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Homologies between the *Salmonella typhimurium* CheY protein and proteins involved in the regulation of chemotaxis, membrane protein synthesis, and sporulation

(OmpR/Dye/Spo0A/membrane receptor)

ANN STOCK*†, DANIEL E. KOSHLAND, JR.†, AND JEFF STOCK*

*Department of Molecular Biology, Princeton University, Princeton, NJ 08544; and †Department of Biochemistry, University of California, Berkeley, CA 94720

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ABSTRACT **Chemotactic receptors at the bacterial cell surface communicate with flagellar basal structures to elicit appropriate motor behavior in response to extracellular stimuli. Genetic and physiological studies indicate that the product of the *cheY* gene interacts directly with components of the flagellar motor to control swimming behavior. We have purified and characterized the *Salmonella typhimurium* CheY protein and have determined the nucleotide sequence of the *cheY* gene. Amino acid sequence comparisons showed CheY to be homologous over its entire length (129 residues) to the N-terminal regulatory domain of another protein involved in chemotaxis, the CheB methyl esterase. The entire CheY protein and the regulatory domain of CheB are also homologous to the N-terminal portions of the *Escherichia coli* OmpR and Dye proteins and the *Bacillus subtilis* Spo0A protein. These homologies suggest an evolutionary and functional relationship between the chemotaxis system and systems that are thought to regulate gene expression in response to changing environmental conditions.**

During bacterial chemotaxis receptors at the cell surface modulate motor behavior in response to binding of extracellular stimulatory ligands (for review, see refs. 1–3). These membrane proteins are also controlled by two intracellular enzymes: a transferase (4), which catalyzes the methylation of specific receptor glutamyl residues (5, 6), and an esterase (7), which demethylates these groups. The regulatory interaction between the receptors and the flagellar motor appears to be indirect. Recent results indicate that the product of the *cheY* gene relays information from the receptors to the flagella by interacting directly with components at the flagellar basal structure (8, 9). For instance, missense mutations in *cheY* may be corrected by allele-specific compensatory mutations in genes that encode flagellar proteins (8).

In this communication, we report the purification and characterization of the *Salmonella typhimurium* CheY protein, together with the sequence of the *cheY* gene. Our results indicate that CheY is a M_r 14,000 monomeric protein present at relatively high concentrations, about 20 μM, in the cytosol of wild-type *S. typhimurium* cells. A search for homologies with other proteins revealed that CheY is homologous to a portion of the CheB methyl esterase, and both CheY and CheB are related to a family of bacterial proteins that are thought to be transcriptional regulators. These include the products of a gene from *Bacillus subtilis* that is required for sporulation, *spo0A* (10, 11); a gene that is involved in the regulation of porin expression in *Escherichia coli* and *Salmonella, ompR* (12); and an *E. coli* gene involved in expression of the sex factor F, *dye* or *sfrA* (13). This finding indicates a common evolutionary origin for all these regulatory systems and suggests that similar molecular mechanisms may be involved.

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MATERIALS AND METHODS

Strains and Plasmids. *Salmonella* strains were derived from the chemotactically wild-type LT2 variant, ST1 (14, 15). The plasmid, pGK24, used to overproduce the CheY protein, is a derivative of pBR322 containing *cheY* and *cheZ* under control of the *lac* promoter (16). *E. coli* MM294*recA* (from B. Ames, Univ. of California, Berkeley) was used as host. The *cheY* gene used for sequencing was from pME1, a pUC12 derivative containing the *Meche* operon as part of a 10-kilobase *Pst* I genomic DNA fragment from *S. typhimurium* ST1. A 4-kilobase *Sma* I fragment of pME1 encoding two-thirds of *tar, cheR, cheB, cheY,* and half of *cheZ* was inserted in both orientations into the polylinker region of M13 mp10 (17) to produce two recombinant M13 phage, M13 melA and M13 melB. An *E. coli* RecA$^-$ strain, JM109 (obtained from J. Messing, Univ. of Minnesota) was used as host for all cloning and sequencing procedures.

Preparation of Anti-CheY Antibodies. *E. coli* MM294-pGK24 was grown at 37°C on low-sulfate 0.1 mM Vogel–Bonner medium (18) containing [^{35}S]sulfate (ICN) and ampicillin at 40 μg/ml. The cells were harvested by centrifugation at 10,000 × *g*, disrupted by freezing and thawing in lysis buffer (19), and subjected to isoelectric focusing in a gradient from pH 4.5 to pH 6.5 (ampholytes from Serva, Heidelberg) on vertical polyacrylamide slab gels. Protein was visualized by autoradiography. Strips containing CheY were excised, equilibrated in 2% $NaDodSO_4$, and electrophoresed in 15% polyacrylamide according to the method of Laemmli (20). Gels were autoradiographed, regions containing CheY were excised, and protein was recovered by electroelution. Approximately 0.2 mg of CheY [from 1 g (wet weight) of cells] was used to raise rabbit anti-CheY antisera (21).

Purification of CheY. *E. coli* MM294pGK24 was grown at 37°C to a density of approximately 10^9 cells per ml in L broth (22) containing ampicillin at 25 μg/ml. The cells were harvested and 50 g (wet weight) was suspended in 150 ml of 0.1 M sodium citrate/1 mM EDTA, pH 6.5, and lysed in a Raytheon model DF101 sonicator. Membranes and large debris were removed by centrifugation at 100,000 × *g* for 90 min. The supernatant fraction was titrated to pH 4.5 with 1 M HCl, and the precipitate was removed by centrifugation at 12,000 × *g* for 15 min. The supernatant was then adjusted to pH 2.9 with 1 M HCl and the precipitate was collected by centrifugation. The precipitate was resuspended in and dialyzed against 10 mM piperizine hydrochloride, pH 5.0, applied to a DE-52 (Whatman) column (2.2 × 26 cm), and eluted with a linear gradient of 0–75 mM NaCl in piperizine buffer. Fractions containing CheY were pooled, concentrat-

ed using a Millipore immersible CX-10 ultrafiltration unit, dialyzed against 0.1 M sodium citrate/1 mM EDTA, pH 6.5, and applied to a Sephadex G-50 (Pharmacia) column (2.2 × 48 cm) equilibrated with citrate buffer. Fractions containing CheY were pooled, dialyzed against 5 mM Tris·HCl, pH 7.5, and applied to a DE-52 column (1 × 18 cm). CheY was eluted with a linear gradient of 0–0.25 M NaCl in Tris buffer. Double-diffusion immunoassays (23) with rabbit anti-CheY antisera were used to assay for CheY protein. All procedures were conducted at 0–4°C.

Determination of the Nucleotide Sequence of the *S. typhimurium cheY* Gene. Processive deletions through the *Meche* inserts in M13 melA and M13 melB were constructed by exonuclease III (New England Biolabs) digestion of the original phage. The *cheY* sequence was determined on 100% of both strands by the dideoxynucleotide chain-termination procedure (24) using DNA polymerase I Klenow fragment (Bethesda Research Laboratories), an M13 universal primer (New England Biolabs), and the deletion phage plus strand DNA as template.

RESULTS

Purification and Characterization of the CheY Protein. CheY was purified to apparent homogeneity (approximately 200-fold with a 20% yield) from a strain containing the *cheY* gene on a multicopy plasmid (Fig. 1). The purified protein had an isoelectric point of 5.0 and a molecular weight of 14,000 as determined by isoelectric focusing and polyacrylamide gel electrophoresis under denaturing conditions. When pure CheY or CheY immunoprecipitated from wild-type *S. typhimurium* was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, two closely spaced bands could be resolved (Fig. 2). The basis for this differential migration has not been determined. There are no cysteine residues in CheY that could account for a tertiary structure resistant to denaturation nor are there any known posttranslational modifications. Neither phosphorylation nor methylation of CheY was detected when cells were grown in the presence of [^{32}P]phosphate or incubated with [*methyl*-^{3}H]methionine in the presence of chloramphenicol. Molecular sieve chromatography of pure CheY under nondenaturing conditions indicated a molecular weight of approximately 14,000, corresponding to that of the CheY monomer. A similar molecular weight was observed when crude cell extracts from *E. coli* or *S. typhimurium* were subjected to molecular sieve chromatography. Affinity columns prepared with rabbit anti-CheY antibodies failed to bind significant quantities of any protein except CheY from extracts prepared from *S. typhimurium* ST1.

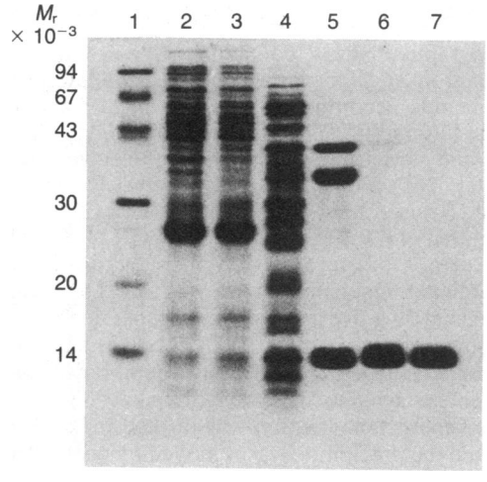

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of fractions from the CheY purification. Lanes: 1, molecular weight markers; 2, 75 μg of crude cell extract; 3, 75 μg of the 100,000 × *g* supernatant; 4, 75 μg of the acid-precipitation pellet; 5, 50 μg of pooled fractions from the pH 5.0 DE-52 chromatography; 6, 25 μg of pooled fractions from Sephadex G-50 chromatography; 7, 25 μg of pooled fractions from the pH 7.5 DE-52 chromatography.

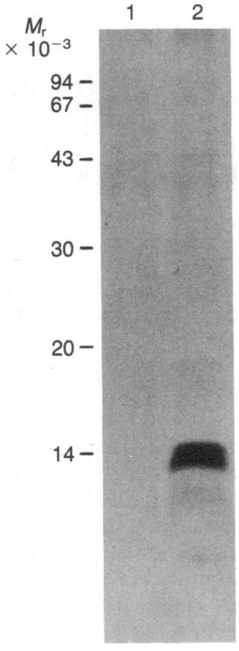

FIG. 2. Autoradiograph of CheY immunoprecipitated from wild-type and CheY deficient strains. A Fla$^-$ strain deleted in *cheY*, ST426 (lane 1), and wild-type *S. typhimurium* (lane 2) were grown in low-sulfate (0.1 mM) Vogel–Bonner citrate medium (18) containing 1% glycerol and $^{35}SO_4$ to a density of 10^9 cells per ml, harvested by centrifugation at 10,000 × *g* for 10 min, lysed by heating at 95°C for 5 min in 1% $NaDodSO_4$ in phosphate-buffered saline (12.5 mM sodium phosphate/0.2 M NaCl, pH 7.5), and diluted 1:30 into phosphate-buffered saline containing 1% Triton X-100. These extracts were used for immunoprecipitation with rabbit anti-CheY antisera and IgGsorb (The Enzyme Center, Malden, MA) as described (25). Immunoprecipitates were solubilized in 2% $NaDodSO_4$ and electrophoresed on a $NaDodSO_4$/15% polyacrylamide gel, and the gel was autoradiographed.

Involvement of CheY in the Methylation of Chemotaxis Proteins. We have previously shown that *cheY* mutants exhibit essentially wild-type increases in receptor methylation in response to attractant stimulation (26). *In vitro* methylation and demethylation assays with extracts prepared from wild-type *Salmonella* and *E. coli* showed that neither transferase nor esterase activity was affected by saturating levels of anti-CheY antibody. Thus, CheY probably does not play a central role in the regulation of receptor methylation. Rates of methylation in *S. typhimurium cheY* mutants did tend to be slightly higher than those in wild type (Fig. 3). This effect may be caused by a rather indirect mechanism, however. For instance, CheY might compete with the transferase for a common binding site on the receptor or for binding of a common small molecule.

Nucleotide Sequence of the *cheY* Gene. The nucleotide sequence of *cheY* was determined as part of the sequence of the *S. typhimurium Meche* operon. The 387-base-pair open reading frame (Fig. 4) was identified as *cheY* by comparison of the predicted N-terminal amino acid sequence with the N-terminal sequence of the purified CheY protein (residues underscored in Fig. 4 starting from alanine-2 of the predicted sequence). The initial formylmethionine appears to be cleaved in the mature protein. The nucleotide sequence predicts a protein with a molecular weight of 13,980 and a pI of approximately 5.0, values consistent with those determined for the purified CheY protein. The amino acid sequence of *S. typhimurium* CheY is 98% homologous with that of *E. coli* CheY (27). Residues phenylalanine-51, isoleucine 54, and serine-76 in *Salmonella* are replaced in *E. coli* by tyrosine, valine, and glycine, respectively. There is also considerable homology at the nucleic acid level. The coding regions of the *S. typhimurium* and *E. coli* genes are 83% homologous with stretches of identical sequence extending as much as 40 base pairs.

The translational initiation region of *cheY* is of special interest because of the relatively high levels of expression of

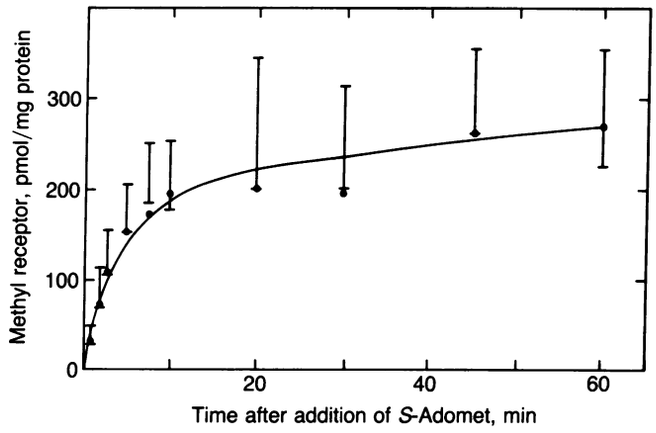

FIG. 3. Rates of receptor methylation in *S. thyphimurium cheY* mutants. Cells were grown in nutrient broth, treated with toluene, and incubated with *S*-adenosyl[*methyl*-^{3}H]methionine (*S*-AdoMet) as described (26). At the indicated times, the reactions were quenched in 10% trichloroacetic acid. The cells were then filtered on Whatman GF/F glass fiber filters, washed with 0.1 M NaCl, and analyzed for [^{3}H]carboxylmethyl groups by the microdistillation procedure (26). Results (I) represent ranges of values obtained with five independently isolated *cheY* mutants (ST112, ST201, ST205, ST305, and ST1001); ● represents the values obtained with wild-type cells (ST1).

this gene (28). From immunoprecipitation assays we estimate that CheY is present at a concentration of roughly 20 μM in the cytosol of wild-type *Salmonella* cells. The intergenic region between the *cheY* initiation codon and the termination codon of the preceding gene, *cheB*, is small in both *E. coli* and *Salmonella*. The *E. coli* sequence, TAAATCAGGAGTGT-GAAATG is not the same as that found in *Salmonella*, TGAACCAGGAGTAGTATTTTATG. Although both contain the same AGGAG Shine–Dalgarno ribosomal binding site (29, 30) three bases distal to the *cheB* termination codon, the remainder of the sequence is different. There are, for instance, three more bases between the ribosomal binding site and the initiation codon in *Salmonella*. This lack of conservation suggests that sequences outside the immediate region of translational initiation may be important for the elevated expression of *cheY*.

Amino Acid Homologies Between CheY and Other Proteins. The predicted amino acid sequence of CheY was first compared to the sequences of the other products of the *S. typhimurium Meche* operon: Tar, CheR, CheB, and CheZ. The only significant relationship detected by the FASTP search algorithm of Lipman and Pearson (31) was between CheY and the receptor demethylating enzyme, the CheB methylesterase. Essentially the entire CheY protein was found to be homologous to the N-terminal third of CheB (Fig. 5 and Table 1). A search was also conducted comparing CheY to the sequences in the Dayhoff collection (32). The only significant relationship was with the *E. coli* OmpR protein (Fig. 5 and Table 1). As in the CheY–CheB relationship, the CheY–OmpR homology involves essentially the entire length of CheY and covers the N terminus of OmpR. Comparison of CheB with OmpR indicated a corresponding relationship between the N termini of these proteins. CheY, CheB, and OmpR have identical residues at 16 positions while 13 residues are specifically shared between CheY and CheB, 18 are specific to the CheY–OmpR homology, and 17 are shared only by CheB and OmpR. Thus, the three proteins seem almost equally related with no specific pair more homologous than any other.

OmpR controls the gross permeability properties of the cell by acting as a positive transcriptional regulator of porin expression (33, 34). OmpR is cotranscribed with one other gene, *envZ* (33), the product of which appears to be a membrane protein with an overall structure analogous to that of the chemotaxis receptors (35). There are two regions of limited homology between EnvZ and the chemoreceptors (36–39): a 60-amino acid stretch at their N termini (20% identity) and a 150-residue region near their C termini (16% identity). Genetic studies indicate that EnvZ regulates the activity of OmpR in response to changes in osmotic pressure (33). Thus, it seems likely that the systems responsible for osmoregulation and chemotaxis in *E. coli* share, at least in part, a common evolutionary origin.

Since this search, new protein sequences have become available, and two of these, predicted from the nucleotide sequences of the *E. coli dye* (13) and *Bacillus subtilis spo0A* genes (10), have been shown to be related to OmpR. Therefore, we compared both CheY and CheB to Dye and Spo0A (Fig. 5 and Table 1). CheY shares approximately the same degree of identity with these proteins as with OmpR, and the homologies extend over corresponding N-terminal regions. Whereas the CheB–Dye relationship is also similar to that between CheB and OmpR, the CheB–Spo0A homology is considerably more extensive. The entire length of Spo0A is homologous to the N-terminal two-thirds of the CheB protein. The relationship between Spo0A and CheB seems to be significantly greater than that between Spo0A and any other protein. In all cases, however, similarities are greatest between N-terminal sequences spanning a region roughly equivalent to the length of CheY. Thus, the CheY protein seems to be homologous to a domain that recurs in modified form in at least four other proteins, each of which plays a central role in a different set of regulatory interactions.

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                                        CGATACGTATTTGAACCAGGAGTAGTATTTT ATG GCG GAT AAA GAG   15
                                                                        Met Ala Asp Lys Glu

CTT AAA TTT TTG GTT GTG GAT GAC TTT TCG ACC ATG CGT CGT ATC GTG CGC AAC CTC TTA   75
Leu Lys Phe Leu Val Val Asp Asp Phe Ser Thr Met Arg Arg Ile Val Arg Asn Leu Leu

AAA GAG CTT GGA TTT AAC AAT GTG GAA GAG GCC GAA GAC GGC GTC GAT GCG CTG AAC AAG  135
Lys Glu Leu Gly Phe Asn Asn Val Glu Glu Ala Glu Asp Gly Val Asp Ala Leu Asn Lys

CTC CAG GCG GGC GGC TTT GGT TTT ATT ATC TCC GAC TGG AAC ATG CCG AAC ATG GAT GGC  195
Leu Gln Ala Gly Gly Phe Gly Phe Ile Ile Ser Asp Trp Asn Met Pro Asn Met Asp Gly

CTG GAG CTG CTG AAA ACC ATT CGC GCC GAT AGC GCC ATG TCG GCG TTA CCC GTG TTG ATG  255
Leu Glu Leu Leu Lys Thr Ile Arg Ala Asp Ser Ala Met Ser Ala Leu Pro Val Leu Met

GTC ACG GCG GAA GCC AAA AAA GAG AAT ATT ATC GCC GCC GCA CAG GCT GGC GCC AGC GGT  315
Val Thr Ala Glu Ala Lys Lys Glu Asn Ile Ile Ala Ala Ala Gln Ala Gly Ala Ser Gly

TAT GTC GTA AAA CCG TTC ACC GCA GCG ACT CTG GAA GAG AAG CTC AAC AAA ATC TTT GAG  375
Tyr Val Val Lys Pro Phe Thr Ala Ala Thr Leu Glu Glu Lys Leu Asn Lys Ile Phe Glu

AAA CTG GGC ATG TGA GGATGCGATGATGATGCAACCATCTATCAAG
Lys Leu Gly Met End
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FIG. 4. Nucleotide sequence of the *S. typhimurium cheY* gene and predicted amino acid sequence of the CheY protein. The termination and initiation codons of the adjacent genes, *cheB* and *cheZ* (unpublished data), are underscored. The sequence of the N terminus of the purified CheY protein (residues underscored) was determined by sequential Edman degradation using an Applied Biosystems gas-phase analyser.

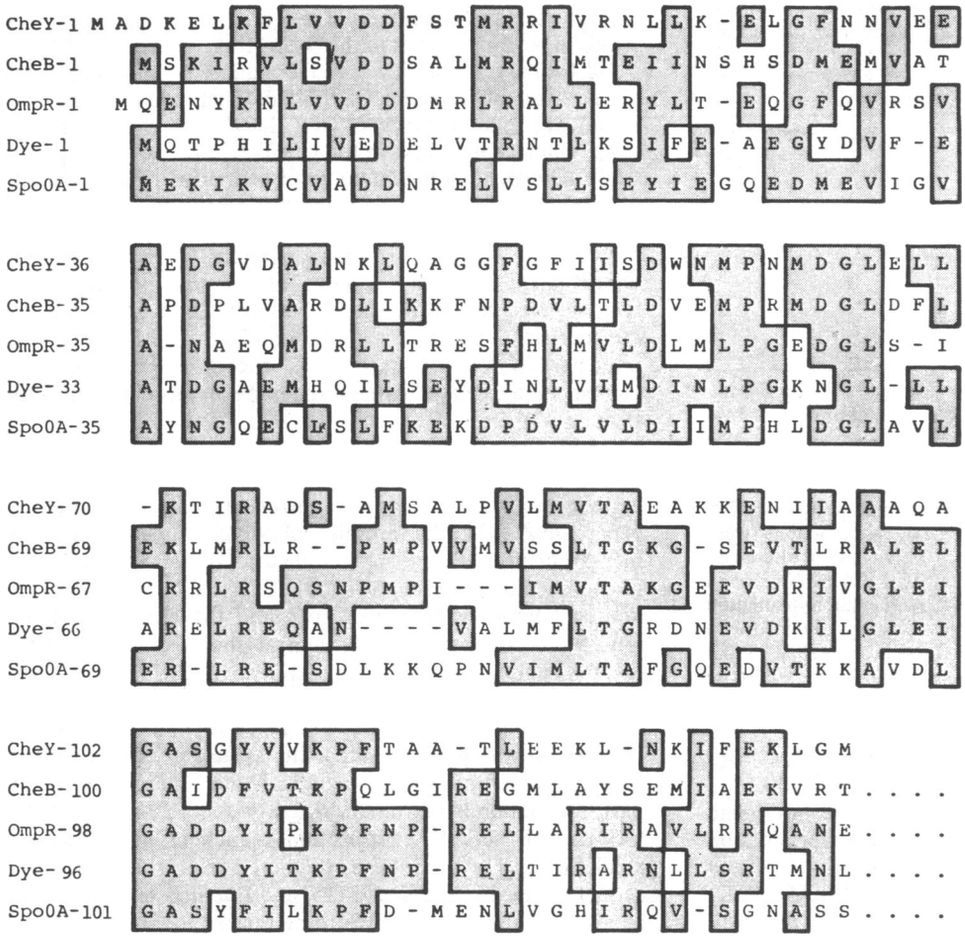

FIG. 5. Amino acid sequence homologies between CheY, CheB, OmpR, Dye, and Spo0A. The standard one-letter code for amino acids is used. Residues that are identical in at least two proteins at a given position are boxed. To display maximal homology, some alignments of individual protein pairs determined by the FASTP program (Table 1) have been slightly altered. Sequences of OmpR, Dye, and Spo0A are from refs. 10–13. The CheB sequence was determined as described in *Materials and Methods* (unpublished data).

DISCUSSION

Our results indicate that CheY is a small protein, M_r = 14,000. The amino acid sequence contains no histidine nor cysteine residues. *Salmonella* CheY is similar to the corresponding protein in *E. coli* (27) with only three conservative amino acid differences. CheY behaves as a monomer during molecular sieve chromatography of cell extracts and is the only cellular component retained by immunoaffinity chromatography. Thus, it seems that the majority of CheY is not involved in a stable complex with any other chemotaxis or flagellar proteins. An analysis of receptor methylation in *cheY* mutants indicates that CheY is not directly involved in regulating levels of receptor modification.

Table 1. Comparison of amino acid sequence homologies between CheY, CheB, OmpR, Dye, and Spo0A

Proteins compared	Region of homology		% identity
	Length	Location	
CheY vs. CheB	105	6–110 vs. 4–108	27
CheY vs. OmpR	124	3–126 vs. 2–122	27
CheY vs. Dye	119	8–126 vs. 6–120	23
CheY vs. Spo0A	115	10–123 vs. 8–122	23
CheB vs. OmpR	104	5–108 vs. 6–106	30
CheB vs. Dye	106	3–108 vs. 2–104	24
CheB vs. Spo0A	225	2–226 vs. 2–224	23
OmpR vs. Dye	229	8–236 vs. 7–235	34
OmpR vs. Spo0A	140	3–139 vs. 2–141	33
Dye vs. Spo0A	235	2–232 vs. 2–236	18

The amino acid sequences of the proteins were aligned using the FASTP computer program (31). The length of each homology is given in total number of aligned residues together with the alignment, counting the formylmethionine encoded by each gene as 1. The statistical significance of the homologies was also evaluated. Similarity scores (31) for each protein pair were compared in terms of standard deviation from the mean to scores obtained when their sequences were randomly shuffled. Values of 3–6 are considered possibly significant, 6–10 probably significant, and >10 significant. The significance scores for the homologies shown here ranged from 5 to 29.

The CheY–CheB sequence homology suggests parallels between the functions of these two proteins. CheB directly interacts with the receptors to catalyze the hydrolysis of glutamyl methyl esters. The enzyme has been shown to be composed of two distinct domains (40). The N-terminal third of the protein, the portion homologous to CheY, comprises a regulatory structure that is not required for esterase activity. Proteolytic removal or genetic deletion of this region results in a greater than 15-fold activation of the enzyme. Repellent stimuli cause a dramatic activation of CheB *in vivo* (41), an effect that parallels the generation of a motor response. Considerable evidence suggests that CheY interacts directly with the flagellar apparatus to control swimming behavior (8, 9). Activation of CheY and CheB by a common receptor-generated signal would provide a mechanism to simultaneously produce a response and initiate its feedback attenuation.

The homology of CheY and CheB to OmpR, Dye, and Spo0A suggests a common mode of action. OmpR acts at the level of transcription to regulate expression of outer membrane proteins in response to changes in extracellular osmotic pressure (33, 34). Dye is thought to function to regulate the expression of several membrane proteins (13). The *B. subtilis* Spo0A protein is required to effect the developmental switch from vegetative growth to endospore formation in response

to conditions of nutrient deprivation (42, 43). Since there is no indication that CheY or CheB acts at the level of transcription, it seems likely that this function of OmpR, Dye, and Spo0A is provided by their C termini. In this respect the proteins may be similar to CheB, each having N-terminal regulatory domains that control C-terminal effector regions. Despite an apparent diversity in effector functions, all five proteins are similar in that they modulate cellular behavior in response to changing environmental conditions. The sequence homologies argue strongly for an evolutionary relationship and raise the possibility that a common mechanism of information processing may be operating in all these systems.

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