For the Gram-positive organism *Bacillus subtilis*, chemotaxis to the attractant asparagine is mediated by the chemoreceptor McpB. In this study, we show that rapid net demethylation of *B. subtilis* McpB results in the immediate production of methanol, presumably due to the action of CheB. We also show that net demethylation of McpB occurs upon both addition and removal of asparagine. After each demethylation event, McpB is remethylated to nearly prestimulus levels. Both remethylation events are attributable to CheR using S-adenosylmethionine as a substrate. Therefore, no methyl transfer to an intermediate carrier need be postulated to occur during chemotaxis in *B. subtilis* as was previously suggested. Furthermore, we show that the remethylation of asparagine-bound McpB requires the response regulator, CheY-P, suggesting that CheY-P acts in a feedback mechanism to facilitate adaptation to positive stimuli during chemotaxis in *B. subtilis*. This hypothesis is supported by two observations: a *cheRBCD* mutant is capable of transient excitation and subsequent oscillations that bring the flagellar rotational bias below the prestimulus value in the tethered cell assay, and the *cheRBCD* mutant is capable of swimming in a Tryptone swarm plate.

Chemotaxis is the process by which bacteria sense their chemical environment and migrate toward more favorable conditions. In *Bacillus subtilis*, chemotaxis toward the attractant asparagine has been shown to be mediated by the methyl-accepting chemotaxis protein McpB (1). When asparagine is added to membranes containing McpB in vitro, the rate of autophosphorylation of the CheA autokinase increases (2). The phosphorylated form of CheA transfers a phosphoryl group to CheY to produce CheY-P (2, 3), which then interacts with switch proteins to cause CCW rotation of the flagella, resulting in smooth swimming behavior (3). CheA-P also donates phosphoryl groups to CheB, which thereby becomes activated to demethylate the MCPs and produce methanol (4, 5). Methylation of the MCPs is known to occur on glutamate side chains (6) through the action of CheR, the chemotactic methyltransferase, which utilizes AdoMet as a substrate (7).

The *B. subtilis* chemotactic machinery also includes CheW, CheC, CheD, and CheV. CheW and CheV are thought to couple CheA activity to the MCPs (8–11). CheC inhibits methylation of the MCPs by an unknown mechanism but does not interfere with the methylesterase, CheB (12, 13). CheD is required to produce a normal prestimulus bias, normal methylation, and azetidine-2-COOH-induced activation of CheA in vivo (12). How these proteins interact to regulate the chemotactic response in *B. subtilis* remains unknown.

The chemotaxis system in *Escherichia coli* has been well characterized and has served as a paradigm for our studies (for reviews, see Refs. 14–17). The *E. coli* system includes homologs of the MCPs, CheA, CheB, CheR, CheW, and CheY. The *E. coli* system also includes CheZ, which facilitates dephosphorylation of CheY-P (18–21), but does not include homologs to CheC, CheD, or CheV. Thus, the *E. coli* and *B. subtilis* chemotactic mechanisms must differ. Indeed, in *E. coli* repellent stimulation is thought to increase CheA activity (18, 22, 24), and CheY-P is thought to interact with the flagellar switch to cause tumbling (25–27).

Regulation of methylation during chemotaxis has been shown to be more complex in *B. subtilis* than in *E. coli*. Both addition and removal of all amino acid attractants result in methanol production (28), as was previously shown to be the case for aspartate (29) and alanine (30). Methanol formation during chemotaxis in *E. coli*, however, increases in response to repellent stimuli and decreases in response to attractant stimuli (31, 32). Second, in *B. subtilis* it has been hypothesized that an acceptor may receive methyl groups from the MCPs during a period of increased turnover due to attractant addition (29, 33–37). Finally, CheC and CheD affect receptor methylation and behavior in *B. subtilis* (12, 13), whereas no homologs to these proteins exist in *E. coli*.

In this study, we have tested the methyl transfer hypothesis in *B. subtilis* by examining asparagine-induced methylation changes on McpB. Our results demonstrate that no methyl group transfer need be postulated to occur during chemotaxis to asparagine in *B. subtilis*. The observed methanol production in response to addition and removal of asparagine (28) is due to two independent demethylation events on McpB catalyzed by CheB-P. After each demethylation event, McpB is remethylated by CheR using AdoMet as a substrate. We also show that remethylation of asparagine-bound McpB requires the response regulator CheY-P, suggesting that a feedback mechanism may exist to bring about adaptation to positive stimuli. This hypothesis is supported by behavioral data showing transient excitation and subsequent oscillation in a *cheRBCD* mutant, which lacks all proteins previously thought to be involved in adaptation during chemotaxis in *B. subtilis*.
Che-dependent Methylation of B. subtilis McpB

TABLE I

| Strain | Relevant genotype | Ref. |
|--------|-------------------|------|
| PS29  | trpC2, unkU29::spc | 44   |
| OI2057| cheY             | 43   |
| OI2680| cheR3::cat       | 45   |
| OI2715| cheB7            | 5    |
| OI2952| cheY54DA         | 3    |
| OI3180| ΔmcpA mcpB tlpA tlpB::101::cat | This work |
| OI3184| ΔmcpA mcpB tlpA tlpB::101::cat amyE5719::mcpB | This work |
| OI3280| mcpC4::erm      | 42   |
| OI3289| ΔcheY8 ΔmcpA mcpB tlpA tlpB::101::cat | This work |
| OI3290| ΔmcpA mcpB tlpA tlpB::101::cat amyE5719::mcpB | This work |
| OI3292| ΔcheY8 ΔmcpA mcpB tlpA tlpB::101::cat amyE5719::mcpB mcpC4::erm | This work |
| OI3349| cheB7 unkU29::spc | This work |
| OI3375| cheB7 unkU29::spc ΔcheC ched501::cat | This work |
| OI3377| cheB7 unkU29::spc ΔcheC ched501::cat cheR3::cat | This work |

*All mutants in this study are derivatives of the parent strain OI1085 (che*, itcC1 leu-1 trpF7 his2 metC1) (39), except strain PS29.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—All bacterial strains and plasmids used in this study are listed in Tables I and II, respectively. All plasmids were propagated in E. coli strain TG-1 (Amer sham Pharmacia Biotech).

Chemicals—[methyl-3H]Methionine (80–85 Ci/mmol) was obtained from Amer sham Pharmacia Biotech. All other chemicals were of reagent grade.

Solutions and Growth Media—Luria-Bertani (LB) medium is 1% tryptone, 0.5% yeast extract, and 1% NaCl. Tryptone broth is 1% tryptone and 0.5% NaCl. Chemotaxis buffer (CB) is 0.1 mM EDTA, 50 mM CaCl2, 0.05% glycerol, 5 mM sodium lactate, 0.3 mM ammonium sulfate, and 20 mM potassium phosphate, pH 7.0 (38). Minimum medium is 50 mM potassium phosphate, 1 mM (NH4)2SO4, 1.2 mM MgCl2, 140 mM CaCl2, 10 mM MnCl2, 50 μg/ml required amino acids, and 20 mM sorbitol, pH 7.0 (38). Protoplasting buffer is 20% sucrose, 25 mM potassium phosphate, 10 mM MgCl2, 30 mM sodium lactate, and 1.0 mM EDTA, pH 7.0 (38).

Construction of an amyE Integration Plasmid Carrying mcpB—A 2.8-kb BglII-XhoI fragment containing mcpB was subcloned from pDW12 (1) into pBlueScript (SK-) at the BamHI-XhoI sites to create pUNK101. Subsequently, a 2.8-kb Smal-XhoI fragment containing mcpB from pUNK101 was subcloned into the SmalI-XhoI sites of pHL007, which is a derivative of the amyE integration plasmid, PAC7 (40), and contains a kanamycin resistance marker. The resulting plasmid (pUNK200) carries mcpB under the control of its natural promoter.

Construction of McpB Expressing Strains—Four mcpl homologs (mcpB, tlpA, mcpA, and tlpB) identified by Hanlon and Ordal (1) are located at 279° on the B. subtilis chromosome. A plasmid was constructed such that the 5' and 3' end of mcpB and the 5' and 3' end of tlpB were separated by a cat cassette to create pMR134. Plasmid pMR134 was linearized and crossed onto the chromosome of the B. subtilis wild-type strain (OI1085). KmR transformants were selected, thereby creating strain OI3180, which has the four mcpB homologs deleted.4 Integration of mcpB into the amyE locus of OI3180 was achieved using linearized pUNK200 to create strain OI3184. KanR transformants were selected, and potential amyE integrants were screened by the starch hydrolysis test. Colonies grown on tryptose blood agar base with 0.2% starch were flooded with Mordant’s solution: 0.294 I2, 0.67% KI in H2O. Integrants, negative for starch hydrolysis, do not form clear zones around the colonies (41). Chromosomal DNA from OI3280 (mcpC4::erm; 42) was then used to transform OI1834 cells, and erythromycin-resistant transformants were selected to generate strain OI3290. OI3290 cells (CmR EmR Amy- ) were shown to express only one band that cross-reacts with anti-McpB antibody,4 and it produces only one methylatable protein that migrates to the expected position in a 10% SDS-polyacrylamide gel, based on the mcpB1::cat phenotype observed by Hanlon and Ordal (1).

Strain OI3294 was constructed in a manner similar to strain OI3290. A strain deleted for cheY (OI2057; 43) was transformed with chromosomal DNA from strain OI3180 to generate strain OI3289 (CmR). This strain was then transformed with linearized pUNK200, selected for KanR, and screened for integration at the amyE locus by the starch hydrolysis assay to create strain OI3292. OI3292 cells (CmR Amy-) were then transformed with chromosomal DNA from strain OI2820 (mcpC4::erm; 42). EmR transformants were selected, and the resulting strain (OI3294) was tested to verify that it produces only one methylated protein at the position to which McpB is expected to migrate, as described above.

Construction of the cheRBCD Mutant—A plasmid was designed to delete the adjacent B. subtilis genes, cheC and cheD. A partially digested 1.8-kb PstI-EcoRI fragment containing cheD and the 5' end of sigD (pMR116)4 was cloned into the PstI-EcoRI sites of pUC18, thereby creating plasmid pAIN500. The 1.8-kb fragment carried an EcoRI site, introduced by site-directed mutagenesis, that is internal to cheD and approximately 8 codons from the normal stop codon. A 1.2-kb PstI-EcoRI fragment from pMR105, containing the 5' end of cheC and a cat cassette (presented in pMR105 containing the EcoRI site internal to cheD. The resulting plasmid (pAIN501) contained a cat cassette flanked by the 5' end of cheC and the 3' end of cheD.

Chromosomal DNA from strain PS29 (44) was used to transform a cheB mutant (O12715) (5) to spectinomycin resistance at a locus immediately upstream of the fla/che operon to create strain OI3349. Linearized pAIN501 was used to transform OI3349 cells. CmR SpcR colonies were selected, and the resulting strain was called OI3357 (cheBCD). The generalized transducing phage PBS1 was grown on OI3375 cells, and the resulting lysate was used to transduce a cheR3::cat strain (O12680) (45), as described previously (46). The resulting cheRBCD mutant was designated strain OI3377 (SpcR CmR). OI3377 SpcR transductants were then back-crossed into strain OI1085 to confirm linkage between the spectinomycin marker and the cat cassette located in the fla/che operon. In an independent assay, the cotransduction frequency between the SpcR marker and the fla/che operon was determined to be 94%.

Pulse Label Methylation—In vivo pulse label methylation experiments were performed as described by Ullah and Ordal (39), with the following changes: cells were grown to 180 Klett units (red filter) in LB, washed three times in CB with 250 μg/ml chloramphenicol, and resuspended at an A600 = 1.0 in the appropriate volume of either CB or protoplast buffer with chloramphenicol. The suspension was then incubated at 37°C with moderate shaking (120 rpm). Methylation was initiated by addition of 50 μCi/ml [3H]methionine (0.6 μM). At the appropriate times, 1.0-ml samples were removed from flasks and frozen in a dry ice/acetone bath. Samples were then thawed and centrifuged at 3000 × g for 30 min at 4°C. The supernatant was either assayed for volatile labeled product ([3H]methanol) or discarded, and the protoplast membranes were resuspended in 100 μl of 4× SDS solubilizer. The samples were then boiled for 7 min and electrophoresed at 15 Vcm on 10% SDS-polyacrylamide gels, pH 8.8 (47). The gels were then treated for fluorography as described previously (48).

steady-state Methylation—For in vivo steady-state methylation reactions, cells were treated as above except that methylation was initiated with 50 μCi/ml of 10 μM [3H]methionine. At the indicated times, 1.0-ml aliquots were frozen in dry ice/acetone baths. For assays requiring the removal of attractant, whole cells were washed by filtration using 0.45-μm-pore cellulose-acetate filters. The cells were resuspended in CB at volumes equal to those before filtration and frozen in dry ice/acetone at the times indicated. After thawing at 4°C, an equal

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3 H. Lu and G. W. Ordal, unpublished data.
4 M. M. L. Rosario and G. W. Ordal, unpublished data.
5 Kirby, J. R., Niewold, T. B., and Ordal, G. W., submitted for publication.
CheY-dependent Methylation of B. subtilis McpB

TABLE II
Plasmids and phage used in this study

| Plasmid or phage | Description | Ref. |
|------------------|-------------|-----|
| PBS1             | B. subtilis generalized transducing phage | 65 |
| pUC18            | Amp<sup>R</sup> | Life Technologies, Inc. |
| pBluescript SK   | Amp<sup>R</sup> | Stratagene |
| pMR134           | 3.7-kb BamHI-EcoRI fragment containing the 5’-end of tlpB (100 bp) in pBluescript, Amp<sup>R</sup>, Cm<sup>R</sup> | Footnote 4 |
| pAC7             | amylE integration plasmid, Amp<sup>R</sup>, Kan<sup>R</sup> | 40 |
| pDB67            | amylE integration plasmid, Amp<sup>R</sup>, Cm<sup>R</sup> | 66 |
| pHLO07           | 1.7-kb EcoRI-BamHI fragment from pDR67 subcloned into EcoRI-BamHI of pAC7, Amp<sup>R</sup>, Kan<sup>R</sup> | Footnote 3 |
| pDW12            | 3.1-kb ClaI fragment containing mcpB in M13mp18 | 1 |
| pUNK101          | 2.9-kb BglII-XhoI fragment from pDW12 subcloned into BamHI-XhoI of pBluescript, Amp<sup>R</sup> | This work |
| pUNK200          | 2.0-kb Smal-XbaI fragment from pUNK101 subcloned into Smal-XbaI of pHLO07, Amp<sup>R</sup>, Kan<sup>R</sup> | This work |
| pMR105           | 2.1-kb PstI-SmaI fragment containing cheW and cheC:cat in M13mp19 | 12 |
| pMR116           | 1.9-kb PstI-EcoRI fragment containing cheW, cheC, cheD, and sigD in M13mp19 | Footnote 4 |
| pAIN500          | 1.8-kb PstI-EcoRI fragment from pMR116 subcloned into PstI-EcoRI of pUC18, Amp<sup>R</sup> | This work |
| pAIN501          | 1.2-kb PstI-EcoRI fragment from pMR105 subcloned into PstI-EcoRI sites of pAIN500, Amp<sup>R</sup>, Kan<sup>R</sup> | This work |

RESULTS

Relationship between McpB Demethylation and Methanol Production—Previous experiments indicated that addition of the attractant aspartate to wild-type B. subtilis (OI1085) caused an immediate (within 24 s) loss of labeled methyl groups from the MCPs under pulse-chase conditions (34). Under the same experimental conditions, methanol production from OI1085 cells in response to aspartate stimulation was observed to reach a maximum after 60 s (34). Those results, however, were obtained from analysis of separate trials, and direct comparison of those results may not have been reliable. Nevertheless, the apparent delay in methanol production relative to methylation changes on the MCPs supported the hypothesis that methyl groups were transferred to a stable intermediate methyl carrier from which methanol was subsequently released (29, 33). That mechanism was proposed to account for the production of methanol that has been observed in response to both positive and negative stimulation for all amino acids in B. subtilis (28–30).

Recent experiments have shown that B. subtilis McpB is required for methanol production in response to addition or removal of asparagine (28). If methyl transfer occurs upon asparagine stimulation, then a delay between McpB demethylation and methanol production might be apparent. However, if no methyl transfer occurs, or if methyl transfer is rapid relative to methanol release, then methanol production and demethylation of McpB would appear to be coincident when asparagine is added to the cells. In order to examine the relationship between methanol production and demethylation of McpB, a time course of methanol production and McpB demethylation was examined in an McpB-expressing strain (OI3184) that lacks McpA, TlpA, and TlpB.

An in vivo pulse label assay was performed such that [3H]methanol produced by the labeled OI3184 protoplasts would be released into the surrounding medium and frozen at the various time points of the experiment. After thawing the aliquots at 4 °C, the supernatant was assayed for (volatile)
CheY-dependent Methylation of B. subtilis McpB

Addition of 0.5 mM asparagine induced rapid net demethylation of McpB. The first time point was taken 5 s after addition of asparagine, by which time a net change in methylation had already taken place. A decrease of approximately 50% in the total level of methylation prior to stimulation occurred and was accompanied by the appearance of a more slowly migrating, presumably less methylated, species (Fig. 2B). The rapid net demethylation was similar to that observed in response to repellent stimulation in E. coli (53). Following this initial demethylation of McpB, however, both methylated species were gradually remethylated such that the total level of methylation returned to near prestimulus levels. This remethylation occurred even in the continued presence of the attractant. By contrast, in E. coli, the net demethylation due to repellent addition persists for at least 30 min (53). Because the data support the hypothesis that methyl groups are released directly as methanol (Fig. 1), the poststimulus remethylation of McpB could be catalyzed by the CheR methyltransferase using AdoMet as a substrate (7).

Effect of Removal of Asparagine on McpB Methylation—Addition and removal of attractants and repellents have opposite effects on the behavior of both B. subtilis and E. coli in tethered cell assays (5, 45, 54). Furthermore, positive and negative stimuli have opposite effects on the final methylation state of the receptors in E. coli (53). Therefore, we hypothesized that removal of asparagine might lead to a transient increase in the overall methylation level of McpB followed by a gradual decrease in the methylation state of the receptor.

However, it is apparent that the same effect occurs upon removal of attractant as upon addition of attractant (Fig. 2A). Both addition and removal of 0.5 mM asparagine induced rapid net demethylation of McpB. The first time point taken after removal of asparagine was 30 s, by which time demethylation was already complete. Again, the extent of demethylation was approximately 50%. After this rapid demethylation, gradual remethylation of McpB occurred over a period of several minutes, as indicated by the disappearance of the more slowly migrating species (Fig. 2C).

To verify that the apparent changes in methylation of McpB are due to net methylation changes and not a net loss of protein, Western blots were performed on the samples over the time course of the experiment shown in Fig. 2. The results
Effect of a cheY Mutation on Methylation of McpB—Recently, it has been shown that methanol production upon removal of asparagine in the continuous flow assay requires the response regulator CheY (28). Because methanol production after removal of attractant can be attributed to a second demethylation event, we hypothesized that a cheY knockout mutation would prevent demethylation of McpB caused by removal of asparagine. A derivative (strain OI3294) of strain OI3290 containing a deletion in the cheY gene was made to test this possibility. The cheY mutation did not significantly alter the extent of initial demethylation of McpB after asparagine addition (Fig. 3). However, remethylation of McpB did not occur when CheY was absent. After removal of asparagine, however, remethylation of McpB occurred over a time course similar to that of strain OI3290 (Figs. 2 and 3). The second demethylation event, which produces methanol when the attractant is removed (Fig. 2), cannot occur in the cheY mutant because remethylation of asparagine-bound McpB does not occur. It should be noted that this behavior in the B. subtilis cheY mutant is similar to that seen in wild-type E. coli upon repellent addition and removal (54). It is also reciprocally related to the results obtained in E. coli upon attractant addition and subsequent removal (53, 54).

Close inspection of the time course of methylation in the cheY mutant indicated that maximal demethylation of McpB was not reached until several minutes after asparagine was added (compare Figs. 2A and 3A). Likewise, the more slowly migrating, presumably less methylated species of McpB only gradually became apparent in strain OI3294, in contrast to the immediate formation of that band in strain OI3290 (compare Figs. 2B and 3B). Thus, CheY accelerates overall methylation changes on McpB by an unknown mechanism.

Effect of the cheY54DA Mutation on Methanol Production—Although CheY is clearly required for the remethylation of asparagine-bound McpB, the assay described above did not indicate that both methylated species cross-react with anti-McpB antibody and that the total amount of protein was the same before and after addition of attractant (data not shown). Anti-McpB antibody does not cross-react with any other protein that comigrates with McpB under the conditions tested. Thus, all methylation changes on McpB can be attributed to net methylation of McpB and not to any loss of protein.

Together, these results provide an explanation for the previous observations that methanol is produced by B. subtilis in response to all stimuli and that the MCPs are relabeled when attractant stimuli are removed (29). No reversible methyl transfer need be postulated to occur during chemotaxis in B. subtilis, in contrast to what was previously hypothesized.

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Together, these results provide an explanation for the previous observations that methanol is produced by B. subtilis in response to all stimuli and that the MCPs are relabeled when attractant stimuli are removed (29). No reversible methyl transfer need be postulated to occur during chemotaxis in B. subtilis, in contrast to what was previously hypothesized.
differentiate between a requirement for the phosphorylated or unphosphorylated form of CheY. In order to determine which form of CheY is required, a strain (OI2952) (3) containing a point mutation (cheY54DA) that renders CheY incapable of being phosphorylated was assayed for its ability to produce methanol. Because methanol production upon asparagine addition and removal can be attributed to the demethylation of McpB, the continuous flow assay for methanol production can be used to track both the demethylation and remethylation events. Strain OI2952 was capable of producing methanol upon addition of asparagine but could not produce methanol when the asparagine was removed (Fig. 4A). The response by strain OI1085 (wild-type for chemotaxis) is shown for comparison (Fig. 4B). Presumably, the alanine substitution simply prevents the mutant CheY54DA protein from becoming phosphorylated and does not create any other significant conformational defects. Therefore, the result of this assay allows us to conclude that the remethylation of asparagine-bound McpB (Fig. 2) specifically requires CheY-P.

Behavior of a cheRBCD Mutant—B. subtilis proteins thought to be involved in adaptation include the methyltransferase (CheR), the methylesterase (CheB), CheC, and CheD (5, 12, 13, 45). The results described above suggest that CheY-P may interact with asparagine-bound McpB, either directly or indirectly. Increased levels of CheY-P after asparagine stimulation could affect the conformation of the signaling complex to lower the level of CheA autophosphorylation, thereby promoting adaptation. To test this hypothesis, a cheRBCD mutant was constructed for behavioral analysis. If CheY-P does not facilitate adaptation and adaptive methylation no longer exists, then the cheRBCD mutant should become 100% CCW (smooth swimming) upon asparagine stimulation and remain smooth swimming. However, if CheY-P can promote adaptation independently of changes in methylation, then we would observe a diminution of CCW flagellar bias after the initial excitation.

The results show that the cheRBCD mutant excites transiently and undergoes subsequent oscillations (Fig. 5A). In contrast, wild-type cells (OI1085), after transiently increasing their CCW bias in response to attractant stimulation, return to their prestimulus bias (about 60% CCW) within 60 s and maintain that bias in the presence of the attractant (Fig. 5B) (3, 5). It should be noted that cheY mutant cells (OII057) are completely tumby (0% CCW) and incapable of increasing their CCW flagellar bias in response to attractant stimulation (3). Thus, the cheRBCD mutant cells, although capable of responding to attractant stimuli, do not return to their prestimulus CCW bias nor maintain a stable, adapted state in the presence of an attractant. After the asparagine is removed from the cheRBCD mutant cells, the oscillation immediately ceases. Why the post-stimulus CCW bias of the mutant remains low relative to its prestimulus level is unknown. However, recent evidence indicates that the flagellar bias of the cheRBCD mutant gradually returns to its prestimulus level over a period of 10–15 min following removal of asparagine.6

Previous behavioral analysis of Salmonella typhimurium cells by Spudich and Koshland (55) demonstrated that cells within a population exhibit considerable individualistic variation. Likewise, our study found that there is considerable heterogeneity among all cells analyzed in the tethered cell assay. Overall, two-thirds of the cheRBCD mutant cells show the oscillating phenotype, and one-third give partial adaptation. These results are highly reproducible. For Fig. 5A three cells were averaged, all within one visual field of our tracking device. Other fields showed similar results. The cells within any given visual field were observed to oscillate synchronously with a relatively long time constant. However, because the exact time when attractant reaches the cells for each field is not known, summation of several fields was not possible without introducing phase variation that smoothes the data. Thus, the fact that a number of these B. subtilis mutant cells show synchronized oscillations implies that a feedback system not requiring CheR, CheB, CheC, or CheD exists. Although other proteins involved in adaptation may exist, these results suggest that the decrease in CCW rotation that follows the initial excitation may be due to CheY-P feedback that results in lowering the rate of CheA autophosphorylation. As a result, CheY-P levels would decrease and feedback inhibition would cease. Because asparagine is still bound to McpB, CheA autophosphorylation would then increase to produce increased levels of CheY-P, allowing the cycle to repeat, thereby producing the observed oscillation (Fig. 5A).

Swarm Plate Analysis for Chemotaxis by the cheRBCD Mutant—In order to test the efficiency of the putative CheY-P
feedback mechanism, the cheRBCD mutant was analyzed for its ability to swarm in a Tryptone swarm plate. The results show that the cheY mutant (OI2057) is unable to produce a swarm in Tryptone semi-solid agar, whereas both the wild-type (OI1085) and the cheRBCD mutant (OI3377) strains are capable of swarming in a Tryptone swarm plate (Fig. 6). Although the possibility remains that adaptation may not be required to produce effective swarming under the conditions tested, we do not believe such a well defined ring, the same size as wild-type, could form without a mechanism for adaptation by the cheRBCD mutant. Furthermore, the swarm cannot be due to oxygen taxis because the swarm diameter is equal through the depth of the agar. Oxygen taxis rings are characteristically larger at the base of the swarm under the conditions tested (56). Thus, we conclude that an adaptational mechanism exists in the cheRBCD mutant that facilitates chemotactic swarming by B. subtilis and that this mechanism may involve feedback of CheY-P onto asparagine-bound McpB. The exact mechanism by which adaptation would be produced remains unknown.

**DISCUSSION**

In this study, we produce evidence that methanol is released directly from the MCPs (Fig. 1), a process known to be mediated through the action of the methylesterase, CheB-P (4, 5). We also show that both addition and removal of asparagine result in transient net demethylation of McpB (Fig. 2). Following both demethylation events, McpB is remethylated (Fig. 2) presumably by CheR using AdoMet as a substrate (7). Together, these results provide an explanation for the previous observations that the MCPs are relabeled upon removal of aspartate and methanol production occurs for both addition and removal of aspartate (29). Thus, no methyl transfer is necessary to account for the observations reported in this study or the previous findings of Thoelke et al. (29). Because it has recently been shown that all 20 amino acid attractants for B. subtilis induce methanol production in the continuous flow assay both upon their addition and removal (28), it is likely that all 20 amino acids induce net demethylation upon binding and release from their receptors. Each demethylation event should be followed by remethylation of the receptor.

We also show that the inability of the cheY null mutant to remethylate asparagine-bound McpB (Fig. 3) accounts for the lack of methanol production in response to asparagine removal from the cheY mutant during the continuous flow assay (28). Methanol production after asparagine removal is also inhibited in cells containing the unphosphorylatable CheY54DA mutant protein (Fig. 4). These results allow us to conclude that remethylation of asparagine-bound McpB requires CheY-P, leading to the hypothesis that CheY-P may participate in a feedback mechanism that promotes adaptation to positive stimuli during chemotaxis in B. subtilis. This hypothesis is supported by the observations that the cheRBCD mutant exhibits transient excitation and subsequent oscillation in the tethered cell assay and that the cheRBCD mutant is capable of swarming in a

**Fig. 5. Behavior of cheRBCD and wild-type tethered cells in response to asparagine stimulation.** The assay was performed as described under “Experimental Procedures.” The downwardly directed arrow indicates the time of addition of 56 μM asparagine. The upwardly directed arrow indicates the time of removal of same. A, the rotational data for 3 cheRBCD mutant cells (strain OI3377) from one visual field were averaged to generate the plot shown. Phase variation between fields prevented summation of several fields. B, the rotational data for 15 wild-type cells (strain OI1085) were averaged to generate the plot shown.

**Fig. 6. Swarm plate assay of a cheRBCD mutant.** The assay was performed as described under “Experimental Procedures.” The plate was inoculated with (clockwise from top) strains OI1085 (wild-type), OI2057 (ΔcheY), and OI3377 (cheRBCD). The Tryptone swarm plate was incubated at 37 °C for 4 h.
Tryptone swarm plate.

The cheRBCD mutant lacks all proteins thought to be involved in adaptation during chemotaxis in B. subtilis (5, 12, 13, 45). Although the possibility remains that other chemotaxis proteins involved in adaptation may exist, there is no evidence to suggest that is the case. No sequence similar to E. coli CheZ has been found in a search of the B. subtilis genome data base in GenBank™. Thus, the ability of the majority of the cheRBCD mutant cells to exhibit transient excitation in an oscillating fashion supports the hypothesis that CheY-P may interact with asparagine-bound McpB to inhibit CheA autophosphorylation. If McpB possesses CheZ-like activity, it might influence the dephosphorylation of CheY-P, and the system could be “re-excited,” as was observed (Fig. 5A). How this potential feedback mechanism would allow the cheRBCD mutant to swarm in a Tryptone swarm plate is unknown (Fig. 6). Furthermore, the nutrient(s) upon which the cells feed and toward which they subsequently migrate in Tryptone swarm plates is unknown, and thus the specific receptor or group of receptors mediating this behavior is also unknown. Nevertheless, it is apparent that the cheRBCD mutant possesses a mechanism that allows for efficient swarming (Fig. 6). One possibility is that the oscillations observed in the tethered cell assay may not occur for the cheRBCD cells in Tryptone swarm plates, and thus, a putative feedback mechanism may result in partial adaptation that allows for chemotactic swarming.

In strain DS280, McpB released methanol upon asparagine stimulation, and the protein immediately appeared as a more slowly migrating, less methylated species (Fig. 2B). In contrast, in the cheY mutant, a more slowly migrating, less methylated species became apparent only several minutes after asparagine stimulation (Fig. 3B). If in the cheY mutant, CheB were phosphorylated to a greater extent, then the more slowly migrating, less methylated form of McpB would be expected to appear more rapidly. Because the band emerged less rapidly instead, we conclude that CheY-P interaction with asparagine-bound McpB facilitates a conformational change influencing these methylation changes in addition to lowering the CheA autophosphorylation rate. The possibility remains, however, that the inability to remethylate the ligand-bound receptor in the cheY mutant (Fig. 3B) may be due to a relative increase in CheB-P levels, thereby preventing a net increase in methylation by CheR. Nevertheless, we speculate that these methylation changes are required for generating the adapted state in the wild-type strain (Fig. 5B), a state that is not generated in the cheRBCD mutant (Fig. 5A).

How could CheY-P feedback influence both CheA autophosphorylation and methylation of asparagine-bound McpB? Methylation of chemotactic receptors in E. coli is thought to affect the interactions between the methylated helices of the MCPs such that CheA activation is enhanced by a more highly methylated receptor (57–62). Because a B. subtilis cheR mutant has a lower prestimulus flagellar rotational bias (reflecting lower CheA activity), we conclude that enhanced CheA activity results from increased methylation for B. subtilis (45). Although enhanced CheA activity leads to tumbling in E. coli (22–27) and smooth swimming in B. subtilis (2, 3), it appears that enhanced CheA activity results from increased methylation for both organisms. In B. subtilis, we speculate that CheY-P interaction with asparagine-bound McpB could affect the topology of the C terminus of the receptor such that CheB-P has access to an otherwise less accessible methylated residue (Fig. 7). The demethylated, asparagine-bound receptor would presumably promote a lower rate of CheA autophosphorylation, thereby generating an adapted state.

The data from this study allow us to account for the unusual changes in methylation of the MCPs and methanol production previously observed in B. subtilis (29). Feedback by CheY-P onto asparagine-bound McpB would provide a simple mechanism to regulate the activity of the sensor kinase, CheA, while also affecting methylation in an unknown way. If CheY-P interacts with McpB, this would be the first example of a response regulator that has two targets (Fig. 7): one upstream of the sensor kinase (asparagine-bound McpB) and one downstream (the flagellar switch) (see Ref. 63 and references therein). Because the archaeal species Halobacterium salinarum, like B. subtilis, produces methanol in response to all stimuli (28, 64), it is possible that CheY-P feedback onto the receptors

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**Fig. 7. Model for CheY-P feedback on asparagine-bound McpB.** This model depicts events leading to an adapted state for ligand-bound McpB. Prior to stimulation, McpB is methylated (Me) at one or more of several sites (solid black lines). Following addition of asparagine, the receptor complex is active, leading to increased production of CheY-P and, therefore, smooth swimming behavior in B. subtilis. Feedback by CheY-P onto the receptor (orange dashed arrow) is thought to alter the conformation of the ligand-bound receptor and results in the following: 1) the rate of CheA autophosphorylation is transiently lowered, thereby promoting adaptation to a positive stimulus, and 2) CheB-P gains accessibility to a methylated residue (black triangle), enhancing demethylation at that site. In the cheY mutant, demethylation of this residue occurs more slowly, as indicated by the more gradual appearance of the more slowly migrating form of McpB (Fig. 3). Furthermore, in the absence of methylation changes in the cheRBCD mutant, oscillation between the active and transiently inactive states would occur due to spontaneous or receptor-enhanced hydrolysis of CheY-P. The brackets around the active and transiently inactive states indicate that CheB-P can remove methyl groups from either form of the receptor complex. Demethylation of ligand-bound McpB would presumably create a stable adapted state, a prerequisite for chemotaxis. Methylation of ligand-bound McpB by CheR does not destroy the adapted state, as indicated by the observation that this event occurs well after behavioral adaptation has occurred. This methylation may occur at a unique site and play a role in presetting the complex to respond to the removal of the attractant (see Footnote 5).
was part of an adaptation mechanism present in a common ancestor of these two highly diverged prokaryotic organisms.

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