Mst*II fragment within *cheC* deleted, end-filled, and religated | this work |
| pMR119 | 1.3-kb *Pst*I-*Bgl*II fragment of pMR118 cloned into *Pst*I-*Bam*HI of pEB112; $amp^R$, $kan^R$ | this work |
| pMR121 | pMR100 with *Hin*dIII site created via site-directed mutagenesis between *cheC* and *cheD* | this work |
| pEBAB | 1.1-kb *Hin*dIII fragment from pMR121 subcloned in coding orientation into pEB112; $amp^R$, $kan^R$ | this work |
| pMR124 | pMR100 with *Nde*I site and a *Hin*dIII site created via site-directed mutagenesis at the start and end of the *cheD* gene, respectively | this work |
| pMR126 | 0.5-kb *Nde*I-*Hind*III fragment containing *cheD* subcloned from pMR124 into pT7-7; $amp^R$ | this work |
| pMR130 | 0.65-kb *Hin*dIII-*Xba*I fragment from pMR 108 cloned into pDR67; $amp^R$, $cm^R$ | this work |

videomicroscopy. Behavior was analyzed as described previously (Kirsch et al., 1993a).

*In Vivo Methylation Assays.* Methylation of MCPs *in vivo* was performed as previously described (Ullah & Ordal, 1981). Briefly, cells were grown to early stationary phase (180 KU), harvested, and diluted to $A_{600} = 1.0$ in protoplast buffer (20% sucrose, 10 mM $MgCl_2$, 25 mM $K_3PO_4$, 30 mM sodium lactate, 1 mM EDTA, pH 7.0; 1 mg/mL lysozyme). Protoplasts were incubated with 100 $\mu$Ci of [*methyl*-$^3$H]-methionine for 5 min. Proteins solubilized in 4× SDS solubilizer (25 mM Tris, 8% (w/v) sodium lauryl sulfate, 40% (v/v) glycerol, 20% (v/v) $\beta$-mercaptoethanol) were separated on a 10% polyacrylamide gel and visualized by fluorography as described in Lasky and Mills (1975).

In investigating methyl group turnover *in vivo*, protoplasts were incubated with 10 $\mu$M methionine at 30 $\mu$Ci/mL. Samples were taken at various time points after addition of methionine and frozen in a dry ice bath. Proteins were solubilized and treated as described above. Labeled bands were quantitated by densitometry. Experiments were done in triplicate.

*Preparation of Crude Soluble Extracts and Membrane Fractions.* Cell fractions were prepared according to Goldman and Ordal (1984) and Kirsch et al. (1993b) with some modifications. Stationary phase cells were diluted 1:100 into 6 liters of LBr and were harvested after 10 h of incubation at 37 °C. Cell pellets were washed twice in 1 M KCl, then twice in FP buffer (10 mM potassium phosphate, pH 7.0, 10 mM $MgCl_2$, 1.0 mM EDTA, 20 mM KCl, 0.1 mM phenylmethanesulfonyl fluoride, 20% (v/v) glycerol, and 0.02% $NaN_3$), and resuspended in a final volume of 30 mL of FP buffer. Cells were broken open by passage through a cell disruptor (Watts Fluidair, Inc.) four times at 100 psi. Unbroken cells and cell debris were removed by centrifugation at 24000*g* for 30 min. Membranes were isolated by centrifugation at 504000*g* in a Beckman 70 Ti rotor. The crude soluble extract (supernatant) was concentrated to 20 mg/mL using an ultrafiltration stirred cell and YM3 ultra-

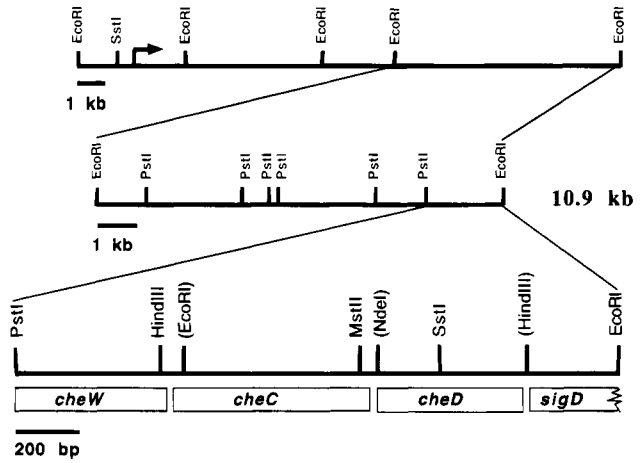


FIGURE 1: Partial restriction map of the chromosomal region containing *cheC* and *cheD*. Their location in the larger 10.9-kb *Eco*RI fragment is shown. Relevant restriction sites are indicated. Sites created by site-directed mutagenesis are in parentheses. The corresponding ORFs are shown as boxes. Transcription of the operon is from left to right.

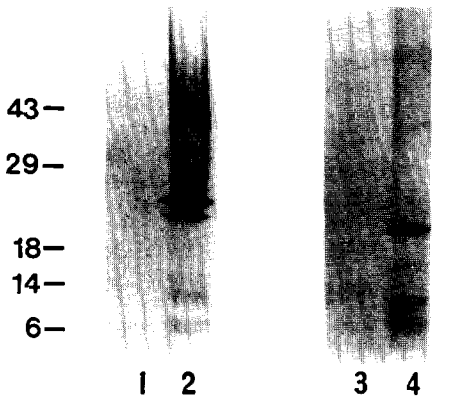


FIGURE 2: Autoradiogram of *B. subtilis* CheC and CheD expressed by the method of Tabor and Richardson (1985) in an *E. coli* strain deleted for chemotaxis genes. Lane 1, pT7-6 (control plasmid); lane 2, pMR113 (pT7-6 expressing CheC); lane 3, pT7-7 (control plasmid); lane 4, pMR126 (pT7-7 expressing CheD). Molecular mass standards from Bethesda Research Laboratories are indicated in kilodaltons.

filtration membrane discs from Amicon. Membranes were washed twice with 1 M KCl, resuspended, and homogenized in MT buffer, pH 7.45 (10 mM potassium phosphate pH 7, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM $\beta$-mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride, 0.02% $NaN_3$) at a protein concentration of 10 mg/mL. Protein concentration was determined using the Coomassie protein assay reagent (Pierce Chemical Co.).

*In Vitro Methylation Assays.* For assay of methyltransferase activity in the soluble extracts, membranes from double mutants OI3154 (*cheRcheC*) and OI2923 (*cheRcheD*) were prepared to be used as the substrate. OI3154 was made by transformation of OI3135 (*cheC*) with chromosomal DNA from OI2680 (*cheR*); OI2923 was made by transformation of OI1100 (*cheR*) with linearized pWN2 (containing *cheD::cat*). For methylesterase assays, membranes from a *cheR-cheC* mutant (OI3154) were prepared as described by Kirsch et al. (1993b). These membrane preparations were labeled with *S*-adenosyl[*methyl*-$^3$H]methionine using soluble extracts of OI2714, an *E. coli* strain that produces *B. subtilis cheR* methyltransferase. Labeled membranes were washed twice in 1 M KCl and once in MT buffer and resuspended in MT buffer at a concentration of 5 mg/mL membrane protein.

*In vitro* methylation assays were carried out as described (Goldman & Ordal, 1984) with slight modifications. The methylation reactions consisted of 10 $\mu$g of membrane protein fraction, 100 $\mu$g of soluble extract, 1 $\mu$L of 0.1 M $MgCl_2$, 5 $\mu$L of 100% (v/v) glycerol, 1.5 $\mu$Ci of *S*-adenosyl-[*methyl*-$^3$H]methionine, and MT buffer to a final reaction volume of 25 $\mu$L. The demethylation reactions contained 25 $\mu$g of radioactively methylated membranes, 125 $\mu$g of soluble extract, 5 $\mu$L of 100% (v/v) glycerol, 1 $\mu$L of 0.1 M $MgCl_2$, and MT buffer in a final volume of 25 $\mu$L. As a control, radioactively methylated membranes were incubated with BSA under the same reaction conditions. Reactions were terminated by addition of 4× SDS solubilizer. Samples were run on 10% polyacrylamide gels and prepared for fluorography. Fluorograms were scanned with an LKB Ultrascan XL laser densitometer.

## RESULTS

*Expression of cheC and cheD.* In order to verify that the two open reading frames *orfA* (renamed *cheC*) and *orfB* (renamed *cheD*) encode proteins, they were expressed in the presence of L-[$^{35}$S]methionine from plasmids pMR113 and pMR126 using the method of Tabor and Richardson (1985). A radiolabeled band with an apparent molecular mass of 23 kDa, the predicted molecular mass of CheC, was seen from expression of pMR113 (Figure 2, lane 2). A lower band running at a molecular mass of 21 kDa and of reduced intensity could have been the result of expression from a second internal start site that is recognized in *E. coli*. It is predicted from the *cheC* DNA sequence that such a protein would be in the same reading frame (beginning with a methionine seven base pairs downstream of a potential ribosome binding site) and 11 amino acids shorter. No radiolabeled band was detected from the parental strain containing pT7-6 alone (Figure 2, lane 1). Using pMR126, a band migrating at the predicted molecular mass of CheD (18 kDa) was seen after induction (Figure 2, lane 4). Expression of the parent plasmid (pT7-7) without any cloned DNA did not reveal any radiolabeled band (Figure 2, lane 3).

*Complementation of the cheD Null Mutant.* When stabbed on a minimal swarm plate containing mannitol, a *cheD* mutant (strain OI2934) formed a diffused swarm that was 50% smaller in diameter than wild-type (Figure 3C, bottom left of plate). In addition, it lacked a distinct ring characteristic of the wild-type OI1085 strain. The mutant, however, formed a larger swarm on tryptone plates, about 30% greater than wild-type (Figure 3A, bottom left of plate). The mutant phenotypes were restored to wild-type phenotype by complementation, demonstrating the non-polarity of the constructed mutation on downstream genes (Figure 3B,D).

*Capillary Assays of the cheD::cat Null Mutant.* We tested the *cheD* mutant's ability to chemotax toward each of the 20 common amino acids by capillary assays. Results revealed that a *cheD* mutant exhibited little chemotaxis to common amino acids and some sugars (Table 3). Its deficiency in chemotaxis was also demonstrated in the analysis of tethered cells (see below and Figure 5).

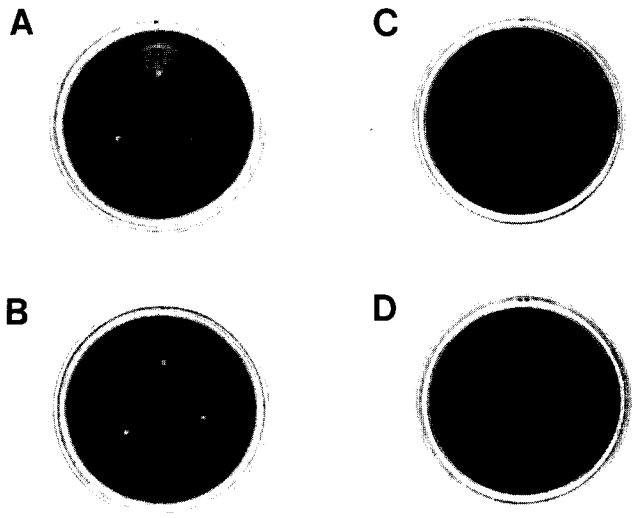


FIGURE 3: Complementation of a *cheD::cat* null mutant. Single colonies were stabbed on tryptone (A and B) and mannitol (C and D) semisolid agar plates and incubated at 37 °C. The swarm plates A and C contain no IPTG. Plates B and D are supplemented with 1.0 mM IPTG for plasmid induction. Colonies were stabbed on the plate as follows: Top, wild-type OI1085 with pEB112; bottom left, OI3140 (*cheD::cat* with pEB112); bottom right, OI3142 (*cheD::cat* with pWN5).

Table 3: Capillary Assays of the *cheD* Mutant

| attractant | accum ratio[a] | attractant | accum ratio[a] |
|---|---|---|---|
| amino acids | | amino acids | |
| alanine | 0.00 | methionine | 0.00 |
| arginine | 0.00 | phenylalanine | 0.069 |
| asparagine | 0.00 | proline | 0.040 |
| aspartate | 0.15 | serine | 0.055 |
| cysteine | 0.00 | threonine | 0.010 |
| glutamate | 0.00 | tryptophan | 0.034 |
| glutamine | 0.00 | tyrosine | 0.00 |
| glycine | 0.10 | valine | 0.031 |
| histidine | 0.050 | sugars | |
| isoleucine | 0.010 | glucose | 0.00 |
| leucine | 0.023 | α-methyl glucoside | 0.00 |
| lysine | 0.031 | mannitol | 0.010 |

[a] Ratio of bacterial accumulation of *cheD::cat* null mutant to wild-type; calculated as $[(c - b)/b]_{cheD}/[(c - b)/b]_{WT}$ where $c$ = average number of colonies in the presence of attractant and $b$ = average number of colonies in buffer only (no attractant).

*Complementation of a cheC null mutant.* A deletion of the *cheC* gene was constructed such that the remaining sequence was in-frame with the coding sequence. This *cheC* mutant (OI3135) formed a small dense swarm on both tryptone (Figure 4A, top right of plate) and mannitol swarm plates (not shown) and lacks the distinct wild-type ring in the latter. Attempts were made to complement the mutant with pMR109, but were unsuccessful (Figure 4B, bottom left of plate) even at high levels (1 M) of IPTG. However, upon "integration" of pMR130 (see Experimental Procedures) into the *amyE* locus (which encodes a protein responsible for degradation of starch) of OI3135, the wild-type phenotype could be restored when induced with IPTG (Figure 4D, bottom right of plate), indicating that the mutation was not polar on downstream genes. Complementation was observed at the minimum of 25 μM IPTG and had a maximal effect at 0.1 mM IPTG (data not shown).

Unable to complement OI3135 (*cheC*) with pMR109, we transformed a plasmid (pEBAB) containing the translationally coupled genes, *cheC* and *cheD*, into the *cheC* mutant strain. The swarm of the resulting strain (OI3165) was similar to that of wild-type upon IPTG induction, but smaller in diameter (50%; Figure 4B, bottom right). Wild-type strains containing plasmids expressing *cheC* (pMR109) or *cheD* (pWN5) alone or together (pEBAB) had no effect on the wild-type swarm phenotype (data not shown).

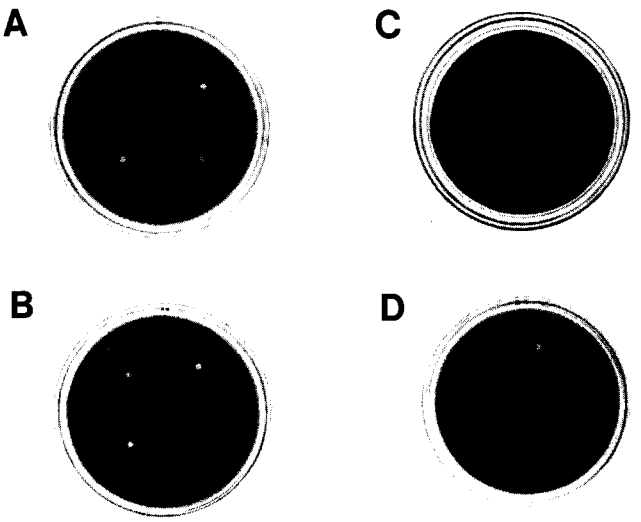


FIGURE 4: Complementation of a *cheC* null mutant. Single colonies were stabbed on tryptone semisolid agar plates and incubated at 37 °C. The swarm plates A and C contain no IPTG. Plates B and D are supplemented with 0.1 mM IPTG for plasmid induction. For plates A and B: Colonies were stabbed on the plate as follows: Top left, wild-type OI1085; top right, OI3138 (Δ*cheC* with pEB112); bottom left, OI3136 (Δ*cheC* with pMR109); bottom right, OI3139 (Δ*cheC* with pEBAB). For plates C and D: *Top*, OI3135 (Δ*cheC*); bottom left, OI1085; bottom right, OI3165 (Δ*cheC amyE::cheC*).

*Tethering Assays of the Null Mutants.* To define the role of CheC and CheD in chemotactic behavior, the excitation and adaptation phases of tethered cells of the mutants on addition and removal of chemoeffectors were observed by videomicroscopy. The wild-type *B. subtilis* strain exhibited a characteristic increase in counterclockwise (CCW) flagellar rotation bias (smooth swimming) upon addition of the nonmetabolizable attractant, azetidine-2-carboxylate (Figure 5A, thick line). Adaptation occurred within 1 min. Upon removal of the attractant, the CCW bias decreased, and then the cells slowly adapted. Analysis of tethered cells of OI3135 (*cheC*) revealed that the bacterial cells could be categorized unambiguously into two different subpopulations; one subpopulation had an extremely high CCW flagellar rotation bias and did not adapt to the addition of attractant; the other subpopulation had a more random bias and did adapt to the addition of attractant, albeit slowly (Figure 5A, dotted lines). The two phenotypes existed at a ratio of 1:1 (200 tethered cells scored) even in cell cultures derived from a single colony which itself was derived from an isolated colony. This phenomenon of two different subpopulations was also observed in a *cheR* mutant (Kirsch et al., 1993b) whose subpopulations had the superimposable behavioral profiles but different prestimulus bias. The behavioral profile of a *cheCcheR* double mutant (OI3154) was identical to that of a *cheR* mutant (Kirsch et al., 1993b), in showing both very tumbly and moderately tumbly subpopulations (Figure 6, dotted lines). In addition, the bacteria partially adapted to the addition of an attractant, the same distinct response exhibited by a *cheR* mutant.

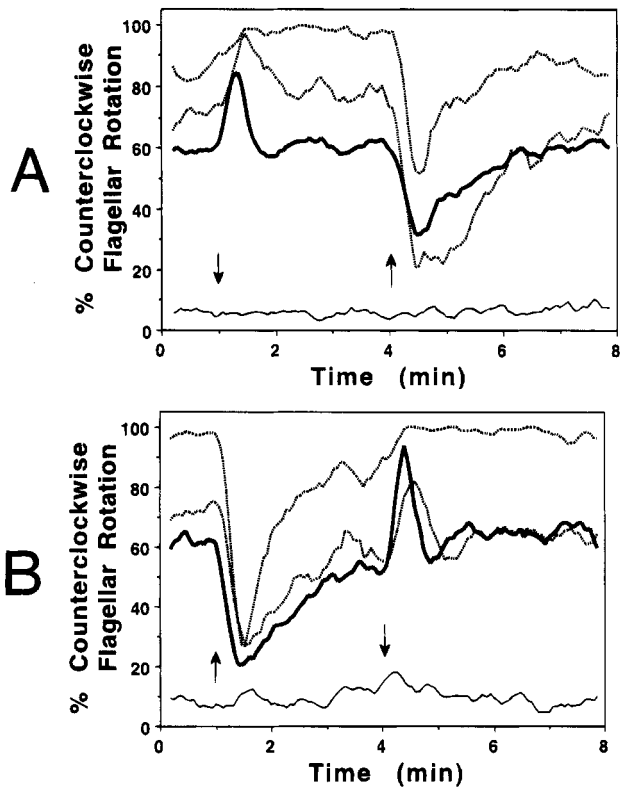


FIGURE 5: Behavior of tethered cells of the null mutants. Tethered cells were subjected to the addition (first arrow) and removal (second arrow) of (A) 10 $\mu$M azetidine-2-carboxylic acid and (B) 3.15 mM indole. Thick line, OI1085 (wild-type); dotted line, OI3135 ($\Delta$*cheC*); thin line, OI2934 (*cheD*). At least 15 cells were observed for each strain over a period of 8 min.

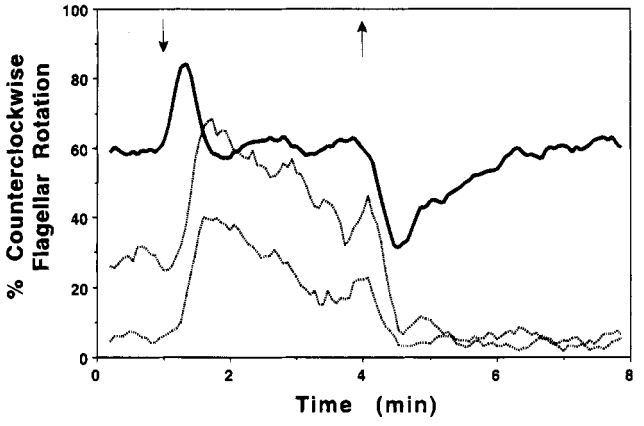


FIGURE 6: Behavior of tethered cells of a *cheCcheR* double mutant. Tethered cells were subjected to the addition (first arrow) and removal (second arrow) of 10 $\mu$M azetidine-2-carboxylic acid. Thick line, OI1085 (wild-type); dotted line, OI3154 ($\Delta$*cheCcheR*). At least 15 cells were observed for each strain over a period of 8 min.

Tethered cells of OI3135 (*cheC*) were also subjected to the addition and removal of the repellent indole (0.82 mM; Figure 5B, dotted line). Two populations were again observed at a ratio of 1:1, with one population that adapted to the removal of repellent and the other that did not. In contrast, the *cheD* mutant strain OI2934 tumbled frequently and did not respond to the addition and removal of attractant or repellent (Figure 5, thin line).

*MCP Methylation.* Chemotactic signaling is regulated at the receptors. To further investigate the role of CheC and CheD in chemotaxis, the methylation of the MCPs were examined after 5 min of protoplast incubation with L-[*methyl*-$^3$H]methionine. *In vivo*, MCPs designated as H1 and H2 were completely unlabeled in a *cheD* mutant strain (OI2934), while H3 was radiolabeled, albeit poorly (Figure 7, lane 1). OI3135 (*cheC*), on the other hand, had MCPs that were labeled 2-fold more than the wild-type (Figure 7, lane 3). This result suggested that the MCPs in the *cheC* mutant might be more methylated compared to wild-type. This expectation was confirmed by following MCP methylation over time until it reached a steady state. The maximal level of methylation in the *cheC* mutant was found to be 70% greater than that of wild-type (Figure 8).

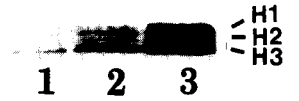


FIGURE 7: Methylation of MCPs *in vivo*. Cells were incubated with L-[*methyl*-$^3$H]methionine for 5 min. Lane 1, OI2934 (*cheD::cat*); lane 2, OI1085 (wild-type); lane 3, OI3135 ($\Delta$*cheC*). MCPs H1, H2, and H3 are indicated.

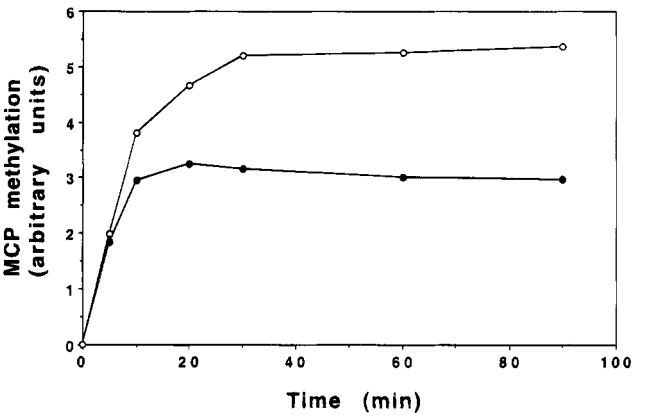


FIGURE 8: Methylation of MCPs in steady state conditions. Cells were incubated with 10 $\mu$M methionine at 30 $\mu$Ci/mL. Samples were taken at various time points, and methylation was quantitated by densitometry of the resulting fluorogram. Filled circles, OI1085 (wild-type); open circles, OI3135 ($\Delta$*cheC*).

Increased methylation of OI3135 MCPs *in vivo* might have been due to changes in CheR methyltransferase activity or CheB methylesterase activity. To determine the level of CheB activity independent of CheR activity in this strain, membrane fractions from strain OI3154 (*cheRcheC*) were radiolabeled with *S*-adenosyl[*methyl*-$^3$H]methionine. Membranes from the double mutant (*cheRcheC*) were used to obtain methylated MCPs with high specific activity and to eliminate effects on methylation caused by the CheC protein that may have cofractionated with the membrane fraction. These radioactively methylated membranes were incubated with OI1085 (wild-type) or OI3135 (*cheC*) soluble fractions, and aliquots were taken at particular timepoints. CheB activity is reflected as a decrease in protein labeling. Densitometric analysis of the fluorogram is shown in Figure 9. Methylesterase activity was unaffected by the absence of CheC (Figure 9A). In contrast, CheR activity, as determined by the ability of cytoplasmic extracts to methylate *cheRcheC* mutant membranes, was considerably faster in a *cheC* mutant compared to wild-type (Figure 9B). Thus, increased labeling in OI3135 (*cheC*) was due to a higher level of methyl groups transferred onto the MCPs. Increased CheR activity coupled with unaffected CheB activity should cause an increase in the number of methyl groups on the MCPs. In fact, the MCPs on the *cheC* mutant had about

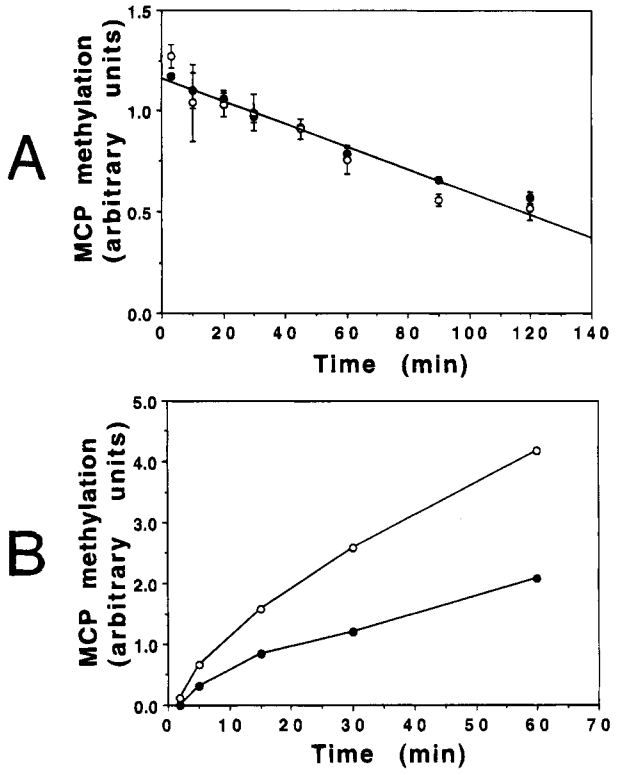


FIGURE 9: Rate of methylation and demethylation of the MCPs in a *cheC* null mutant, *in vitro*. (A) Radioactively methylated OI3154 (Δ*cheCcheR*) membranes were incubated with wild-type OI1085 (*filled circles*) or OI3135 (Δ*cheC*) (open circles) soluble extracts. Reactions were terminated at certain time points, and methylation was quantitated by densitometry of the resulting fluorogram. (B) OI3154 membranes were incubated with OI1085 or OI3135 soluble extracts and analyzed as described above.

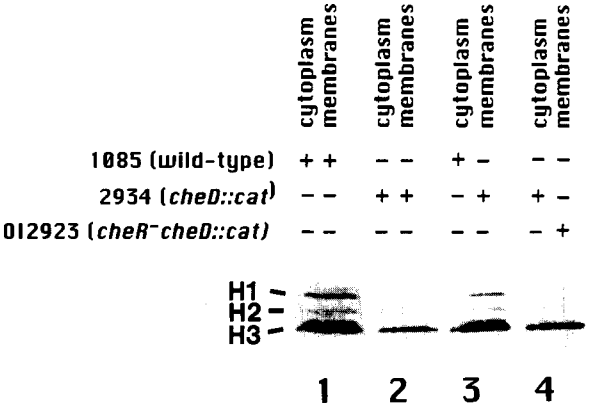


FIGURE 10: *In vitro* methylation of MCPs of a *cheD* null mutant. Null mutant and wild-type cytoplasms and membranes were isolated and incubated with *S*-adenosyl[*methyl*-$^3$H]methionine for 1 h at 28 °C. Labeled proteins were separated on a 15% polyacrylamide gel and visualized by flourography. Membranes and cytoplasms used for each reaction are shown. MCPs H1, H2, and H3 are indicated.

1.7 times as many methyl groups as the wild-type (Figure 8).

The methylation profile of the MCPs of OI2934 (*cheD*) as seen in Figure 7 (lane 1) indicates that the slower migrating MCPs are not methylated. This might be due to (1) very slow turnover of methyl groups during the methylation experiment, (2) failure to methylate these MCPs, or (3) failure to express them. To test whether the MCPs were being expressed, wild-type cytoplasmic extracts were incubated with OI2934 (*cheD*) membrane fractions. MCPs were found to be methylated normally (Figure 10, lane 3). Thus, the MCPs are present in the mutant strain but are not methylated in the absence of CheD (Figure 7, lane 1; Figure 10, lane 1). It is possible that the MCPs in the *cheD* mutant are not methylated because of a deficiency in methylesterase activity. If the mutant has fully methylated MCPs, methylation by *S*-adenosyl[*methyl*-$^3$H]methionine will not be detected. To determine this, we obtained membrane fractions from the double mutant strain OI2923 (*cheRcheD*, which has unmethylated MCPs) and incubated them with OI2934 (*cheD*) cytoplasmic fractions. H1 and H2 were not labeled in this case (Figure 10, lane 4), suggesting that CheD is required for CheR to methylate these MCPs, rather than for CheB to demethylate them.

## DISCUSSION

In this study, we have characterized the effects of mutations in two genes located at the distal region of the *che/fla* operon, *cheC* and *cheD*. Mutants in either gene affected both chemotactic behavior and chemoreceptor methylation. These genes have no known homologs in *E. coli* or any other bacteria and hence form part of the basis of the unique mechanism of chemotaxis in *B. subtilis*.

The behavioral response of a strain to the addition and removal of stimuli is dependent upon the levels of CheY-P in the strain. In *E. coli*, CheY-P causes the flagella to rotate CW while CheY-P in *B. subtilis* causes the flagella to rotate CCW (Bischoff & Ordal, 1991). Levels of CheY-P in both species are controlled by the state of the reversibly methylated receptors, the MCPs (Parkinson, 1978). Binding of a repellent to *E. coli* MCPs increases levels of CheY-P while the same effect is obtained in *B. subtilis* upon binding of an attractant. Since a *cheR* mutant shows a considerably lower CCW bias than wild-type (i.e., gives chronic underactivation of CheA), it appears that increased methylation of the MCPs sends strong CCW signals to the flagella, a behavior opposite that of the *E. coli* system (Kirsch et al., 1993b). Thus, in both *B. subtilis* and *E. coli*, increased MCP methylation enhances CheA activity.

A *cheC* mutant has highly methylated MCPs as a result of the increased activity of CheR in transferring methyl groups. This higher degree of methylation correlates well with the high CCW bias of flagellar rotation of the *cheC* mutant when tethered cells of this strain were analyzed (Figure 5). The fact that a *cheCcheR* double mutant behaved identically to a *cheR* mutant suggests that CheC affects behavior only by regulating methylation on the MCPs: if the MCPs are unmethylated, the presence or absence of CheC does not affect behavior.

Very interestingly, the *cheC* mutant is like the *cheR* mutant of *B. subtilis* in showing two subpopulations when grown from a single cell. In the case of the *cheR* mutant, the bacteria were either somewhat tumbly or very tumbly, the former phenotype resembling that of a *cheRcheB* double mutant. Indeed, CheB was found to be reduced to 20% normal levels in the *cheR* mutant (Kirsch et al., 1993b). Thus some glutamine residues in the MCPs that are normally deamidated by CheB might remain intact. These residues can function similarly to methylated glutamates (Dunten & Koshland, 1991) and consequently promote higher CheA activity. It was hypothesized that the amount of CheB varied from cell to cell and that, in bacteria exhibiting the *cheRcheB*

phenotype, this amount was below some threshold value (Kirsch et al., 1993b).

In the case of the *cheC* mutant, one of the two subpopulations was about 85−95% bias (wild-type being 60%) and the other was about 70% bias (Figure 5). The former did not adapt to addition of attractant, and the latter adapted slowly. Both subpopulations responded to negative stimuli. About half of the bacteria grown from a single cell were in each subpopulation.

We suspect that the subpopulations in the *cheC* mutant are due to differential proteolysis of some chemotaxis protein, much like the CheB methylesterase in the *cheR* mutant. One candidate might be CheV. Loss of CheV would lead to poor phosphorylation of CheB and hence to defective adaptation (Rosario et al., 1994). Further work will be required to explore for deficiency in some chemotaxis protein that might lead to the phenotype of two subpopulations in a *cheC* mutant.

An additional puzzling result in the course of these experiments was the unusual complementation of mutants in *cheC*. A *cheC* mutant was complemented by expression of both *cheC* and *cheD* on a plasmid but not by *cheC* alone. However, when *cheC* was expressed in single copy at the *amyE* locus, complementation of the *cheC* mutant *did* occur. Moreover, expression of *cheC* alone does not itself cause deleterious effects since expression of *cheC* from a plasmid in wild-type does not interfere with taxis (data not shown). At this point, we have to say that there is no good evidence for interaction of CheC and CheD and that the failure of complementation when *cheC* was on the plasmid must be due to an artifact. Of course, both CheC and CheD obviously intimately interact with MCPs and may in fact interact with each other; future experiments will have to determine this.

Perhaps the most striking characteristic of CheC is its ability to interfere with MCP methylation without at the same time interfering with demethylation, as shown *in vitro*. We imagine that the poor adaptation in the *cheC* mutant reflects the overmethylation of the MCPs, presumably a result of a lack of interference with CheR activity. Adaptation of this mutant to negative stimuli, at least to addition of indole and removal of azetidine-2-carboxylic acid, is normal. Thus, the primary problem with the *cheC* mutant is poor adaptation to positive stimuli. It is not altogether clear why CheC interferes with CheR; it is reasonable to speculate that the purpose is to regulate CheR. One possibility is that CheC might help regulate tumbling frequency by preventing CheR from overmethylating the MCPs and hence overactivating CheA, so that the bacteria remain random. In effect, the bacteria are kept in an optimum state to respond to chemotactic stimuli that causes enhanced CW (negative stimuli) or CCW (positive stimuli) flagellar rotation. The fact that absence of CheC leads to overmethylation and a high smooth swimming bias gives some support to this idea. It may be that CheC regulation is governed by some factor, like CheY-P, which would constitute a means of feedback from the state of the motor. In this conception, CheY-P would interact with CheC and cause it to inhibit CheR. In time, the MCP methylation would fall and, afterward, CheA-P and then CheY-P concentrations would also fall. Experiments are in progress to investigate these possibilities.

[2] M. L. Kirsch, unpublished results.

Unlike the *cheC* mutant, the *cheD* mutant is very tumbly and does not respond to addition or removal of attractant; it is comparable to a *cheA* null mutant.[2] In addition, its MCPs H1 and H2 are unmethylated. The chemotactic phenotype of this mutant is very interesting. In capillary assays, it is defective in taxis to each amino acid and to a number of sugars. However, it swarms even better than wild-type on a tryptone swarm plate. It might be that CheD is required for some but not all amino acid taxes and that the poor accumulations in the capillary assays might reflect the strong tumbly bias in the *cheD* mutant. The *cheA* mutant has a similar bias and also accumulates poorly in capillary assays but, by contrast, swarms poorly on tryptone swarm plates. The tumbly bias of the *cheD* mutant might, in fact, enhance swarming by helping to keep the bacteria from getting stuck in the agar.

The interesting phenotype of the *cheD* mutant is not limited to its tumbly behavior and lack of excitability. Remarkably, the CheR methyltransferase requires CheD to methylate some of the MCPs. This is the only instance we are aware of where CheR alone did not suffice for MCP methylation. We presume that CheD is required to put these MCPs in a conformation that makes them now subject to methylation by CheR. It also seems likely that this conformation is necessary for interaction with CheA and, in strains lacking CheD, the activation of CheA might be very poor. Thus, the bacteria would be tumbly and hard to excite. Under the proper circumstances, however, excitation does occur since swarming on tryptone swarm plates occurs very well.

In general, it seems to be true that phosphorylation reactions bring about excitation and methylation reactions bring about adaptation. CheD obviously plays an important role in methylation, probably by facilitating and, one might surmise, regulating methylation of some of the MCPs. One unique feature of *B. subtilis* chemotaxis is that methyl transfer may occur between the MCPs and a yet unidentified methyl acceptor (Thoelke et al., 1989). Presumably, adaptation is the purpose of that transfer. It cannot be excitation, for strains lacking CheB, which is required for this transfer (Kirsch et al., 1993b), excite normally. Were the methyl group to be transferred to CheD and, as the result, were CheD to dissociate from a complex that included MCPs and CheA, that dissociation would cause a strong reduction of CheA activity and might help bring about adaptation. Experiments are currently underway to test this possibility.

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