Purification and Characterization of *Bacillus subtilis* CheY†

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ABSTRACT: Amino acid sequence comparison suggests that numerous proteins are common to the signal transduction pathways controlling chemotaxis in *Bacillus subtilis* and *Escherichia coli*. However, previous work has indicated several differences between the two systems. We have undertaken a comparative study of the roles of the CheY protein in chemotaxis by *B. subtilis* and *E. coli*. Although CheY from the two species share only 36% amino acid sequence identity, purified *B. subtilis* CheY was phosphorylated *in vitro* by *E. coli* CheA, and dephosphorylation of CheY-P was enhanced by *E. coli* CheZ. Alteration of the putative site of phosphorylation in *B. subtilis* CheY, Asp54, eliminated chemotaxis *in vivo*, further confirming that phosphorylation is important for *B. subtilis* chemotaxis. Loss of CheY function resulted in tumbling behavior in *B. subtilis*. Introduction of positively charged residues in place of Asp10 of *B. subtilis* CheY abolished function, whereas the corresponding changes in *E. coli* CheY apparently result in constitutive activation. The *B. subtilis* CheY Asp10 mutant proteins also failed to cause tumbling in *E. coli*, consistent with a different interaction between CheY and the flagellar switch in the two species. Finally, *B. subtilis* adapted more rapidly to positive stimuli than negative stimuli, whereas the opposite is true of *E. coli*. We conclude that *B. subtilis* regulates its response to positive chemotactic stimuli by enhancing phosphorylation of chemotaxis proteins, whereas *E. coli* reduces phosphorylation in the same circumstance.

Sequence analysis of a large *Bacillus subtilis* operon containing many chemotaxis and motility genes has given strong indications of similarity of the *B. subtilis* and *Escherichia coli* chemotaxis mechanisms (Zuberi et al., 1991a,b; Bischoff & Ordal, 1991; Fuhrer & Ordal, 1991; Bischoff & Ordal, 1992; Hanlon et al., 1992a). In particular, homologs of *E. coli cheA* (Fuhrer & Ordal, 1991), *cheW* (Hanlon et al., 1992b), *cheB* (Kirsch et al., 1993), *cheY* (Bischoff & Ordal, 1992), *cheR* (Kirsch et al., manuscript in preparation), *fliM* (Zuberi et al., 1991a), *fliG* (Albertini et al., 1991), and *fliN* (Bischoff & Ordal, 1992) have been identified. In *E. coli*, CheA, an autophosphorylating kinase, is believed to be modulated in its activity by the binding of attractants or repellents to the methylated receptors (Borkovich et al., 1989; Borkovich & Simon, 1990; Ninfa et al., 1991). CheA-P donates its phosphoryl group to CheY and to CheB (Hess et al., 1988b; Wylie et al., 1988). CheY-P binds at the flagellar switch, consisting of FliG, FliM, and FliN, to cause clockwise rotation of the flagella (Parkinson et al., 1983; Yamaguchi et al., 1986). In the absence of CheY-P, the flagella rotate counterclockwise (Parkinson, 1978). As an adaptation reaction, CheB-P demethylates the receptors in order to reduce CheA activity (Lupas & Stock, 1989; Stewart et al., 1990). CheY-P rapidly autodephosphorylates and additionally is subject to dephosphorylation stimulated by CheZ (Hess et al., 1988a,b). In this way, the level of CheY-P reflects the "current" degree of activation of CheA.

Table I: Bacterial Strains

strain	relevant genotype	reference
E. coli		
RP437	*che*$^+$	Parkinson (1978)
RP5232	Δ*cheY6021*	Liu and Parkinson (1989)
HCB721	Δ (*cheA–cheY*) 1590::*Xho*I (Tn5), Δ*tsr*7021 *trg*::Tn10	Wolfe (1988)
B. subtilis		
OI1085	*che*$^+$	Ullah and Ordal (1981)
OIB055	*cheY*::*cat*	Bischoff and Ordal (1991)
OIB057	Δ*cheY*	Bischoff and Ordal (1991)
OI2894	*cheYDK10*	this work
OI2895	*cheYDR10*	this work
OI2896	*cheYDT54*	this work
OI2897	*cheYDS54*	this work
OI2952	*cheYDA54*	this work
OI3021	*che*$^+$ (pDB21)	this work
OI3023	Δ*cheY* (pDB21)	this work

There are indications, however, that the mechanism regulating chemotaxis is considerably different in the two organisms. For instance, in *B. subtilis* methyl groups are apparently transferred from the methylated receptors (methyl-accepting chemotaxis proteins, MCPs) to an acceptor rather than liberated directly as methanol from the receptors as in *E. coli* (Thoelke et al., 1988, 1989, 1990). The differences are underscored by finding two unique genes apparently required for *B. subtilis* chemotaxis—*orfA* and *orfB* (Bischoff & Ordal, 1992). It is a matter of considerable interest to elucidate a signal transduction mechanism that is thought to be considerably different and yet utilize so many homologous proteins.

It is important to determine whether *B. subtilis* CheY is indeed subject to phosphorylation. In this article, we show that *E. coli* CheA phosphorylates *B. subtilis* CheY. To understand the action of *B. subtilis* CheY, site-directed mutations were created and the behavioral phenotypes of the corresponding mutants were compared with those of similar mutants in *E. coli* CheY. The results are consistent with the

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Table II: Plasmids

plasmid	description	reference
pEB112	*B. subtilis/E. coli* shuttle vector, contains IPTG-inducible *tac* promoter, Kan^r	Leonhardt and Alonso (1988)
pSI-1	*B. subtilis/E. coli* shuttle vector, contains IPTG-inducible "*spac*" promoter, Cm^r	Yansura and Henner (1983)
pT7-6	*E. coli* expression plasmid with cloned insert under the control of the T7 promoter, Amp^r	Tabor and Richardson (1985)
pDB16	0.6-kb *Sau*3A fragment cloned into pT7-6 in the direction that expresses *cheY*, Amp^r	Bischoff and Ordal (1991)
pDB21	0.55-kb *DraI/PstI* fragment cloned into pEB112, *cheY* under control of *tac* promoter, Kan^r	Bischoff and Ordal (1991)
pDB43	0.55-kb *DraI/PstI* fragment cloned into pEB112, expressing CheYDK10	this work
pDB51	0.55-kb *DraI/PstI* fragment cloned into pEB112, expressing CheYDR10	this work
pDB52	0.55-kb *DraI/PstI* fragment cloned into pEB112, expressing CheYDT54	this work
pDB53	0.55-kb *DraI/PstI* fragment cloned into pEB112, expressing CheYDS54	this work
pDB56	0.55-kb *DraI/PstI* fragment cloned into pEB112, expressing CheYDA54	this work
pBR322	*E. coli* expression plasmid	Bolivar et al. (1977)
pNT201	Tar-encoding fragment in pBR322	Borkovich et al. (1989)

hypothesis that CheY-P causes smooth swimming in *B. subtilis*, whereas it causes tumbling in *E. coli*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. Tables I and II list *E. coli* and *B. subtilis* strains and plasmids used in this study. Plasmids pDB16, which has *B. subtilis cheY* under the control of the T7 promoter in pT7-6 (Tabor & Richardson, 1985) and pDB21, which has *cheY* under the control of the *tac* promoter in pEB112 (Leonhardt & Alonso, 1988) have been described previously (Bischoff & Ordal, 1991). Plasmids pDB43, pDB51, pDB52, pDB53, and pDB56 contain 0.55-kb *DraI/PstI* fragments cloned into pEB112 and express mutant alleles of *cheY*. Plasmid pNT201 expresses *tar* from pBR322 and has been described previously (Borkovich et al., 1989).

Purification of CheY. A T7 expression system was used to express *B. subtilis cheY* by the method of Tabor and Richardson (1985) in RP5232, an *E. coli* strain that is deleted for *cheY*. Under these conditions, CheY forms insoluble aggregates which precipitate with the membrane pellet. The method of McCleary and Zusman (1990) was used to separate the inclusion bodies from the membranes. Insoluble protein aggregates were dissolved by denaturation in 6 M guanidine hydrochloride and then renatured in a 50% glycerol solution overnight. Following renaturation, the sample was dialyzed in TE buffer (10 mM Tris Cl (pH 8.0) and 1 mM EDTA) to remove the glycerol and then concentrated using Aquacide. The sample was loaded on a Bio-Rad Bio-Gel P-30 column and the proteins were separated using size-exclusion chromatography. The purification of CheY was monitored by Coomassie Blue staining after SDS–PAGE.

In Vitro Phosphorylation of B. subtilis CheY with E. coli CheA. In vitro phosphorylation experiments were performed as described (Bourret et al., 1990). *E. coli* CheY and CheZ were purified as described (Hess et al., 1991). Membranes were prepared as described previously (Borkovich & Simon, 1990). Reactions were carried out in TEDG buffer [50 mM Tris, pH 7.5, 1 mM EDTA, 2 mM DTT, and 10% (V/V) glycerol] supplemented with 5 mM $MgCl_2$ in a final volume of 12.2 μL at room temperature with 0.5 mM [γ-^{32}P]ATP (specific activity = 8400 cpm/pmol). The reaction was stopped by addition of 25 μL of SDS sample buffer supplemented with EDTA to a final concentration of 5 mM. The products of the reactions were separated on a 15% polyacrylamide gel and visualized by autoradiography.

Mutagenesis of cheY. Mutations were introduced into *cheY* using the oligonucleotide-directed *in vitro* mutagenesis system version 2.0 from Amersham using primers complementary to the coding strand of *cheY* in M13mp18.

The plasmid-borne *cheY* mutant alleles were transferred onto the chromosome of OIB055 using gene conversion selecting for kanamycin resistance (Kan^R) (from the plasmid) and screening for chloramphenicol sensitivity (Cm^S) (due to loss of the *cheY*::*cat* allele from the chromosome). Chromosomal DNA from the mutants was prepared as described by Marmur (1961). Southern blots were performed on nitrocellulose. Probes were radiolabeled using a random primer kit from Bethesda Research Laboratories and [α-^{32}P]-dCTP from ICN.

Swarm Plate and Microscopic Analysis of Bacteria. Bacterial swarming and swimming behavior were analyzed as previously described (Bischoff & Ordal, 1991).

Tethering Assays. The method used to tether bacteria has been described (Berg & Tedesco, 1975; Berg & Block, 1984). In order to analyze the behavior, a computer program was written to obtain a time-dependent behavioral profile of a collection of bacteria. Each bacterium was subjected to the addition and removal of the attractant azetidine 2-carboxylic acid (Ordal et al., 1978), a nonmetabolizable proline analogue, over a period of 8 min. The behavior was videotaped on a Panasonic 1960 VCR from images seen under phase-contrast microscopy. Each bacterium was then analyzed separately by manually entering the behavior observed into a computer database. Each time the bacterium changed the direction of rotation, a key was pressed indicating the new direction. This continued throughout the given time period. Thus each keystroke recorded the time of change and the direction of rotation of the cell body. An observed clockwise rotation of the cell body results from counterclockwise rotation of the tethered flagellum. The behavior was digitized by giving clockwise (CW) rotation of the cell body (smooth swim) a value of 1 and counterclockwise (CCW) rotation (tumble) a value of 0. After an entire set of bacteria was entered, the program averaged the behavior of the set at every 0.25–s interval. Four-second intervals of this data set were then averaged. Counterclockwise flagellar rotation bias, corresponding to the observed clockwise rotation of the cell body, was plotted against time.

Nomenclature. Chemotaxis proteins from *B. subtilis* and *E. coli* are identified in the text by the use of B and E subscripts, respectively.

RESULTS

Purification of CheY. B. subtilis cheY was expressed under a T7 expression system in *E. coli* RP5232, which is deleted for *cheY* to ensure that no *E. coli* CheY ($CheY_E$) copurified with the *B. subtilis* protein ($CheY_B$). $CheY_B$ formed inclusion bodies under these conditions, which were separated from *E. coli* cytoplasmic proteins by pelleting with the membranes. After denaturation and renaturation of the inclusion bodies, the concentrated sample was fractionated by size-exclusion chromatography. $CheY_B$ is a rather small protein (14 kDa),

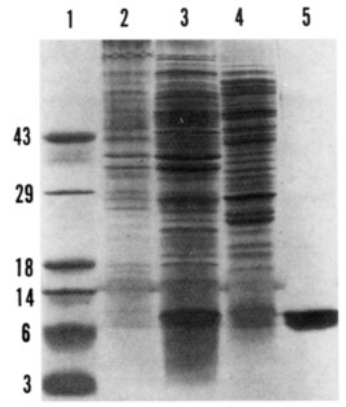

FIGURE 1: SDS–PAGE analysis of $CheY_B$ purification. Purification was performed as described under Experimental Procedures. *cheY* was expressed under the T7 promoter in pDB16 in *E. coli* strain RP5232, which is deleted for *E. coli cheY*. Lane 1, BRL molecular weight standards; lane 2, French-pressed RP5232 with the parent plasmid pT7-6; lane 3, French-pressed RP5232 expressing *cheY* from pDB16; lane 4, protein fraction after renaturation; lane 5, pooled P-30 fractions containing CheY.

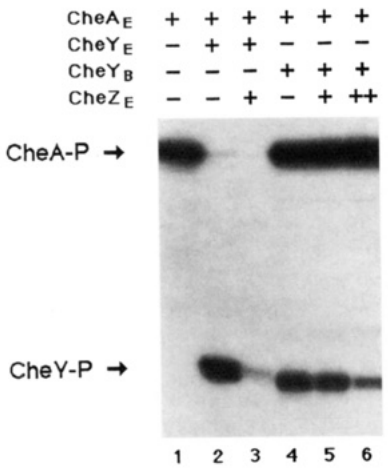

FIGURE 2: *In vitro* phosphorylation of $CheY_B$ by $CheA_E$. The experiment was performed as described in Experimental Procedures. Each reaction contained 15 pmol of $CheA_E$ and 0.5 mM [γ^{32}P]ATP and lasted 2.5 min. Lanes 2 and 3 contained 60 pmol of $CheY_E$, whereas lanes 4–6 contained 60 pmol of $CheY_B$. $CheZ_E$ was present at 15 pmol in lanes 3 and 5 and 60 pmol in lane 6.

so it is easily separated from the other *E. coli* proteins on a Bio-Rad Bio-Gel P-30 column. The purification was monitored by Coomassie staining after SDS–PAGE (Figure 1). N-Terminal amino acid sequence analysis confirmed that the protein purified was $CheY_B$. This procedure yielded approximately 1.5 mg of pure $CheY_B$ from 2 L of cells.

Phorphorylation of B. subtilis CheY by E. coli CheA in Vitro. To test whether $CheY_B$ could be phosphorylated, it was incubated with *E. coli* CheA ($CheA_E$) and [γ-^{32}P]ATP. In the absence of CheY, $CheA_E$ autophosphorylated and was labeled with radioactive phosphoryl groups (Hess et al., 1978, 1988b) (Figure 2, lane 1). In the presence of $CheY_E$, the label was transferred from $CheA_E$ to $CheY_E$ (Hess et al., 1988a; Wylie et al., 1988) (Figure 2, lane 2). $CheA_E$-P was no longer observed under these reaction conditions, which employed a 4-fold molar excess of $CheY_E$. Inclusion of $CheZ_E$ in the reaction stimulated $CheY_E$ dephosphorylation and thus reduced the level of $CheY_E$-P (Hess et al., 1988a, b) (Figure 2, lane 3).

$CheA_E$ phosphorylated $CheY_B$ (Figure 2, lane 4). The amount of $CheA_E$-P observed in the presence of $CheY_B$ increased significantly in comparison to that observed in the

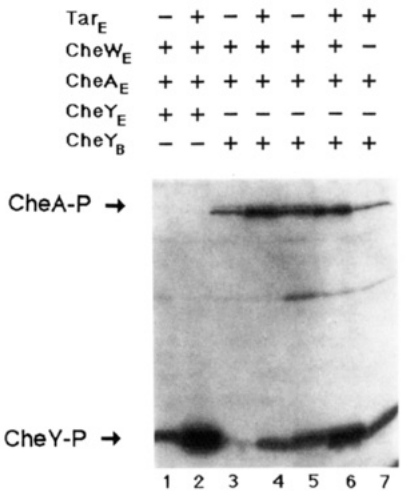

FIGURE 3: *In vitro* phosphorylation of $CheY_B$ by $CheA_E$ in the presence of Tar_E and $CheW_E$. The experiment was performed as described in Experimental Procedures. Each lane contained 1 pmol of $CheA_E$ and 0.5 mM [γ^{32}P]ATP, and 20 pmol of $CheW_E$ was present in all but lane 7. Each reaction also contained 60 pmol of CheY ($CheY_E$ in lanes 1 and 2 and $CheY_B$ in lanes 3–7) and membranes with 6 μg of protein (–Tar_E, i.e., HCB721/pBR322, in lanes 1, 3, and 5; +Tar_E, i.e., HCB721/pNT201, in lanes 2, 4, 6, and 7). The reactions in lanes 1–4 were for 10 s, whereas those in lanes 5–7 were 60 s. Note the higher background of $CheY_B$-P formed by unstimulated $CheA_E$ in the longer reactions.

presence of $CheY_E$ (Figure 2, cf. lanes 2 and 4), suggesting that phosphotransfer to the $CheY_B$ protein is less efficient. Increasing the reaction time from 2.5 to 10 min did not increase the amount of $CheY_B$ labeled (data not shown). $CheZ_E$ stimulates dephosphorylation of $CheY_B$-P (Figure 2, lanes 5 and 6). An increased amount of $CheZ_E$ was necessary to easily observe the dephosphorylation reaction, because it was less efficient than with $CheY_E$-P.

Formation of $CheY_E$-P by $CheA_E$ was enhanced in the presence of CheW and the methyl-accepting chemotaxis protein Tar from *E. coli* (Borkovich et al., 1989) (Figure 3, lanes 1 and 2). Phosphorylation of $CheY_B$ was also enhanced by the addition of Tar_E and $CheW_E$ (Figure 3, lanes 3 and 4). Prolonged incubation (60 s) increased the label in $CheY_B$, but the extent of labeling was still less than that observed in a 10-s reaction with $CheY_E$ (Figure 3, cf. lanes 2 and 6). Stimulation of $CheY_B$-P formation was dependent on both Tar_E and $CheW_E$ (Figure 3, lanes 5–7).

Mutagenesis of cheY. To further investigate the role of CheY in *B. subtilis* chemotaxis, mutations were created in two residues of $CheY_B$ whose corresponding residues are important for function of $CheY_E$. *Salmonella typhimurium* CheY ($CheY_S$), which shares 98% amino acid sequence identity with $CheY_E$ (Matsumura et al., 1984; Stock et al., 1985), is phosphorylated at Asp57 (Sanders et al., 1989). All eight amino acid substitutions tested to date that alter Asp57 of $CheY_E$ or $CheY_S$ result in the null phenotype; i.e., they eliminate chemotaxis and cause exclusively CCW flagellar rotation (Sanders et al., 1989; Bourret et al., 1990, 1993). Amino acid sequence comparison suggests that the likely site of $CheY_B$ phosphorylation is Asp54 (Bischoff & Ordal, 1991). Asp54 was therefore changed to Ala (CheYDA54), Ser (CheYDS54), or Thr (CheYDT54). The mutant alleles were each transferred to the *B. subtilis* chromosome using gene conversion and verified by Southern hybridization (data not shown). The $CheY_B$ Asp54 mutants all exhibited behavior indistinguishable from the *B. subtilis* Δ*cheY* mutant OIB057: no chemotaxis on swarm plates, tumbly swimming behavior, and exclusively CW flagellar rotation as determined

by tethering assays (data not shown). These results strongly suggest CheY phosphorylation is essential for *B. subtilis* chemotaxis.

Replacement of Asp13 with either Arg or Lys activates $CheY_E$ to cause CW flagellar rotation in the absence of phosphorylation (Bourret et al., 1990, 1993). The analogous mutations (CheYDK10 and CheYDR10) were created in $CheY_B$, transferred to the *B. subtilis* chromosome, and verified by Southern hybridization. In contrast to the $CheY_E$ case, the $CheY_B$ mutants exhibited the null phenotype rather than the opposite, activated behavior. The Asp10 mutants tumbled, showed exclusively CW flagellar rotation, and showed no chemotaxis on swarm plates. In $CheY_E$, the DK13 and DR13 mutant proteins are activated but are clearly not as efficient as wild-type $CheY_E$-P. The activated phenotype is stronger when the mutant *cheY*$_E$ alleles are expressed from multicopy plasmids than from a single copy on the chromosome (Bourret et al., 1993). Plasmids expressing $CheY_B$DK10 and $CheY_B$-DR10 were introduced into the mutant strains containing the corresponding mutations. Swarm plate and tethering analyses both confirm that these mutant forms of CheY exhibit the null rather than the activated phenotype, even when expressed at higher levels (data not shown). The failure of the Asp10 mutations to activate $CheY_B$ suggests the interaction between CheY and the flagellar switch is different in *B. subtilis* and *E. coli* or that $CheY_B$DK10 is not in an "active" conformation.

Expression of Mutant B. subtilis cheY Alleles in Wild-Type E. coli. Overproduction of $CheY_E$ in wild-type *E. coli* results in tumbling behavior (Clegg & Koshland, 1984). Overproduction of $CheY_B$ in wild-type *E. coli* also inhibits chemotaxis but results in smooth swimming behavior (Bischoff & Ordal, 1991). To further investigate the properties of the mutant $CheY_B$ proteins, DNA carrying the mutant alleles of *B. subtilis cheY* was subcloned into the IPTG-inducible expression vector pEB112. The wild-type *E. coli* strain RP437 was transformed with these plasmids and the transformants tested on TBr (Tryptone broth: 1% Tryptone, 0.5% NaCl, and 0.27% agar) swarm plates with varying concentrations of IPTG. All five $CheY_B$ mutants inhibited *E. coli* chemotaxis in a manner similar to wild-type $CheY_B$, starting at 10 μM IPTG and reaching maximal effect at 316 μM IPTG (Figure 4). Microscopic observation revealed that the transformants exhibited smooth swimming behavior (data not shown). The ability of the Asp54 mutants to inhibit *E. coli* chemotaxis suggests the interaction responsible does not require phosphorylation. The failure of the Asp10 mutants to cause tumbling behavior indicates $CheY_B$ cannot interact with the *E. coli* flagellar switch in the same manner that $CheY_E$ does.

Expression of B. subtilis cheY in the B. subtilis ΔcheY Strain OIB057. Tethering assays were performed to further examine the role of CheY in *B. subtilis* chemotactic signal transduction. The Δ*cheY* mutant tumbled incessantly (Bischoff & Ordal, 1991) and exhibited exclusively CW flagellar rotation (Figure 5). Furthermore, the deletion mutant did not respond to the addition or removal of the attractant azetidine 2-carboxylic acid. A plasmid expressing wild-type *B. subtilis cheY* complements the defect in this strain, so the resulting bacteria approximate wild-type behavior, including response to the addition and removal of attractant (Figure 5).

Expression of B. subtilis cheY in Wild-Type B. subtilis Strain OI1085. Expression of wild-type *B. subtilis cheY* in the wild-type *B. subtilis* strain OI1085 did not appear to inhibit chemotaxis on a swarm plate (Bischoff & Ordal, 1991). This strain responded to addition of attractant in a tethering assay by rotating its flagella CCW; however, the bacteria did not recover prestimulus behavior during the observation period (Figure 6).

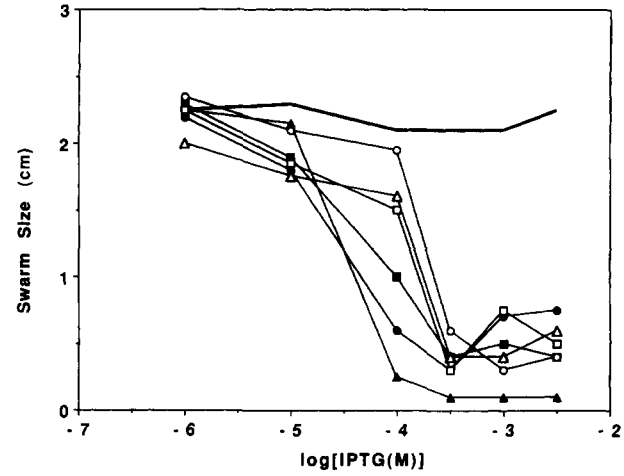

FIGURE 4: Effect of expression of *B. subtilis cheY* tumbly alleles on swarm size of wild-type *E. coli* strain RP437. The experiment was performed as described under Experimental Procedures. Strains are as follows: RP437 expressing wild-type *cheY*$_B$ from pDB21 (▲), CheYDK10 from pDB43 (■), CheYDR10 from pDB51 (●), CheYDT54 from pDB52 (△), CheYDS54 from pDB53 (□), CheYDA54 from pDB56 (○), and the control with pEB112 (heavy line with no symbol).

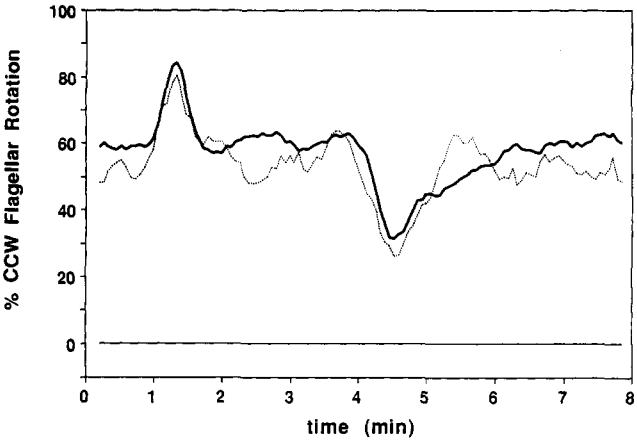

FIGURE 5: Tethering assays of various *B. subtilis* strains. The experiment was performed as described under Experimental Procedures. The attractant 10 μM azetidine 2-carboxylic acid was added at 1 min and removed at 4 min. Strains used are as follows: wild-type strain OI1085 (bold line), Δ*cheY* strain OIB057 (thin line), and Δ*cheY* strain OI3023 expressing *cheY* from pDB21 (dotted line). IPTG (1 mM) was added during the growth of strains to induce the expression of plasmid-encoded genes.

DISCUSSION

Phosphorylation of CheY$_B$. This study shows that purified $CheY_B$ can be phosphorylated by $CheA_E$ (Figure 2). The reaction appeared to be less efficient than phosphorylation of $CheY_E$ by $CheA_E$; however, the degree of which renaturation of the $CheY_B$ preparation was successful is unknown. Phosphorylation of $CheY_B$ was enhanced by the addition of Tar_E and $CheW_E$, similarly to $CheY_E$ phosphorylation (Figure 3). In *E. coli*, $CheA_E$ exists in a ternary complex with $CheW_E$ and the receptors (MCPs) so that events at the receptors affect the rate of $CheA_E$ autophosphorylation (Borkovich & Simon, 1990; Ninfa et al., 1991; Gegner et al., 1992). It should be noted that although $CheW_E$ was required for stimulated phosphorylation of $CheY_B$ by $CheA_E$, $CheW_B$ is not required for activation of $CheA_B$ (Hanlon et al., 1992a,b). Presumably any substrate for CheA would become more phosphorylated in the presence of $CheW_E$ and the receptors.

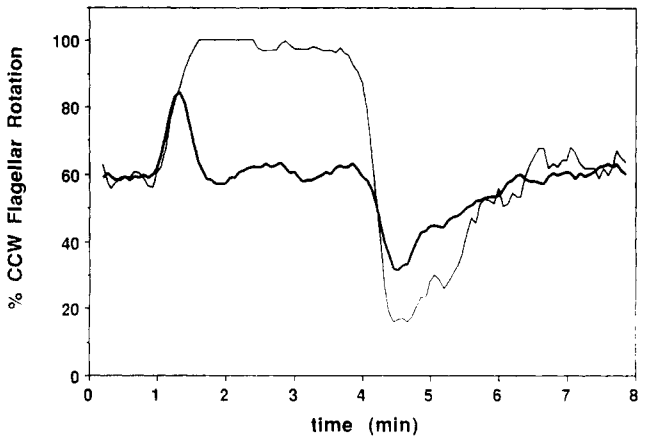

FIGURE 6: Tethering assays of wild-type *B. subtilis* strains with and without expression of *cheY*. The experiment was performed as described under Experimental Procedures. The attractant 10 μM azetidine 2-carboxylic acid was added at 1 min and removed at 4 min. Strains used are as follows: wild-type strain OI1085 (bold line) and wild-type strain OI3021 expressing $cheY_B$ from pDB21 (thin line). IPTG (1 mM) was added during the growth of strains to induce the expression of plasmid-encoded genes.

Some 26 kb of *B. subtilis* DNA encoding flagellar and chemotaxis proteins has been sequenced to date, but a *B. subtilis cheZ* gene has not yet been identified (Bischoff & Ordal, 1992). Nevertheless, $CheY_B$ is susceptible to dephosphorylation stimulated by $CheZ_E$ (Figure 2). $CheY_B$ shares only 36% amino acid sequence identity with $CheY_B$ (Bischoff & Ordal, 1991); yet $CheY_B$ can productively interact with both $CheA_E$ and $CheZ_E$. Biochemical reactions utilizing heterologous components, in conjunction with amino acid sequence comparison, may help identify the portions of each protein necessary for various interactions.

Role of CheY Phosphorylation in B. subtilis Chemotaxis. $CheA_E$ and $CheY_E$ null mutants exhibit smooth swimming behavior (Parkinson, 1978). In contrast, $CheA_B$ and $CheY_B$ are each involved in the smooth swimming response to *B. subtilis*, inasmuch as the respective null mutants exhibit tumbly swimming behavior (Bischoff & Ordal, 1991; Fuhrer & Ordal, 1991). Elimination of the putative site of phosphorylation in $CheY_B$, Asp54 by site-directed mutagenesis similarly results in tumbly behavior. This result further suggests that phoshorylated $CheY_B$ is the species required for smooth swimming by *B. subtilis*.

Role of CheY in B. subtilis Swimming Behavior. Behavioral experiments using tethered cells were carried out to further explore the role of CheY in *B. subtilis* behavior. The *cheY* null mutant OIB057 exhibited exclusively CW flagellar rotation, even upon stimulation by sufficient attractant, azetidine 2-carboxylic acid, to titrate 50% of the receptors (Ordal et al., 1978) (Figure 5). In view of the extreme degree of CW rotation, it is surprising that OIB057 tumbled and showed absolutely no forward progress when observed in the microscope (Bischoff & Ordal, 1991). Inverse swimming, typical of extreme CW mutants of *S. typhimurium* and the result of bundling of CW rotating flagella (Kahn et al., 1978), apparently does not occur in *B. subtilis*.

The *B. subtilis cheY* null mutant was complemented by expressing $cheY_B$ from a plasmid. The response to addition or removal of an attractant was similar to wild type (Figure 5). By contrast, a wild-type *B. subtilis* host induced for expression of $cheY_B$ from a plasmid exhibited unusual behavior upon tethering. These bacteria did not adapt to addition of an attractant during the 3-min exposure but did adapt to removal of attractant (Figure 6). This is reminiscent of the behavior of a *B. subtilis cheB* null mutant (Kirsch et al., 1993) and may be explained by assuming that in addition to an abundance of CheY-P, excess CheY titrates phosphoryl groups from CheA so that a CheB-P deficiency results. In this scheme CheB-P would promote adaptation to attractant stimuli (Kirsch et al., 1993).

The strategy and signaling mechanism of chemotaxis in *B. subtilis* thus appears to be inverted in comparison to *E. coli*. The default behavior in *B. subtilis* is apparently tumbling, which can be changed to smooth swimming by CheY-P. The default behavior in *E. coli* is smooth swimming, which can be changed to tumbling by CheY-P. In both cases, CheY-P is thought to interact with the flagellar switch to implement an appropriate response to environmental conditions. The *B. subtilis* flagellar switch consists of FliG and FliM, as in *E. coli*, but also of FliY, which is similar in amino acid sequence near its carboxyl terminal end to *E. coli* FliN but is much larger (42 vs 14 kDa). The failure of the $CheY_B$ mutants substituting Arg or Lys for Asp10 to cause smooth swimming, in contrast to the tumbling (activation) caused by the analogous $CheY_E$ Asp13 mutants, is consistent with a different interaction between CheY and the switch in the two species.

An additional difference between the two organisms is that attractants cause an increase in methyl group turnover on the *B. subtilis* MCPs. This appears to be required for adaptation because a null mutant lacking CheB swims smoothly upon addition to attractant but adapts poorly (Kirsch et al., 1993). Phosphorylation of CheB by CheA may enhance the removal of methyl groups from the MCPs. The methyl groups are believed to be transferred from the MCPs to an acceptor, from which methanol eventually arises. The methanol release may occur at the flagellar switch (Bischoff & Ordal, 1992) and it is conceivable that the unique part of the switch (FliY) is involved.

We imagine that during evolution a seminal event (or events) occurred in a progenitor of *E. coli* in which the methylation system was simplified, and the switch was reduced in size (FliY to FliN) and inverted. It would seem advantageous, in fact, for CheY-P to cause tumbling rather than smooth swimming, as in the "*E. coli* scheme". Under deenergizing conditions, when the ATP concentration is low, such bacteria would tend to swim and thus leave, rather than tumble and remain.

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