Chemotactic Methylesterase Promotes Adaptation to High Concentrations of Attractant in *Bacillus subtilis*

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The *Bacillus subtilis* gene encoding CheB (cheBB), the chemotactic methylesterase, has been sequenced. The 39-kDa protein which resulted from the expression of cheBB, using a T7 expression system was consistent with the predicted open reading frame. CheBB shares 39.5% identity with *Escherichia coli* CheB and can complement a cheB null mutant. CheBB is required for removal of methyl groups from the receptors upon attractant stimulus and appears to play an important role in adaptation to the addition of attractants, whereas CheB plays an important role in adaptation to the addition of repellents. Unlike the cheB and cheE mutants of *E. coli*, which show extreme flagellar rotational biases, the unstimulated cheBB mutant showed a normal (wild type) bias. Upon addition of attractant, the cheBB null mutant showed a counter-clockwise bias that was higher than for wild type and demonstrated only partial adaptation. In the capillary assay for the attractant azetidine-2-carboxylic acid, the mutant gave a wild type response at low concentrations but a very reduced response at high concentrations. We conclude that *B. subtilis* has an effective methylation-independent adaptation system but must utilize the methylation system for adaptation to high concentrations of attractant.

Adaptation to chemotactic stimuli is a prerequisite for bacteria to carry out chemotaxis since excitatory stimuli would otherwise persist and permanently bias behavior. In *Escherichia coli*, binding of repellent at the receptors, the methyl-accepting chemotaxis proteins (MCPs), is thought to cause increased autophosphorylating activity of the CheA kinase (1, 2). The phosphorylated form of CheA (CheA-P) phosphorylates CheY, which then interacts with the flagellar switch to cause clockwise (CW) rotation of the flagella. CheA-P also phosphorylates CheB, which in turn is activated to demethylate the receptors. Demethylated receptors, with repellent still bound, no longer augment CheA autophosphorylation, but rather maintain a level of CheA activity as found in unstimulated cells (for a review, see Refs. 2 and 3). The importance of CheB in performing this function is underscored by the fact that mutants in cheBB always tumble (4) due to a strong bias in clockwise flagellar rotation (5).

Like *E. coli*, *Bacillus subtilis* has CheA, CheB, CheY, and MCPs. However, null mutants in cheY and null mutants in cheB are very tumbly whereas the corresponding mutants of *E. coli* are very smooth swimming. Therefore, unlike *E. coli*, it appears that attractants activate CheA to lead to smooth swimming behavior. Furthermore, the MCPs of *B. subtilis* are larger than those of *E. coli*, having apparent molecular masses greater than 70 kDa, compared with 58–60 kDa for *E. coli* (6). Addition of attractants such as aspartate causes an increased turnover of methyl groups on all three MCPs (7) in a reaction requiring CheA. That is, methyl groups are transferred away and replaced by new methyl groups from S-Adenosylmethionine. The identity of the methyl acceptor is unknown. However, on removal of the attractant, the lost methyl groups may return to the MCPs (8).

In *E. coli* adaptation to positive and negative stimuli involves changing the degree of methylation of the MCPs. The importance of the degree of methylation of these proteins is clear from the phenotype of strains unable to methylate or demethylate them. cheB mutants are smooth swimming, and cheE mutants are tumbly. The double mutant is able to move on a swarm plate and shows some degree of chemotaxis in capillary assays. These bacteria do not adapt very well to moderate or large stimuli but do adapt to weak stimuli (9–11). It is thus apparent that, although the methylation system is very important, it is to some extent dispensable.

In this article, we report the sequence of *B. subtilis* chemotactic methylesterase, cheBB, and characterization of a null mutant in cheBB. Our results indicate that CheBB plays a significant role in adaptation, but not in excitation, in relation to attractants. There appears to be a very effective methylation-independent adaptation system in *B. subtilis* that operates for low concentrations of attractant. Indeed, chemotaxis toward low concentrations of attractant, as measured in the capillary assay, the cheBB mutant was as efficient as wild type.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—The bacterial strains and plasmids used in this investigation are described in Tables I and II, respectively.

**Growth Media**—Tryptone broth is 1% Tryptone and 0.5% NaCl. Minimal medium is 50 mM potassium phosphate buffer, pH 7.0, 1 mM MgCl₂, 1 mM (NH₄)₂SO₄, 0.14 mM CaCl₂, 0.01 mM MnCl₂, 50 μg/ml required amino acids (His, Met, Trp), and 20 mM sorbitol.

**DNA Sequence and Analysis**—A 2.1-kb PstI fragment of *B. subtilis*...
DNA from pG0104 (12) was subcloned into M13 phages M13mp18 and M13mp19. Additional templates were derived from exonuclease III-generated deletions of these constructs (13). Both strands were sequenced in their entirety using the Sequenase kit (U. S. Biochemicals) and 35S-labeled ATP (Amersham Corp.). Analysis of DNA sequences and homology alignments were performed using DNASTAR, Madison, WI.

Mutagenesis—Two mutations in cheB were created, resulting in strains O12715 and O12836. Strain O12715 was created by digesting pMK100 at a unique SpeI restriction site, (Fig. 1) located in the first third of cheB and filling in the 3' recessed ends with Sequenase enzyme in the presence of 20 μM deoxynucleotide triphosphates. The resulting blunt ends were subsequently religated, resulting in a nonsense mutation. The mutation was then crossed onto the B. subtilis chromosome by gene conversion (14) yielding a truncated cheB.

Strain O12838 was created by first introducing two StuI restriction sites, using oligonucleotide-directed in vitro mutagenesis, at each end of cheB in pMK100. The resulting 1.1-kb StuI fragment containing cheB in its entirety was removed. The vector with the adjacent B. subtilis DNA was religated to create pMK113. The oligonucleotides used in the site-directed mutagenesis were designed so that after religation, the expression of the adjacent genes would not be affected. In order to facilitate the transfer of the mutated DNA onto the B. subtilis chromosome, an EcoRI restriction site was introduced between orf298 and cheA (Fig. 1). The resulting 1.1-kb EcoRI fragment was then subcloned into pMK116, to create pMK117. A promoterless chloramphenicol resistance marker (chloramphenicol acetyltransferase, cat gene) (15) was inserted into the EcoRI restriction site of pMK117, to create pMK118. The mutation was then crossed onto the B. subtilis chromosome by gene conversion (14).

Expression of cheB—The method of Tabor and Richardson (16) was used to express cheB. pT7-5 derivatives were introduced into competent E. coli K38 cells. Subcloned genes were expressed after induction at 42 °C in the presence of rifampicin and L-[3H]methionine. The labeled proteins were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by autoradiography.

Complementation of cheB Mutants—Complementation of the mutants was performed by transforming pMK108, which is an expression vector containing wild type cheB, into competent O12836. Complementation was assayed on semisolid agar swarm plates containing 0.27% agar (17). Iacpropyl-1-thio-β-galactopyranoside (IPTG) was added in varying concentrations to induce the expression of cheB.

The swarm plates were incubated for 9 h at 37 °C. The swarm size of the complemented strain was compared to that of the wild type.

In Vivo Methylation—In vivo methylation experiments were performed as previously described (18). The in vivo cold chase experiment was performed as previously described (8).

Capillary Assay—Capillary assays have been described (17). Cells were grown overnight on tryptose blood agar base plates, resuspended at 10⁶ cells/ml in tryptone broth, and diluted 50-fold into minimal media. After 4 h, growth cells were supplemented with 0.05% glycerol and 5 mM sodium lactate, grown 15 min longer, and harvested. They were resuspended at OD₅₇₀ = 0.001 and assayed for chemotaxis. Cells accumulated in the capillary tubes were plated out and counted. The assay was performed in triplicate.

This study describes the first flagellar operon of B. subtilis. The 2.1-kb Psil fragment containing cheB from pG0104 has been expanded. Open boxes below the map indicate open reading frames. Direction of transcription of the operon is designated by the arrow.
homologous to FrzG of M. xanthus (25) with 25.7% identity over a 334 amino acid sequence.

Expression of cheB~—The 2.1-kb *PstI* fragment was subcloned in the coding orientation of pT7-5, resulting in pMK110 (Table II). Expression of this fragment produced a protein having an apparent molecular mass of 40 kDa (Fig. 4). This agrees well with the predicted molecular mass of 39.2 kDa, based on the amino acid sequence, deduced from the DNA sequence. Another expression plasmid was constructed from the expression of the truncated gene, a protein of approximately 12.5 kDa is visualized (Fig. 4). This agrees well with the predicted molecular mass of 12.5 kDa, based on the amino acid sequence, deduced from the DNA sequence.

Complementation of the cheB~ Mutants—Two cheB~ mutants were made. One, strain OI2715, allowed the expression of the first 34% of cheB~ (Fig. 4). The other, OI2836, contained no DNA encoding the methylesterase but left the upstream and downstream genes intact, as confirmed by complementation. Both mutants showed identical in *vivo* methylation of MCPs (Fig. 5, data not shown), and swarm plate morphologies (Fig. 6, data not shown), indicating that the two mutations caused the same defects. The corresponding mutations were verified by sequence analysis and Southern hybridization (data not shown).

In the absence of CheBE, MCPs cannot undergo enzymatically catalyzed demethylation (26). Thus in an *in vivo* methylation experiment, in a cheB~ mutant, the only way that methyl groups can replace the existing unlabeled glutamate methylesters: radiolabeled methyl groups can replace the existing unlabeled methylester groups is by the spontaneous demethylation of the glutamate methylesterases. As expected, the level of labeling of MCPs was significantly reduced but not absent in both strain OI2836 and strain OI2715 (Fig. 5, data not shown).

The complementation as observed by MCP methylation in strain OI2837 was 88% of wild type when 1 mM IPTG was added during the growth of the culture. This is a large increase over the amount of methylation in OI2836, which was only 17% of the wild type level. (Fig. 5).

To ensure that the mutations created in cheB~ were nonpolar on other genes required for chemotaxis, the mutations were complemented with pMK108 (Table II), which expressed only cheB~. The swarm of the complemented strain (OI2837) was similar to that of wild type but smaller (Fig. 6).

The complementation was also verified by tethering analysis. The complemented strain demonstrated wild type behavior (data not shown).
The addition of repellent or removal of attractant resulted in a decrease in the bias of counterclockwise flagellar rotation. Negative stimuli (either addition of repellent or removal of attractant) caused an increase in the bias of counterclockwise flagellar rotation. The adaptation period to positive stimuli was shorter than the adaptation period to negative stimuli by about a factor of four. The *B. subtilis* population never reached a 100% counterclockwise bias in response to the addition of attractant. Finally, there appeared to be a slight overshoot, a period of excess clockwise rotation following adaptation.

In the absence of stimuli, the *cheB* mutant showed a baseline level of counterclockwise rotation similar to the wild type. Addition of attractant caused a higher than wild type level of counterclockwise rotation of the flagella (Fig. 9). After the 96% counterclockwise bias was achieved (compared with 85% for wild type), the bacteria partially adapted. The extent of adaptation depended inversely on stimulus strength. The bacteria adapted much more completely to the lower concentration of attractant than to the higher concentration (Fig. 9). Prolonged exposure to 10 μM azetidine-2-carboxylic acid showed that a 75% bias was maintained, even after 8 min (data not shown). Upon removal of the attractant, the bacteria showed a counterclockwise bias far below the base-line value. Interestingly, the onset of this low counterclockwise bias was slower and less extreme for removal of the higher concentration of attractant. The *cheB* mutant was also subjected to repellent stimuli. When 3.15 mM indole was added to the *cheB* mutant, it responded like the wild type. However, when indole was removed, the mutant adapted more slowly (Fig. 10). Thus, *cheB* appears to be required for normal adaptation to positive stimuli.

**Capillary Assay**—To further analyze the *cheB* mutants ability to perform chemotaxis at different concentrations of attractant, capillary assays were performed. At low concentrations of attractant, the mutant was capable of performing chemotaxis as well as the wild type (*011085*) (Fig. 11). However, as the concentration of attractant was increased, the efficiency of chemotaxis decreased greatly.

**DISCUSSION**

**Homology of Methylesterases from Several Bacteria**—In this article, we report the identification of a gene encoding the chemotactic methylesterase in *B. subtilis* and show that it is homologous to the *E. coli* chemotactic methylesterase in both structure and function. The amino acid sequences of *cheB* and the *E. coli* and *M. xanthus* counterparts were compared (Fig. 3). There are conserved regions among all three of the distantly related proteins. While there are regions that share identity between any two of the proteins, the regions where all three show identity can be indicative of regions that are likely to be essential for proper functioning. These highly conserved regions occur uniformly throughout all three proteins, in both the regulatory (NH2-terminal) and in the enzymatic (COOH-terminal) regions (31).

**Requirement of *cheB* for Removal of Methyl Groups from MCPs**—A *cheB* mutant would be expected to show a deficiency in the level of radioactive labeling of the MCPs in an *in vivo* methylation experiment. In order to radiolabel the MCPs, non-radioactive groups added during growth must first be removed. Thus, if the enzymatic machinery to remove these is disabled, then the MCPs will remain unlabeled following adaptation.
**TABLE II**

| Plasmid | Description of plasmid | Refs. |
|---------|------------------------|-------|
| pGP1-2  | Contains gene encoding T7 polymerase, *kan*<sup>+</sup> | (11)  |
| pEB112  | *B. subtilis*/E. coli shuttle vector, contains IPTG inducible "tac" promoter, *kan*<sup>+</sup> | (12)  |
| pT7-6   | *E. coli* expression plasmid with clones insert under the control of T7 promoter, *amp*<sup>+</sup> | (11)  |
| pGO104  | 10.9 EcoRI fragment, containing *B. subtilis* chemotaxis DNA, cloned into pUC18, *amp*<sup>+</sup> | (7)   |
| pBluescript | 2.1 PstI fragment, containing cheB, cloned into M13mp19 | Stratagene |
| pMK100  | 2.1 PstI fragment, containing cheB, cloned into pEB112, *kan*<sup>+</sup> | This work |
| pMK108  | 2.1 PstI fragment, containing cheB, cloned into pT7-6, *amp*<sup>+</sup> | This work |
| pMK110  | 2.1 PstI fragment, containing cheB, cloned into pT7-6, *amp*<sup>+</sup> | This work |
| pMK111  | 2.1 PstI fragment, with unique SphI end filed and religated, containing cheB, cloned into pT7-6, *amp*<sup>+</sup> | This work |
| pMK113  | pMK100 with cheB deleted | This work |
| pMK116  | pBluescript with EcoRI site removed | This work |
| pMK117  | 1.1 PstI fragment from pMK100 subcloned into pMK116 | This work |
| pMK118  | pMK117 with cat gene inserted in EcoRI site | This work |

**Fig. 4.** SDS-polyacrylamide gel electrophoresis analysis after induction of expression of pT7 inserts. Autoradiogram of cheB<sub>E</sub> expression in *E. coli* K38 after a 16-h exposure. Lane 1, pMK110; lane 2, pMK111. Molecular mass standards are indicated in kDa.

**Fig. 5.** *In vivo* MCP methylation. Methylations were performed for 5 min with 10 μCi of [methyl-<sup>3</sup>H]methionine/sample. Lane 1, OI1085; lane 2, OI2836; lane 3, OI2837; and lane 4, OI2838.

Following addition of radioactive methionine. As expected, the cheB<sub>E</sub> mutant showed poor labeling of the MCPs (Fig. 5). The low level (17% of wild type) of methylation that does occur can be attributed to spontaneous demethylation of the MCPs. A 10% level of methylation has been observed in cheB<sub>E</sub> mutants. It is unlikely that there is a "back-up" methylesterase since in a continuous flow assay (32, 33), no increase in methanol production was seen in response to the addition of attractant to the cheB<sub>E</sub> null mutant strain (OI2836) as occurs in the wild type strain (data not shown).

CheB<sub>S</sub>, like its *E. coli* counterpart, is presumably activated by phosphoryl transfer, for it shares the conserved Asp residue which is phosphorylated in *E. coli*. In *B. subtilis*, addition of attractant causes an increase in methyl group turnover on the MCPs. If this turnover occurs due to activation of CheB<sub>S</sub> by
subtilis Methylesterase

3.

FIG. 7. Effect of expression of cheB on the swarm size of RP4310. Single colonies of RP437 (○), RP4310::pMK108 (□), and RP4310 (×) were stabbed on tryptone swarm plates containing varying concentrations of IPTG. The swarm size was measured after 15 h at 30 °C.

FIG. 8. Cold chase in vivo methylation. Cells were given a 100-fold excess of nonradioactive methionine after being labeled for 5 min with 10 μCi of [methyl-3H]methionine, and then incubated for an additional 2 min. Samples were removed at times before and after a 10-s exposure to 100 mM aspartate. Densitometer scans of SDS-polyacrylamide gel electrophoresis fluorogram provided relative values of MCP methylation. Solid bars represent MCP methylation before addition of aspartate. Hatched bars represent MCP methylation after addition of aspartate.

Role of CheB in Adaptation—Tethering analysis indicates that CheB plays a role in adaptation to attractant (Fig. 9) as the cheB mutant remained somewhat biased to smooth swimming until attractant was removed. Thus, the removal of methyl groups from the MCPs by CheB contributes to the adaptive response to addition of attractants, rather than to the addition of repellents as in E. coli (26). The role of CheB in adaptation to attractant stimuli is consistent with the fact that the null mutants of cheA and cheY are tumbly (23, 27), whereas they are smooth swimming in E. coli (34). This result implies that CheY-P causes smooth swimming, whereas CheY-P is widely held to cause tumbling (35–37). As in E. coli, activation of CheA may cause increased phosphorylation of both CheY and CheB. In B. subtilis, however, this may be due to addition of attractant rather than addition of repellent. Adaptation to positive stimuli is more rapid than to negative stimuli in B. subtilis. This probably reflects the activation of CheB by phosphorylation. By analogy, CheR, which is not known to be activated in E. coli (38), presumably helps bring about the slower adaptation to removal of attractants in B. subtilis.

The capillary assay (Fig. 11) shows that for lower concentrations of azetidine-2-carboxylic acid (0.1 mM and below), CheB is not needed for chemotaxis. In fact, in this concentration range, chemotaxis is equal in the wild type and in the cheB mutant. Concomitantly, addition of lower concentrations of azetidine-2-carboxylic acid to tethered cells shows more nearly complete adaptation than does addition of higher concentrations. It would seem that there exists in B. subtilis a methylation-independent adaptation that is very successful at low concentrations of attractant, but not at high concentrations. Indeed, at 0.1 M azetidine-2-carboxylic acid, the accumulation in the capillary assay was 50-fold less than for wild type. It thus appears that the purpose of the adaptation system involving methylation in B. subtilis is to allow the
bacteria to reach very high concentrations of attractant. However, for reaching more modest concentrations of attractant, this system is not necessary.

CheB-independent Adaptation—The existence of CheB-independent adaptation is supported by the following. First, as mentioned above, the bias of the unstimulated cheB3 mutant is the same as for wild type (Figs. 9 and 10). By contrast, the cheB3 mutant led to a counterclockwise bias upon the addition of attractant. Al- though previously we had speculated that methyl transfer was obligatory for excitation (39) this hypothesis was based on the properties of the cheB3null mutant, which showed a very low (10%) counterclockwise bias and does not excite (data not shown). It is likely that CheB3null somehow interfered with the normal excitatory process but in the absence of CheB3 as in cheB3 null mutant, no interference was seen.

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Fig. 11. Capillary assays to azetidine-2-carboxylic acid. OI1085 is indicated by O and OI2836 by •. Each strain was assayed over the range of attractant concentrations indicated. The background value for OI1085 was 13 and of OI2836 was 25 and were subtracted from the values in the figure.