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CheY-dependent Methylation of the Asparagine Receptor, McpB, during Chemotaxis in *Bacillus subtilis**

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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 swarming 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[1] rotation of the flagella, resulting in smooth swimming behavior (3). CheA-P also donates phosphoryl groups to CheB,[2] 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*.

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[1] The abbreviations used are: CCW, counterclockwise; AdoMet, *S*-adenosylmethionine; *cat*, chloramphenicol acetyltransferase; MCP, methyl-accepting chemotaxis protein; Tlp, transducer-like protein; CB, chemotaxis buffer; bp, base pair(s); kb, kilobase pair(s).

[2] Garrity and Ordal, unpublished data.

TABLE I
B. subtilis strains used in this study

Strain	Relevant genotype[a]	Ref.
PS29	*trpC2, unkU29::spc*	44
OI2057	*ΔcheY8*	43
OI2680	*cheR3::cat*	45
OI2715	*cheB7*	5
OI2952	*cheY54DA*	3
OI3180	*Δ(mcpA mcpB tlpA tlpB)101::cat*	Footnote 4
OI3184	*Δ(mcpA mcpB tlpA tlpB)101::cat amyE5719::mcpB*	This work
OI3280	*mcpC4::erm*	42
OI3289	*ΔcheY8 Δ(mcpA mcpB tlpA tlpB)101::cat*	This work
OI3290	*Δ(mcpA mcpB tlpA tlpB)101::cat amyE5719::mcpB mcpC4::erm*	This work
OI3292	*ΔcheY8 Δ(mcpA mcpB tlpA tlpB)101::cat amyE5719::mcpB*	This work
OI3294	*ΔcheY8 Δ(mcpA mcpB tlpA tlpB)101::cat amyE5719::mcpB mcpC4::erm*	This work
OI3349	*cheB7 unkU29::spc*	This work
OI3375	*cheB7 unkU29::spc Δ(cheC cheD)501::cat*	This work
OI3377	*cheB7 unkU29::spc Δ(cheC cheD)501::cat cheR3::cat*	This work

[a] All mutants in this study are derivatives of the parent strain OI1085 (*che*$^+$, *ilvC1 leu-1 trpF7 hisH2 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 (Amersham Pharmacia Biotech).

Chemicals—L-[*methyl*-^{3}H]Methionine (80–85 Ci/mmol) was obtained from Amersham 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 μM $CaCl_2$, 0.05% glycerol, 5 mM sodium lactate, 0.3 mM ammonium sulfate, and 20 mM potassium phosphate, pH 7.0 (38). Minimal medium is 50 mM potassium phosphate, 1 mM $(NH_4)_2SO_4$, 1.2 mM $MgCl_2$, 140 μM $CaCl_2$, 10 μM $MnCl_2$, 50 μg/ml required amino acids, and 20 mM sorbitol, pH 7.0 (38). Protoplast buffer is 20% sucrose, 25 mM potassium phosphate, 10 mM $MgCl_2$, 30 mM sodium lactate, and 1.0 mM EDTA, pH 7.0 (39).

Construction of an amyE Integration Plasmid Carrying mcpB—A 2.8-kb *Bgl*II-*Xba*I fragment containing *mcpB* was subcloned from pDW12 (1) into pBluescript (SK$^-$) at the *Bam*HI-*Xba*I sites to create pUNK101. Subsequently, a 2.8-kb *Sma*I-*Xba*I fragment containing *mcpB* from pUNK101 was subcloned into the *Sma*I-*Xba*I sites of pHL007, which is a derivative of the *amyE* integration plasmid, pAC7 (40), and contains a kanamycin resistance marker.[3] The resulting plasmid (pUNK200) carries *mcpB* under the control of its natural promoter.

Construction of McpB Expressing Strains—Four *mcp* 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′-end of *mcpB* (about 300 bp) and 3′-end of *tlpB* (about 100 bp) 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). Cm^R transformants were selected, thereby creating strain OI3180, which has the four *mcp* homologs deleted.[4] Integration of *mcpB* into the *amyE* locus of OI3180 was achieved using linearized pUNK200 to create strain OI3184. Kan^R 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: 0.33% I_2, 0.67% KI in H_2O. 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 OI3184 cells, and erythromycin-resistant transformants were selected to generate strain OI3290. OI3290 cells (Cm^{R}Kan^{R}Em^{R}Amy$^-$) were shown to express only one band that cross-reacts with anti-McpB antibody,[5] 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 (Cm^R). This strain was then transformed with linearized pUNK200, selected for Kan^R, and screened for integration at the *amyE* locus by the starch hydrolysis assay to create strain OI3292. OI3292 cells (Cm^{R}Kan^{R}Amy$^-$) were then transformed with chromosomal DNA from strain OI3280 (*mcpC4::erm*; 42). Em^R 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 *Pst*I-*Eco*RI fragment containing *cheD* and the 5′-end of *sigD* (pMR116)[4] was cloned into the *Pst*I-*Eco*RI sites of pUC18, thereby creating plasmid pAIN500. The 1.8-kb fragment carried an *Eco*RI site, introduced by site-directed mutagenesis, that is internal to *cheD* and approximately 8 codons from the normal stop codon. A 1.2-kb *Pst*I-*Eco*RI fragment from pMR105, containing the 5′-end of *cheC* and a *cat* cassette (12), was cloned into *Pst*I-*Eco*RI sites of pAIN500 utilizing the *Eco*RI 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 (OI2715) (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. Cm^{R}Spc^R colonies were selected, and the resulting strain was called OI3375 (*cheBCD*). The generalized transducing phage PBS1 was grown on OI3375 cells, and the resulting lysate was used to transduce a *cheR3::cat* strain (OI2680) (45), as described previously (46). The resulting *cheRBCD* mutant was designated strain OI3377 (Spc^{R}Cm^R). OI3377 Spc^R 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 Spc^R marker and the *fla*/*che* operon was determined to be 94%.

Pulse Label Methylation—In vivo pulse label methylations 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 A_{600} = 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 [^{3}H]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 ([^{3}H]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 V/cm 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 [^{3}H]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

[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.

TABLE II
Plasmids and phage used in this study

Plasmid or phage	Description	Ref.
PBS1	*B. subtilis* generalized transducing phage	65
pUC18	Amp^R	Life Technologies, Inc.
pBluescript SK^-	Amp^R	Stratagene
pMR134	3.7-kb *Bam*HI-*Eco*RI fragment containing the 5′-end of *mcpB* (300 bp), *cat*, and 3′-end of *tlpB* (100 bp) in pBluescript, Amp^R, Cm^R	Footnote 4
pAC7	*amyE* integration plasmid, Amp^R, Kan^R	40
pDR67	*amyE* integration plasmid, Amp^R, Cm^R	66
pHL007	1.7-kb *Eco*RI-*Bam*HI fragment from pDR67 subcloned into *Eco*RI-*Bam*HI of pAC7, Amp^R, Kan^R, Cm^S	Footnote 3
pDW12	3.1-kb *Cla*I fragment containing *mcpB* in M13mp18	1
pUNK101	2.8-kb *Bgl*II-*Xba*I fragment from pDW12 subcloned into *Bam*HI-*Xba*I of pBluescript, Amp^R	This work
pUNK200	2.8-kb *Sma*I-*Xba*I fragment from pUNK101 subcloned into *Sma*I-*Xba*I of pHL007, Amp^R, Kan^R	This work
pMR105	2.1-kb *Pst*I-*Sma*I fragment containing *cheW* and *cheC::cat* in M13mp19	12
pMR116	1.9-kb *Pst*I-*Eco*RI fragment containing *cheW*, *cheC*, *cheD*, and *sigD* in M13mp19	Footnote 4
pAIN500	1.8-kb *Pst*I-*Eco*RI fragment from pMR116 subcloned into *Pst*I-*Eco*RI of pUC18, Amp^R	This work
pAIN501	1.2-kb *Pst*I-*Eco*RI fragment from pMR105 subcloned into *Pst*I-*Eco*RI sites of pAIN500, Amp^R, Cm^R	This work

volume of 2× protoplast buffer with 5 mg/ml lysozyme was added to each tube, and the tubes were incubated for 1 h at 4 °C. The resulting protoplasts were then centrifuged and treated as described above.

Detection of Methanol from Protoplasts—Volatile radiolabeled product was detected using a continuous flow assay based on the method described by Kehry *et al*. (32) and Thoelke *et al*. (33). Protoplasts were labeled *in vivo* as described above for 15 min. 1.0-ml aliquots were frozen in dry ice/acetone baths at the times indicated. After thawing at 4 °C, the protoplasts were pelleted by centrifugation, and 400-μl aliquots of the supernatants were placed in 0.5-ml microcentrifuge tubes (without lids). The microcentrifuge tubes containing the supernatants were then placed in scintillation vials containing 2 ml of Liquiscint mixture (National Diagnostics), allowed to equilibrate for 24 h, and counted.

Continuous Flow Assay for Methanol Production—An assay based on that described by Kehry *et al*. (32) has been described previously (33). Cells were methylated as described above. Labeled cells were loaded onto a 0.45-μm-pore cellulose-acetate filter and had CB pumped past them at a constant rate of 15 ml/min. The buffer contained excess methionine (10 μM). When the buffer reservoirs were changed, the cells experienced addition and removal of the effector. The effluent was fractionated and assayed for volatile labeled product ([^{3}H]methanol). A 400-μl aliquot of each fraction was placed in a 0.5-ml microcentrifuge tube (without a lid), and the microcentrifuge tubes were then placed in scintillation vials containing 2 ml of scintillation fluid, allowed to equilibrate for 24 h, and counted in a Beckman LS1701 scintillation counter.

Quantitation of Labeled Membrane Proteins—The ^{3}H-labeled proteins were visualized by fluorography (48), and the entire region containing [^{3}H]McpB was quantified in units of absorbance with a PDI 420oe scanning densitometer with 42 μm resolution, using Quantity One® software.

Tethered Cell Assay—The strain to be analyzed was grown from a single colony without shaking in 1.0 ml of Tryptone broth. After overnight growth, the culture was pelleted and resuspended in 250 μl of Tryptone broth. The entire suspension was used to inoculate 25 ml of minimal medium, and the culture was grown for 4.5 h at 37 °C with vigorous shaking (250 rpm). Fifteen min prior to harvesting, 250 μl of a 5% glycerol/0.5 M sodium lactate solution was added to the culture. The cells were placed in a 300-ml prechilled Waring blender for 10 min at 4 °C. In order to shear the majority of flagella, the cells were blended twice for 15 s, with a 45-s intervening pause to allow cooling. A 250-μl aliquot of the blended cells was then placed on a glass coverslip preincubated with 25 μl of anti-flagellin antibody and incubated at room temperature for 30–45 min. The coverslip was then inverted and placed within a laminar flow chamber to be observed by phase-contrast microscopy in a system similar to those described previously (5, 49). Chemotaxis buffer with or without attractant was pumped through the laminar flow chamber at a constant rate such that consumption of an attractant did not significantly affect the concentration of the attractant. Rotating cells were videotaped and analyzed by a Hobson Tracker, Bacterial Edition (Hobson Tracking Systems Ltd., Sheffield, United Kingdom), which generates text files containing continuous-time rotational data. The data were processed by programs written with Matlab software (The Mathworks, Inc.). For each cell, the continuous-time rotational data was converted to discrete-time data with a step size of 0.1 s. All the data points within a 4-s window were then averaged to generate a data set with a step size of 4 s that contained the probability of CCW rotation (smooth swimming behavior). The data for all cells in the sample population were then pooled and averaged, and the resulting data were smoothed over a five data point window to generate the final graph.

Swarm Plate Assay for Chemotaxis—Strains were grown overnight on tryptose blood agar base plates with the appropriate drug. A single colony was transferred to a Tryptone semi-solid agar plate (1% Tryptone, 0.5% NaCl, 1× Spizizen salts, 5 μg/ml required amino acids, and 0.27% agar) and incubated at 37 °C for 4 h (38).

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 [^{3}H]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)

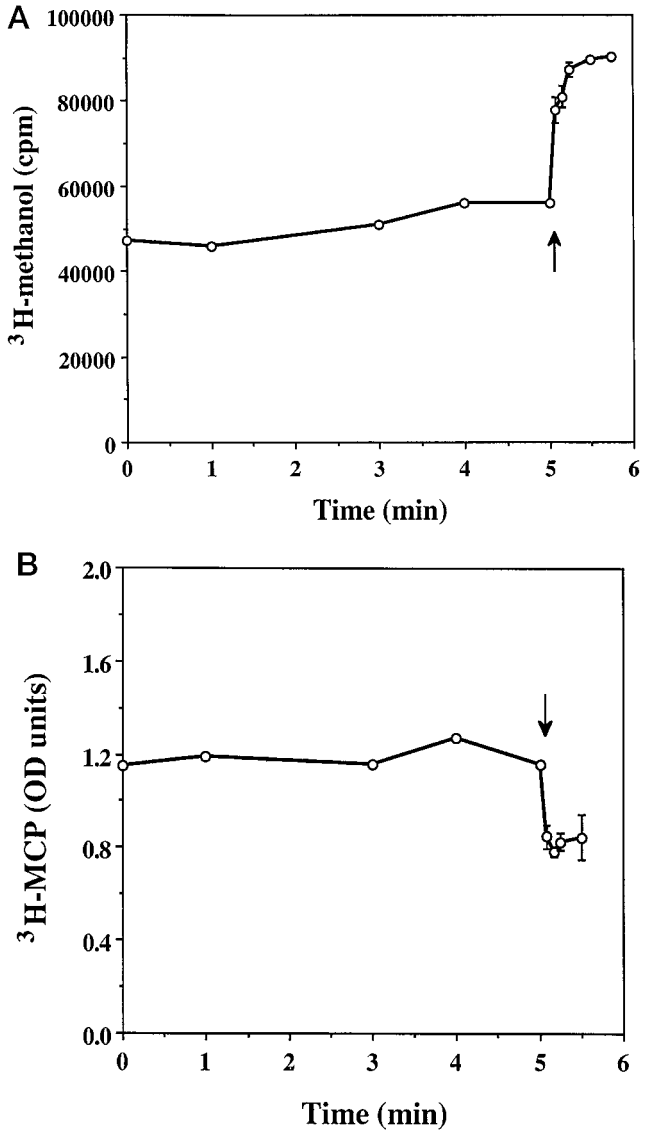

FIG. 1. **Time course of methanol evolution and net demethylation of McpB in response to asparagine stimulation.** The assay was performed as described under "Experimental Procedures." The *arrow* indicates the time of addition of 0.5 mM asparagine to strain OI3184. *A,* methanol production. *B,* net demethylation of MCPs. The data represent the average of three trials. *Error bars* represent the S.D.

[^{3}H]methanol (see under "Experimental Procedures"). The OI3184 protoplast membranes from those samples were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by fluorography to detect changes in McpB methylation. The results show that methanol production increased dramatically within 5 s of addition of 0.5 mM asparagine (calculated to be enough asparagine to titrate 90% of receptors based on the experimentally determined K_D; 50) (Fig. 1*A*). Demethylation of McpB also occurred within 5 s of asparagine addition during this assay (Fig. 1*B*). These data demonstrate that there is no significant delay in methanol production following addition of asparagine, in contrast to what was previously reported for aspartate (34). Although the results of this experiment do not eliminate the possibility of rapid methyl transfer to an intermediate carrier, no methylated intermediate is necessary to account for the data in this assay. Thus, it seems likely that methyl groups removed from McpB upon asparagine addition are released directly as methanol. This methanol production can therefore be attributed to the action of the CheB methylesterase (4, 5), which is activated by CheA-P (2, 51) and is similar to the process seen in *E. coli* in response to repellent addition (31, 32).

Effect of Addition of Asparagine on McpB Methylation—Previous studies on the *B. subtilis* OI1085 strain (wild-type for chemotaxis) also indicated that the attractant aspartate caused increased turnover of methyl groups on the MCPs and that net methylation changes did not occur (34, 37). In conjunction with that finding, a return of ^{3}H label to the MCPs after removal of the aspartate under pulse-chase conditions was interpreted to mean that reversible methyl transfer occurred during chemotaxis in *B. subtilis* (29). In order to test the methyl transfer hypothesis specifically for McpB, a strain was constructed that lacked chromosomal copies of *mcpA*, *mcpB*, *mcpC*, *tlpA*, and *tlpB* at their normal loci and had *mcpB* cloned into the *amyE* locus (strain OI3290). A strain lacking *mcpA*, *mcpB*, *mcpC*, *tlpA*, *tlpB,* and *tlpC* (OI3281; 42) has no radiolabeled bands at the position where [^{3}H]McpB migrates in strain OI3290.[5] TlpC migrates much faster during SDS-polyacrylamide gel electrophoresis than does McpB (52), and it therefore does not interfere with the analysis of McpB in strain OI3290. Thus, in strain OI3290 it is possible to follow methylation changes specifically in McpB that occur in response to asparagine stimulation.

OI3290 cells were labeled with 10 μM [^{3}H]methionine. The methylation reached a steady-state level by 30 min (Fig. 2*A*). 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. 2*B*). 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. 2*A*). 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. 2*C*).

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

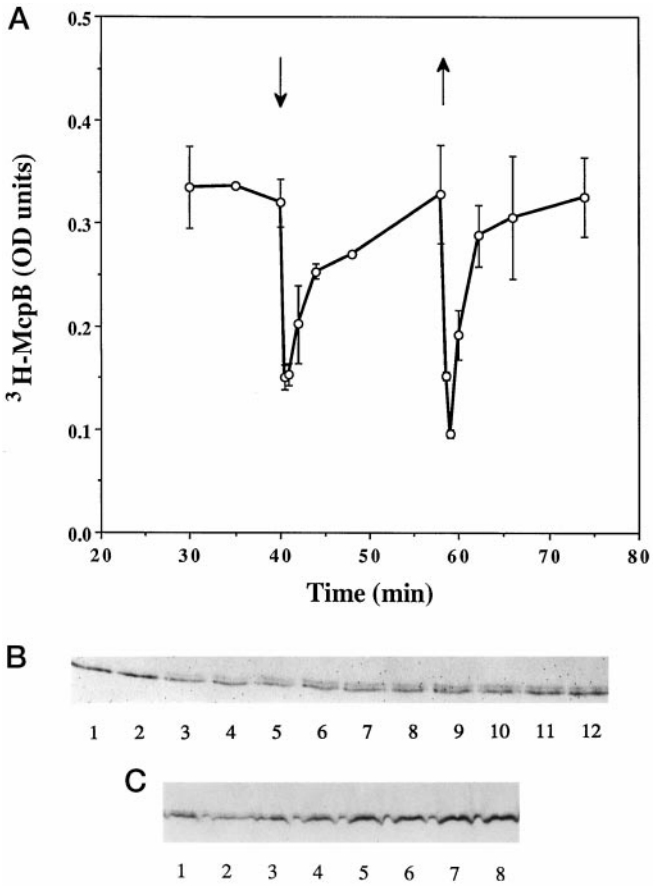

FIG. 2. **Methylation changes on McpB in response to addition and removal of asparagine.** The assay was performed as described under "Experimental Procedures." *A,* The *downwardly directed arrow* indicates the time of addition of 0.5 mM asparagine to strain OI3290. The *upwardly directed arrow* indicates the time of removal of same. The data represent the average of three trials. *Error bars* represent the S.D. *B,* fluorograph of [^{3}H]McpB showing methylation changes in response to addition of asparagine. *Lanes 1* and *2* were sampled 40 min after addition of [^{3}H]methionine and prior to addition of asparagine. Following addition of asparagine, samples were taken at 15 s (*lanes 3* and *4*), 2 min (*lanes 5* and *6*), 4 min (*lanes 7* and *8*), 8 min (*lanes 9* and *10*), and 16 min (*lanes 11* and *12*). *C,* fluorograph of [^{3}H]McpB showing methylation changes due to removal of asparagine. *Lane 1,* 16 min after addition of asparagine and prior to removal of asparagine (as in *B, lanes 11* and *12*). Following removal of asparagine, samples were taken at 35 s and 1, 2, 4, 8, 16, and 24 min (*lanes 2–8,* respectively).

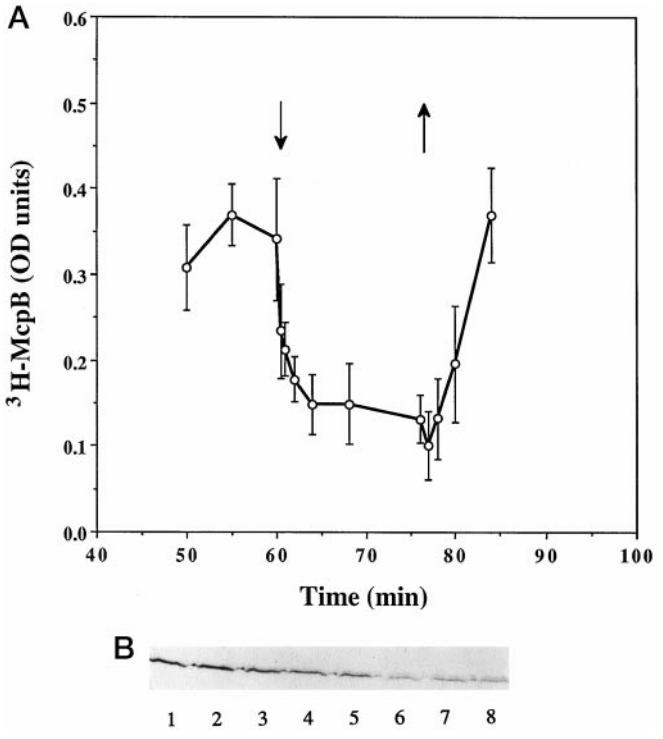

FIG. 3. **Methylation changes in McpB in a *cheY* mutant in response to addition and removal of asparagine.** The assay was performed with strain OI3294 as described under "Experimental Procedures." *A,* the *downwardly directed arrow* indicates the time of addition of 0.5 mM asparagine to strain OI3294. The *upwardly directed arrow* indicates the time of removal of same. The data represent the average of three trials. *Error bars* represent the S.D. *B,* fluorograph of [^{3}H]McpB showing methylation changes following addition of asparagine. *Lanes 1* and *2* were loaded with samples taken 40 and 50 min, respectively, after addition of [^{3}H]methionine and prior to addition of asparagine. *Lanes 3–8* were loaded with samples taken 30 s and 1, 2, 4, 8, and 16 min after addition of asparagine, respectively.

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.[6] 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.

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. 2*A* and 3*A*). 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. 2*B* and 3*B*). 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

[6] M. M. Saulmon and G. W. Ordal, unpublished data.

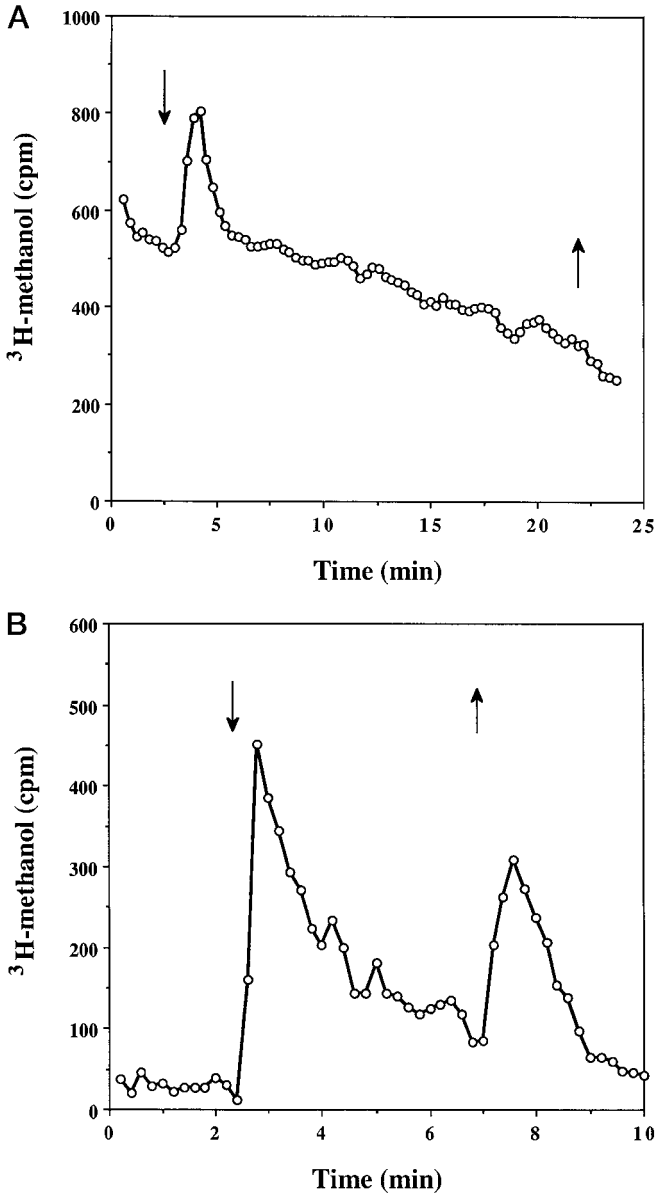

FIG. 4. **Methanol production by *cheY*D54A mutant and wild-type cells following stimulation with asparagine.** The assay was performed as described under "Experimental Procedures." The *downwardly directed arrow* indicates the time of addition of 0.5 mM asparagine. The *upwardly directed arrow* indicates the time of removal of same. *A,* response by the *cheY*54DA mutant (strain OI2952). *B,* response by the wild-type (strain OI1085).

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 (*cheY*54DA) 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. 4*A*). The response by strain OI1085 (wild-type for chemotaxis) is shown for comparison (Fig. 4*B*). 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. 5*A*). 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. 5*B*) (3, 5). It should be noted that *cheY* mutant cells (OI2057) are completely tumbly (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 poststimulus 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. 5*A* 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. 5*A*).

Swarm Plate Analysis for Chemotaxis by the cheRBCD Mutant—In order to test the efficiency of the putative CheY-P

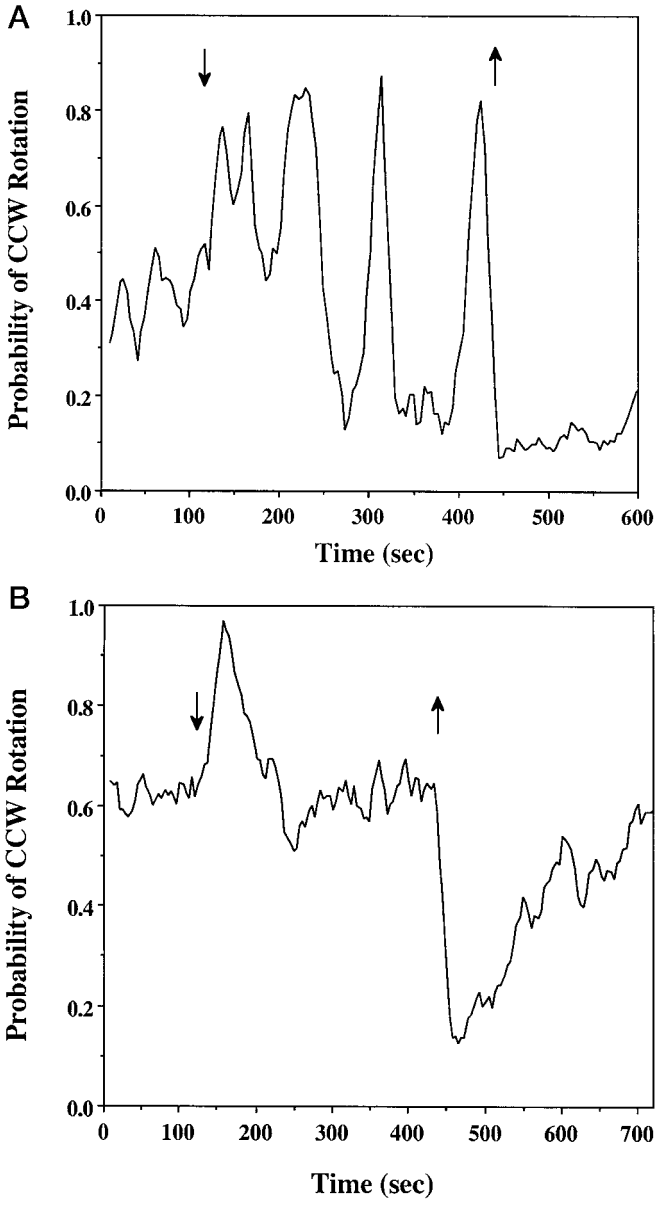

FIG. 5. **Behavior of *cheRBCD* and wild-type tethered cells in response to asparagine stimulation.** The assay was performed as described under "Experimental Procedures." The *downwardly directed arrow* indicates the time of addition of 56 μM asparagine. The *upwardly directed arrow* indicates the time 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.

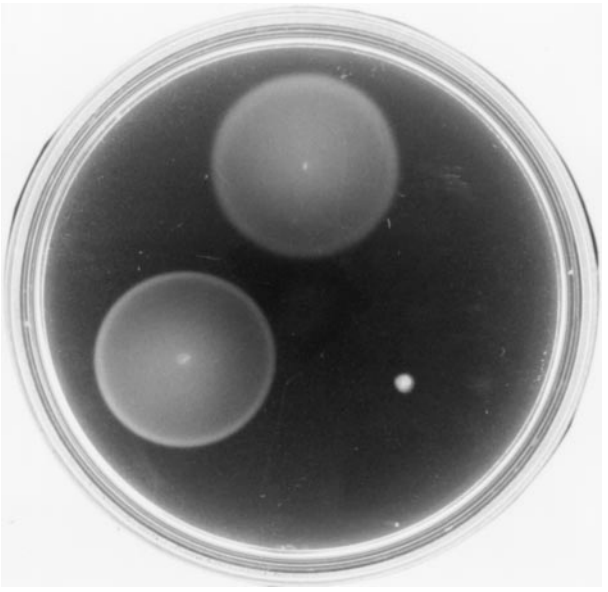

FIG. 6. **Swarm plate assay of a *cheRBCD* mutant.** The assay was performed as described under "Experimental Procedures." The plate was inoculated with (*clockwise from top*) strains OI1085 (wild-type), OI2057 (Δ*cheY*), and OI3377 (*cheRBCD*). The Tryptone swarm plate was incubated at 37 °C for 4 h.

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

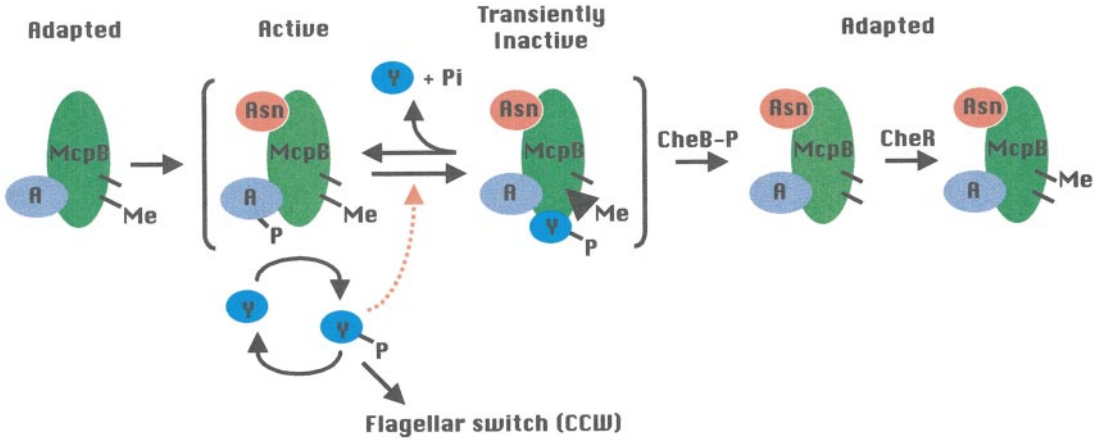

FIG. 7. **Model for CheY-P feedback on asparagine-bound McpB.** This model depicts events leading to an adapted state for ligand-bound McpB. Prior to stimulation, McpB is methylated (Me) at one or more of several sites (*solid black lines*). Following addition of asparagine, the receptor complex is active, 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).

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. 5*A*). 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 OI3290, McpB released methanol upon asparagine stimulation, and the protein immediately appeared as a more slowly migrating, less methylated species (Fig. 2*B*). In contrast, in the *cheY* mutant, a more slowly migrating, less methylated species became apparent only several minutes after asparagine stimulation (Fig. 3*B*). 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. 3*B*) 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. 5*B*), a state that is not generated in the *cheRBCD* mutant (Fig. 5*A*).

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 archael species *Halobacterium salinarium*, like *B. subtilis*, produces methanol in response to all stimuli (28, 64), it is possible that CheY-P feedback onto the receptors

was part of an adaptation mechanism present in a common ancestor of these two highly diverged prokaryotic organisms.

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