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Characterization of the *Thermotoga maritima* Chemotaxis Methylation System that Lacks Methyltransferase CheR:MCP Tethering

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Summary

Sensory adaptation in bacterial chemotaxis is mediated by covalent modifications of specific glutamate and glutamine residues within the cytoplasmic domains of methyl-accepting proteins (MCPs). In *Escherichia coli* and *Salmonella enterica*, efficient methylation of MCPs depends on the localization of methyltransferase CheR to MCP clusters through an interaction between the CheR β-subdomain and a pentapeptide sequence (NWETF or NWESF) at the C terminus of the MCP. *In vitro* methylation analyses utilizing *S. enterica* and *Thermotoga maritima* CheR proteins and MCPs indicate that MCP methylation in *T. maritima* occurs independently of a pentapeptide-binding motif. Kinetic and binding measurements demonstrate that despite efficient methylation, the interaction between *T. maritima* CheR and *T. maritima* MCPs is of relatively low affinity. Comparative protein sequence analyses of CheR β-subdomains from organisms having MCPs that contain and/or lack pentapeptide-binding motifs identified key similarities and differences in residue conservation, suggesting the existence of two distinct classes of CheR proteins: pentapeptide-dependent and pentapeptide-independent methyltransferases. Analysis of MCP C-terminal ends showed that only ~10% of MCPs contain a putative C-terminal binding motif, the majority of which are restricted to the different proteobacteria classes (α, β, γ, δ). These findings suggest that tethering of CheR to MCPs is a relatively recent event in evolution and that the pentapeptide-independent methylation system is more common than the well characterized pentapeptide-dependent methylation system.

Introduction

Most proteins undergo post-translational modifications that alter their function, influencing properties such as localization, lifetime, or activity. To date more than 200 distinct post-translational modifications have been identified, most often coming in the form of proteolytic cleavage events or covalent modifications at specific amino acid residues (Blom *et al.*, 2004; Khidekel and Hsieh-Wilson, 2004). While proteolytic cleavage is an irreversible modification, covalent modifications in many instances are reversible. In prokaryotes, two common reversible modifications are protein phosphorylation and carboxyl methylation. These modifications, performed by a protein kinase or methyltransferase, may be reversed

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by the action of an antagonistic enzyme, a phosphatase or methylesterase. A recent comprehensive census of signal transduction proteins from 167 completed bacterial and archaeal genomes has shown protein methylation to be a key post-translational modification in bacterial signaling, with methyl-accepting chemotaxis proteins (MCPs) being the third most abundant transmembrane signaling proteins ranking behind only histidine kinases and diguanylate cyclases (Galperin, 2005).

In the well-studied chemotaxis systems of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, several homologous transmembrane receptors (MCPs) sense extracellular stimuli, producing signals that are transmitted to their cytoplasmic domains. These domains regulate an associated two-component phosphotransfer signal transduction system that controls flagellar rotation (Bourret and Stock, 2002; Falke and Kim, 2000; Falke and Hazelbauer, 2001). Chemotaxis sensory systems are able to adapt to persistent stimuli, returning cells to their prestimulus behavior and allowing for highly sensitive detection of stimuli over a wide dynamic range. Adaptation is in part mediated by reversible covalent modifications. Specific glutamate residues within the cytoplasmic domains of receptors are methylated by AdoMet-dependent methyltransferase CheR and demethylated by methylesterase/deamidase CheB (Kehry and Dahlquist, 1982; Springer and Koshland, 1977; Stock and Koshland, 1978; Terwilliger and Koshland, 1984). The molecular mechanisms of chemotaxis signaling and adaptation have been most extensively characterized in *E. coli* and *S. enterica* (Bren and Eisenbach, 2000; Falke *et al.*, 1997; Wadhams and Armitage, 2004). Studies of chemotaxis and MCP methylation in other organisms (Armitage and Schmitt, 1997; Astling *et al.*, 2006; Martin *et al.*, 2001; Perazzona and Spudich, 1999; Szurmant and Ordal, 2004; Ward and Zusman, 1997) have revealed both similarities and differences to the *E. coli*/*S. enterica* chemotaxis pathway.

One such difference is the presence of a conserved pentapeptide sequence (NWETF or NWESF) located at the extreme C-terminal end of some transmembrane receptors. While this motif is required for efficient methylation, demethylation, and deamidation in *E. coli* and *S. enterica* (Barnakov *et al.*, 1999; Li and Hazelbauer, 2005; Wu *et al.*, 1996), it is absent from many bacterial MCPs (Shiomi *et al.*, 2002b). In *E. coli* and *S. enterica*, high abundance receptors Tar (aspartate), Tsr (serine), and Tcp (citrate) possess a pentapeptide sequence, while low abundance receptors Trg (ribose-galactose) and Tap (dipeptide) do not. Studies involving truncation and/or mutation of key residues within this pentapeptide motif have demonstrated lowered methylation levels and ineffective tactic responses (Feng *et al.*, 1997; Le Moual *et al.*, 1997; Li *et al.*, 1997; Okumura *et al.*, 1998). Conversely, addition of this pentapeptide sequence to low abundance receptors has been shown to greatly improve methylation (Barnakov *et al.*, 1998; Feng *et al.*, 1999). The crystal structure of *S. enterica* CheR bound to the pentapeptide NWETF identified the binding site within CheR to be the small β-subdomain that is inserted into the C-terminal methyltransferase catalytic domain (Djordjevic and Stock, 1998). This motif provides a high affinity binding interaction (~2 μM K_d) (Wu *et al.*, 1996) that increases CheR concentration at receptor clusters, facilitating methylation of nearby high and low abundance receptors (Le Moual *et al.*, 1997; Li *et al.*, 1997; Li and Hazelbauer, 2005). Thus in *E. coli* and *S. enterica*, interactions between CheR and receptors involve two distinct sets of contacts: the tethering of CheR to the pentapeptide through the β-subdomain and the catalytically productive contact between the methylation regions of the receptor and the active site of CheR. The α2 helix within the N-terminal domain of CheR has been shown to be critical for the latter interaction (Perez *et al.*, 2004; Shiomi *et al.*, 2002b).

Thermotoga maritima is a thermophilic bacteria that thrives at high temperatures (Swanson *et al.*, 1996), making laboratory studies of bacterial physiology difficult. Furthermore, the organism lacks a tractable genetic system. However, *T. maritima* has become a model

system for structural characterization of chemotaxis proteins and signaling complexes. Structures of many *T. maritima* chemotaxis proteins have been determined, in several notable cases where structures of homologs from mesophilic organisms have been unobtainable (Bilwes *et al.*, 1999; Bilwes *et al.*, 2001; Chao *et al.*, 2006; Griswold *et al.*, 2002; Park *et al.*, 2006; Park *et al.*, 2004a; Park *et al.*, 2004b; Quezada *et al.*, 2004; Usher *et al.*, 1998). Recently, studies addressing methylation in *T. maritima* showed four different receptors to be efficiently methylated by *T. maritima* CheR (Perez *et al.*, 2006), despite none possessing a recognizable pentapeptide-binding sequence (Nelson *et al.*, 1999). Receptor methylation in *B. subtilis* and *H. salinarium*, organisms that also lack the pentapeptide, has also been reported (Hanlon and Ordal, 1994; Kirby *et al.*, 1999; Perazzona and Spudich, 1999). Thus, while CheR and CheB interactions with pentapeptide are critical for methylation, demethylation, and deamidation in *E. coli* and *S. enterica*, this is likely not the case for the majority of bacteria.

Interestingly, although MCPs of many bacteria do not contain the C-terminal binding motif, all CheR homologs possess a β-subdomain (Shiomi *et al.*, 2002b). What is the role of the β-subdomain in organisms with receptors that all lack pentapeptide-binding motifs? Are there differences between a β-subdomain that interacts with pentapeptides and one that does not? Can CheR proteins from organisms with receptors that lack pentapeptides recognize the C-terminal binding motif in MCPs from different organisms? In this study, utilizing *S. enterica* and *T. maritima* CheR proteins and receptors, we have begun to address some of these questions. Our results demonstrate that *T. maritima* CheR, unlike *S. enterica* CheR, is not dependent upon pentapeptide binding for efficient methylation. Despite the likelihood that *T. maritima* CheR interacts with receptors only at the sites of methylation, a combination of binding studies and kinetic analyses revealed this to be a low affinity interaction, dissimilar to the high affinity binding exhibited between CheR and the pentapeptide. Finally, comparative protein sequence analyses of the CheR β-subdomain from organisms with receptors containing pentapeptide motifs with those from organisms with receptors lacking pentapeptide-binding motifs indicate key differences between the two groups, defining predictive features and providing further insight into CheR-pentapeptide interactions.

Results

Characterization of cross-species methylation between S. enterica and T. maritima

Cross-species methylation and demethylation of *E. coli*/*S. enterica* receptors was previously demonstrated with CheR proteins from *B. subtilis* and *Rhodobacter sphaeroides* (Burgess-Cassler and Ordal, 1982; Martin *et al.*, 2001) and *T. maritima* CheB protein (Anand and Stock, 2002). Moreover, methylation in *T. maritima* has recently been characterized, with determination of methylation rates for both receptor cytoplasmic domains and full-length transmembrane receptors catalyzed by *T. maritima* CheR (Perez *et al.*, 2006). To further characterize *T. maritima* receptor methylation and to gain insight into methylation in organisms lacking the pentapeptide, we performed cross-species *in vitro* methylation assays utilizing *S. enterica* and *T. maritima* CheR proteins and receptors (wild-type *S. enterica* Tar, WT Tar; *S. enterica* Tar lacking the five C-terminal residues, TarΔpp; and *T. maritima* TM1428; TM1428). Methylation rates, determined at 30°C and 37°C, indicated that both *S. enterica* and *T. maritima* CheR efficiently methylate WT Tar, with rates for *S. enterica* CheR being ~3-fold higher (Table 1). Methylation rates for *T. maritima* CheR versus WT Tar are slightly lower than those observed with its cognate receptor, TM1428 (Perez *et al.*, 2006). Conversely, methylation rates exhibited by *S. enterica* CheR versus TM1428 were significantly lower (~25-fold) compared to *T. maritima* CheR (Table 1). These low methylation rates for *S. enterica* CheR versus TM1428 appear to be due to the absence of the pentapeptide sequence in these receptors, since a similar drop in the rate of methylation was observed when the pentapeptide was eliminated from Tar (TarΔpp) (Table 1). Interestingly,

methylation rates for *T. maritima* CheR with TarΔpp, were no different than those obtained with WT Tar (Table 1). Thus, despite possessing a β-subdomain, *T. maritima* CheR does not appear to interact with the NWETF motif, suggesting that methylation in *T. maritima* is pentapeptide independent.

Effects of C-terminal deletion of TM1428 on methylation

Based on primary sequence alignments, *T. maritima* receptors do not contain a recognizable NWETF motif at the end of a flexible linker that extends beyond the predicted coiled-coil motif. However, to investigate whether a different C-terminal motif might mediate interactions with *T. maritima* CheR, sequence alignments of all six *T. maritima* transmembrane chemoreceptors were generated, revealing strong conservation of residues located at the C termini (Fig. 1A). Previously, alanine scanning mutagenesis of the NWETF binding sequence in *E. coli* Tar suggested that the hydrophobicity and size of the aromatic residues Trp and Phe were critical for interaction with CheR and for efficient methylation (Shiomi *et al.*, 2000). Close examination of the last five residues of *T. maritima* receptors showed the presence of two conserved hydrophobic residues (at the third and fifth positions), including one position containing a bulky aromatic residue (Trp or Tyr) (Fig. 1A). Despite some similarity between *E. coli/S. enterica* and *T. maritima* receptor C termini, the structures adopted by the residues that lead up to the C-terminal ends are different. *E. coli/S. enterica* high abundance receptors possess C-terminal tails (30–35 residues in length) that are predicted to be flexible and are not observed in the crystal structure (Kim *et al.*, 1999; Le Moual and Koshland, 1996). This tail presumably acts as a flexible tether, allowing CheR to methylate neighboring receptors (Le Moual *et al.*, 1997; Li *et al.*, 1997; Li and Hazelbauer, 2005). Also, it has been shown that deletion of all or part of the tail reduces the activity of CheR as well as the other adaptational modifications, indicating its importance for proper chemoreceptor function (Le Moual *et al.*, 1997; Li *et al.*, 1997; Li and Hazelbauer, 2006). However, for *T. maritima* receptors, secondary structure predictions (data not shown) and the recent determination of the crystal structure of the cytoplasmic domain of TM1143 (Park *et al.*, 2006) indicate that an alpha-helical structure is maintained throughout the cytoplasmic domain up to the C terminus, which ends prior to the 30-residue flexible extension of the major *E. coli/S. enterica* receptors. Although *T. maritima* receptors do not possess a flexible tail, the importance of the receptor C terminus for methylation by CheR was investigated. A mutant TM1428 receptor lacking five C-terminal residues, ERFKI, was generated (TM1428Δ5) and methylation rates for this truncated receptor were measured. Results showed that methylation rates for TM1428Δ5 (0.34 ± 0.04 mol CH_3·min-1·mol CheR-1) were similar to those for TM1428 (0.35 ± 0.02 mol CH_3·min-1·mol CheR-1) and that these residues, as expected, did not play any perceptible role in receptor methylation (Fig. 1B).

Characterization of binding and kinetic parameters of methyltransferase CheR and TM1143c

A consensus methylation sequence within *T. maritima* MCPs has been established (Perez *et al.*, 2006). For *T. maritima* and other organisms that contain receptors all lacking a C-terminal binding motif, CheR interactions with MCPs are likely limited to the conserved methylation regions, which could potentially serve dual roles, both as high affinity binding sites and as sites of modification. A precedent exists for this in the case of the *T. maritima* receptor-modifying deamidase/demethylase CheD. Pulldown assays utilizing His-tagged CheD and the cytoplasmic domain of TM1143 (TM1143c) demonstrated high affinity interactions, with CheD-TM1143c complex formation visualized at relatively low concentrations (1.2 μM CheD and 10 μM TM1143c) (Chao *et al.*, 2006). CheD also forms a relatively high affinity complex (K_d = 0.9–1.4 μM) with the α2′ helix of CheC, which mimics the receptor methylation region (Park *et al.*, 2004b, Chao, 2006 #3075). To assess

binding between CheR and TM1143c, pulldown experiments were performed utilizing His-tagged TM1143c. Unlike the high affinity CheD-TM1143c interaction, complex formation of CheR with TM1143c was not observed (data not shown). Attempts to promote complex formation with higher concentrations of CheR and TM1143c (up to 60 μM of each) were unsuccessful. Based on conservative estimates of the detection limits in these assays, we conclude that CheR binding to TM1143c is a low affinity interaction with a theoretical dissociation constant (K_d) >0.6 mM.

To further assess the interactions of *T. maritima* CheR with receptors, initial velocities of methylation were determined over a range of concentrations of TM1143c. Previous kinetic studies of *S. enterica* CheR methylation of Tar receptor-enriched membrane fractions exhibited typical Michaelis-Menton kinetics (Simms *et al.*, 1987). We have shown that soluble *T. maritima* receptor cytoplasmic domains are methylated similarly to their full-length membrane counterparts in *in vitro* methylation reactions (Perez *et al.*, 2006). The kinetic analyses performed in the present study take advantage of using a soluble substrate that can be examined at substantially higher concentrations than are achievable with receptor-enriched membrane fractions. Our results yielded K_m and V_{max} values of 1.14 ± 0.2 μM, and 104 nmol·min^{-1}·mg CheR^{-1}, which is the equivalent to a K_{cat} of 3.6 ± 0.4 mol CH_3·min^{-1}·mg CheR^{-1} for reactions at 30°C (Fig. 2). These kinetic parameters are surprisingly similar to those previously determined for *S. enterica* CheR and Tar, (K_m = 4.2 μM, K_{cat} = 10 mol CH_3·min^{-1}·mg CheR^{-1}) (Simms *et al.*, 1987; Simms, 1991 #428). However, the K_m value for CheR and TM1143c was not reflective of the K_d for CheR-receptor binding. In contrast, binding studies with *S. enterica* CheR and Tar receptor cytoplasmic fragments containing the pentapeptide-binding motif showed it to be a high affinity interaction with a K_d of ~2 μM, similar to the K_m values for both *S. enterica* and *T. maritima* receptor methylation. Consistent with these affinity measurements, CheR was detected in Ni-NTA affinity pulldown assays utilizing a His-tagged Tar receptor cytoplasmic fragment (Wu *et al.*, 1996), in contrast to the negative results obtained with *T. maritima* CheR and TM1143c. Thus, we conclude that the methylation regions of the receptors are not high affinity binding sites for *T. maritima* CheR. Despite weak binding, *T. maritima* receptor methylation proceeds with rates comparable to those observed for *S. enterica*.

Screening and analysis of MCP C-terminal ends

It was previously reported that MCPs of many bacteria lack the C-terminal pentapeptide-binding motif (Shiomi *et al.*, 2002b). To investigate which organisms possess putative pentapeptide sequences, we utilized a database of 167 completed bacterial and archaeal genomes to screen MCP C-terminal ends (Galperin, 2005). In total, 1,121 MCPs were analyzed from 78 organisms, of which only ~10% (113 MCPs total) were found to contain the putative binding motif (Table 2). Also, except for two MCPs identified in phylum Spirochetes, the overwhelming majority of the MCPs identified with a putative pentapeptide sequence (111 of 113) were restricted to the different proteobacteria classes (α, β, γ, δ) (Table 2). To examine the residues constituting the selected putative pentapeptide sequences and their conservation, a weblogo (Schneider and Stephens, 1990) was generated (inset to Table 2). Clearly, as expected based on the criteria we used to screen for putative binding motifs (see Experimental Procedures), the strongest conservation was seen at positions 2 (Trp - 83%, Phe - 17%) and 5 (Phe - 99%) of the pentapeptide. Notably, there was strict conservation of Phe at position 5 and also a paucity of Tyr at either position. Position 3 was also conserved, with 87% of residues at this position being either Glu (65%) or Gln (22%) (inset to Table 2). The crystal structure of a complex of *S. enterica* CheR and an NWETF pentapeptide demonstrated the structural importance of these three residues of the pentapeptide for interactions with the CheR β-subdomain (Djordjevic and Stock, 1998). The strong conservation displayed among these residues likely reflects their importance in

receptors of all organisms that are reliant on CheR-pentapeptide interactions for efficient methylation.

Comparative protein sequence analysis of the CheR β-subdomain

The β-subdomain is composed of an α helix and a short antiparallel three-stranded β sheet (Djordjevic and Stock, 1997) (Fig. 3A) and is present in all CheR homologs despite the absence of MCPs containing a C-terminal binding motif in the majority of organisms (Shiomi *et al.*, 2002b). Our screen for putative pentapeptide-binding motifs not only confirmed the finding that they were absent from most MCPs, but also served as a basis for further analysis of the β-subdomain of CheR. Utilizing the TIGR database (www.tigr.org), a total of 107 homologous CheR proteins were selected from the MCP-containing bacterial genomes that were screened (MCP containing organisms from phylums Cyanobacteria and Deinoccus did not contain any CheR homologs). A multiple sequence alignment was generated from the 107 β-subdomain sequences (Fig. 3B, C, D). Each β-subdomain was then separated into one of three groups, based on the following criteria: group I consisted of CheR β-subdomains from organisms containing only 1 CheR protein and ≥1 MCP with a putative pentapeptide sequence; group II consisted of CheR β-subdomains from organisms containing ≥2 CheR proteins and ≥1 MCP with a putative pentapeptide sequence; and group III consisted of CheR β-subdomains from organisms containing ≥1 CheR and only MCPs that lacked putative pentapeptide sequences. The groups of sequences were examined for key similarities and/or differences within each group, as well as for universal conservation of residues over the entire set of β-subdomain sequences.

Group I members include CheR proteins from *E. coli* and *S. enterica*, which have been the major focus for study of β-subdomain-pentapeptide interactions. Previous analysis of the *S. enterica* CheR-NWETF crystal structure revealed extensive interactions between the β-subdomain and the highly conserved residues of the binding motif. Specifically, β-subdomain residue Arg187 forms a salt bridge with the Glu of the pentapeptide and His192 and Arg197 interact with the Trp residue of the NWETF binding motif (Djordjevic and Stock, 1998). In addition, the C-terminal carboxylate of the pentapeptide forms a hydrogen bond with the amide nitrogen of the side chain of Gln182 (Djordjevic and Stock, 1998). Moreover, comparison to the CheR-AdoHcy structure solved in the absence of the pentapeptide (Djordjevic and Stock, 1997) demonstrated that side chains of Gln182, Arg187, His192, and Arg197 underwent the most significant conformational changes upon pentapeptide binding (Djordjevic and Stock, 1998). Examination of β-subdomain sequences confirmed the importance of these residues for CheR binding to pentapeptide, since three of the four (excluding His192) were highly conserved in group I β-subdomain sequences (Fig. 3B) and were poorly conserved in group III β-subdomain sequences from organisms lacking receptors with pentapeptide-binding motifs (Fig. 3D). Further support for the importance of these residues in binding the pentapeptide is illustrated by a CheR His192Ala/Arg197Ala double mutant that exhibited significantly slower rates of migration in soft agar chemotaxis assays compared to wild type and that did not exhibit CheR localization to cell poles (Shiomi *et al.*, 2002b).

In addition to Gln182, Arg187, and Arg197, our sequence analysis also identified three glycine residues (Gly188, Gly190, and Gly194) in the β-subdomain β-loop that are highly conserved in group I sequences (Fig. 3B), but are not well conserved in those of group III (Fig. 3D). The first study of sequence alignments of a small number of MCP-methytransferase pairs from different organisms, suggested that the β-subdomain possessed either a long or short β-loop, and that this difference might influence binding of the pentapeptide (Djordjevic and Stock, 1998). Subsequent studies, utilizing 8 CheR and CheR-related protein sequences, confirmed this difference in the length of the β-loop (Shiomi *et al.*, 2002b). Furthermore, Shiomi et al. found that CheR proteins from organisms containing

MCPs with pentapeptide-binding motifs possessed longer β-loops and proposed that this difference in length might reflect differences in the mode of receptor recognition by CheR (Shiomi *et al.*, 2002b). Our analysis indicated that indeed, β-subdomain sequences from organisms containing MCPs with binding motifs had longer β-loops (Fig. 3B, 3C), while the majority of β-subdomains from organisms lacking MCPs with binding motifs had shorter β-loops (Fig. 3D). Thus the longer β-loop and the three highly conserved glycines embedded within it are likely to be important in enabling pentapeptide binding. Notably, phi/psi angles for all three glycine residues were between 110° and 115°, exhibiting backbone conformations unattainable by most other residues.

Inspection of Group II sequences and surface analysis of the β-subdomain

Structural, mutational, and sequence analyses have revealed the importance of six residues, Gln182, Arg187, Arg197, and β-loop residues Gly188, Gly190, and Gly194, for CheR-pentapeptide interactions. Utilizing these key residues we examined the group II β-subdomain sequences to investigate if they fit the profile of a CheR protein that recognized a C-terminal pentapeptide (i.e. similar to group I) or lacked these residues (i.e. similar to group III). Inspection of these β-subdomain sequences demonstrated that both types were present, with 12 of the 38 β-subdomain sequences exhibiting conservation of at least five of the six key residues (Fig. 3C). Interestingly, the eight organisms (*C. violaceum, G. sulfurreducens, P. aeruginosa, S. oneidensis, V. cholerae, V. vulnificus, X. axonopodis,* and *X. campestris*) that possessed CheR proteins with sequence conservation similar to group I, in all cases, also contained at least one other CheR protein that exhibited poor conservation of those same residues (Fig. 3C). This suggests that in these organisms, two different types of CheR proteins exist: some that utilize a pentapeptide-dependent mechanism for methylation and others that do not. For example, in *S. oneidensis*, based on residue conservation, CheR1 and CheR3 likely require binding to a pentapeptide for efficient methylation of MCPs, similar to the *E. coli* and *S. enterica* CheR proteins. In contrast, CheR2, similar in sequence conservation to group III CheR proteins, presumably methylates MCPs without recognizing the C-terminal binding motif, similar to the methylation system in *T. maritima*.

Analysis of residue conservation in the β-subdomains identified certain residues to be universally conserved and revealed differences that distinguish pentapeptide-dependent from pentapeptide-independent CheR proteins. Differences between these two groups were visualized by mapping sequence conservation onto the surface of a structural model of the β-subdomain (Djordjevic and Stock, 1998) using the Consurf server (Glaser *et al.*, 2003). Two multiple sequence alignments were generated from sequences that were predicted to be either pentapeptide-dependent or pentapeptide-independent β-subdomains. Sequences from groups I and II that displayed high conservation of Gln182, Arg187, Arg197, Gly188, Gly190, and Gly194 were selected to represent pentapeptide-dependent β-subdomains (27 sequences). The remaining sequences from groups I and II and all sequences from group III (80 sequences) constituted the set of pentapeptide-independent β-subdomains.

The β-subdomain can be considered to contain two distinct surfaces: an inner face that packs against the rest of the C-terminal domain of CheR and an outer face that is exposed to solvent and potentially available for interactions with other proteins. Several residues that are highly conserved in both pentapeptide-dependent and pentapeptide-independent β-subdomains (Gly166, Tyr168, Tyr184, Phe185 and Val198) all map to a subset of the inner face (Fig. 4A and 5). The outer face of pentapeptide-dependent β-subdomains contains many highly conserved residues, whereas this surface shows little conservation in pentapeptide-independent β-subdomains (Fig. 4B and 5). This patch of conserved residues (Gln182, Arg187, Arg197, Gly188, Gly190, and Gly194) in pentapeptide-dependent β-subdomains lines the pentapeptide-binding pocket. Notably, outside of the surface that

interfaces with the catalytic domain of CheR and the pentapeptide-binding pocket in one class of β-subdomains, there are no other strongly conserved surfaces that might be implicated in other functions such as interactions with the conserved methylation regions of receptor substrates.

Discussion

Covalent modifications of proteins have a profound impact at both the molecular and cellular level. One such example is observed in bacterial chemotaxis where methylation and demethylation of specific glutamate residues within the cytoplasmic domains of MCPs counterbalance the signaling effects of ligand binding to the sensory domain, allowing adaptive behavior in bacterial swimming. While signaling and chemotaxis have been most extensively studied in *E. coli* and *S. enterica*, studies of other organisms have identified many complex variations on the streamlined *E.* coli/*S. enterica* system (Szurmant and Ordal, 2004). Over the past decade, one organism in particular, *T. maritima*, has emerged as a valuable source of chemotaxis proteins and receptor signaling complexes for structural analysis (Bilwes *et al.*, 1999; Bilwes *et al.*, 2001; Chao *et al.*, 2006; Griswold *et al.*, 2002; Park *et al.*, 2006; Park *et al.*, 2004a; Park *et al.*, 2004b; Quezada *et al.*, 2004; Usher *et al.*, 1998). As part of the characterization of *in vitro* methylation of *T. maritima* MCPs (Perez *et al.*, 2006), we investigated a key difference between *E. coli/S. enterica* receptors and the majority of other bacterial and archaeal MCPs, the presence and absence of a C-terminal binding motif for methyltransferase CheR.

We have shown that *T. maritima* CheR efficiently methylates *T. maritima* MCPs and is also capable of efficiently methylating *S. enterica* Tar with or without the C-terminal pentapeptide. Moreover, analysis of the residues preceding the C-terminal ends of *T. maritima* receptors and methylation assays with TM1428 containing a truncation of the last five residues (TM1428Δ5) indicate that MCP methylation in *T. maritima*, unlike the *E. coli/S. enterica* paradigm, is performed independently of a C-terminal binding motif. Utilizing a database of transmembrane signal transduction proteins (Galperin, 2005), we screened and analyzed MCP C-terminal ends as well as CheR β-subdomains from a large number of completed bacterial and archaeal genomes. Results showed that only ~10% of MCPs possessed a putative pentapeptide-binding motif, despite all CheR proteins possessing a β-subdomain (Djordjevic and Stock, 1997; Shiomi *et al.*, 2002b). Furthermore, key similarities and differences in residue conservation of the β-subdomain were identified when comparing pentapeptide-dependent and pentapeptide-independent CheR methyltransferases.

All CheR β-subdomains contain a set of highly conserved residues (Gly166, Tyr168, Leu173, Leu176, Tyr184, Phe185 and Val198, Fig. 3) that map to the interface formed between the β-subdomain and the rest of the CheR C-terminal catalytic domain (Fig. 4A and 5). Buried surface analysis (Brünger *et al.*, 1998) of the CheR-NWETF pentapeptide crystal structure (Djordjevic and Stock, 1998) confirmed that all of the universally conserved residues are buried at the interface comprising 213.1 $Å^2$ of the 596.9 $Å^2$ total buried surface of the β-subdomain. Further examination of this interface revealed several H-bonds that bridge the interface, formed between Gly166, Tyr168, Tyr184 and residues within the catalytic domain. The β-subdomain, as its name implies, appears to be an integral part of the C-terminal domain. Attempts to delete the β-subdomain by engineering constructs that replaced the β-subdomain with a large variety of small linkers designed to join αB and αB2, helices of the catalytic domain that anchor the β-subdomain endpoints, resulted in misfolded protein (Djordjevic and Stock, unpublished data).

Thus it appears that the β-subdomain and the conserved residues at the interface with the catalytic domain play a role in folding and/or stability of the protein. However, it should be

noted that the β-subdomain is unique to CheR methyltransferases and is not present within the common catalytic domain fold of any other class of methyltransferases. Thus, it is logical to speculate that the β-subdomain evolved for some function other than structural integrity. Interaction with the receptor methylation region is a plausible role, as the upper face of the β-subdomain together with a surface of the N-terminal domain form a large C-shaped cleft surrounding the active site that could potentially cradle the receptor coiled coil. However, the lack of sequence conservation in residues that comprise this surface argues against such a role.

Although bacteria lack discrete cellular compartments, many proteins and protein complexes are able to navigate the bacterial cell and ultimately localize to specific destinations (Lybarger and Maddock, 2001). For example, in *E. coli/S. enterica*, MCPs, CheW and CheA form a ternary complex at the cell pole (Maddock and Shapiro, 1993; Sourjik and Berg, 2000). Similar polar localization of MCPs has also been observed in *C. crescentus* (Alley *et al.*, 1992), *B. subtilis* (Kirby *et al.*, 2000), *P. aeruginosa* (Bardy and Maddock, 2005) and *R. sphaeroides*, that in addition, has a discrete cluster of chemotaxis proteins localizing within the cytoplasm as well (Porter *et al.*, 2002; Wadhams *et al.*, 2002; Wadhams *et al.*, 2005). Proper localization of the other components of the chemotaxis pathway to receptor clusters is also necessary. Localization of response regulator CheY, CheZ phosphatase and CheB methylesterase to receptor clusters in *E. coli/S. enterica*, is dependent on CheA, $CheA_S$ (a short form of CheA) and the CheA P2 domain, respectively (Banno *et al.*, 2004; Cantwell *et al.*, 2003; Sourjik and Berg, 2000). CheR targeting to the cell pole occurs via the high affinity interaction of the CheR β-subdomain with the MCP pentapeptide. This was demonstrated with a GFP-CheR (H192/R197A) β-subdomain mutant, that unlike WT CheR, did not localize to the cell pole (Shiomi *et al.*, 2002b). The vast majority of MCPs identified as containing a putative pentapeptide sequence (111 of 113) are almost exclusively restricted to the different proteobacteria classes (α, β, γ, δ) (Table 2). From its limited spread among bacteria, it is likely that tethering of CheR to MCPs through a β-subdomain-C-terminal pentapeptide interaction is a relatively recent event in evolution. However, the possibility that differences in CheR-MCP interactions represent a loss of function from the majority of organisms rather than a gain of function in proteobacteria cannot be excluded.

An obvious question is whether CheR localizes to receptor clusters in the absence of a C-terminal binding motif and if so, how? Does localization occur through another conserved region of the MCP cytoplasmic domain? The greatest sequence conservation among MCPs occurs at a region designated the highly conserved domain (HCD) (Zhulin, 2001), which serves as the locus of protein-protein interactions with CheA and CheW. The next most highly conserved segments correspond to regions containing the methylation sites, which are all located within a consensus sequence (Perez *et al.*, 2006; Terwilliger and Koshland, 1984). Either of these are logical sites for possible high affinity interactions with CheR, but no high affinity binding between *T. maritima* CheR and TM1143c was detected in our studies. If CheR is localized to receptor clusters through high affinity interactions, other proteins must facilitate these interactions.

Two other proteins, CheC and CheD, absent from *E. coli/S. enterica* but present in many of the organisms that contain peptide-independent CheR (Szurmant and Ordal, 2004), have been shown to play a role in MCP methylation and adaptation (Kristich and Ordal, 2002; Rao *et al.*, 2004; Rosario *et al.*, 1995; Rosario and Ordal, 1996). An additional difference between methylation systems in *E. coli/S. enterica* and some organisms with pentapeptide-independent methylation is the specific effect of methylation on the signaling activity of the receptors. In *E. coli/S. enterica* receptor signaling is influenced by the total number of sites methylated (Bornhorst and Falke, 2001), while in *B. subtilis* and *M. xanthus* the receptor signaling state is dictated by the specific site methylated (Astling *et al.*, 2006; Kirby *et al.*,

1999; Zimmer *et al.*, 2000). Results in *B. subtilis* suggest that CheC and CheD coordinate CheY-dependent selective methylation by protecting one methylation site and exposing another using phosphorylated CheY as the cue (Rao *et al.*, 2004). This may indicate that archaea and bacteria lacking a C-terminal binding motif, instead utilize a more complex pathway combining CheC, CheD, and CheY, in conjunction with CheB and CheR to effectively methylate and demethylate specific sites *in vivo* to regulate CheA activity. Conversely, for the comparatively simple *E. coli/S. enterica* chemotaxis system, continuous localization of CheR to the receptor clusters via the pentapeptide, would seem to be more advantageous, since total receptor methylation levels and not the specific site methylated controls kinase activity.

BLAST searches using the *S. enterica* CheR C-terminal catalytic domain identified several CheR-related proteins FrzF (*M. xanthus*), SMb20515 (*S. meliloti*) and CheW3 (*B. burgdorferi*) that also contained a β-subdomain (Shiomi *et al.*, 2002b). These β-subdomains have sequences similar to the pentapeptide-independent type. Interestingly all of these proteins contain additional domains that are capable of facilitating protein-protein interactions; FrzF contains several tetratrico peptide repeats (TPR), SMb20515 possesses several PAS domains, and CheW3 has a CheW domain in its N terminus (Blatch and Lassle, 1999; Das *et al.*, 1998; Lamb *et al.*, 1995; Liu and Parkinson, 1989; Shiomi *et al.*, 2002b; Taylor and Zhulin, 1999). Furthermore, recent studies with FrzF have demonstrated that the TPR motifs mediate multiple interactions with several other chemotaxis proteins (Bustamante *et al.*, 2004). The presence of other protein-protein interaction motifs in pentapeptide-independent CheR proteins suggests that other mechanisms of molecular recognition may have been utilized by CheR prior to the evolution of the pentapeptide-binding function of the β-subdomain. Whether the β-subdomain ever played, or currently plays, a role outside of structural integrity of the methyltransferase catalytic domain in pentapeptide-independent CheR proteins remains a mystery.

CheR localization to receptor clusters through a C-terminal binding motif has been extensively studied, yet appears to be used by only a very small percentage of bacterial organisms. Moreover, with the current array of completed genomes over-representing proteobacteria classes and under-representing organisms from other phylums, the percentage of organisms with pentapeptide-independent methylation systems is likely to be even greater. In this study, we have utilized *T. maritima* and *S. enterica* to begin to address the fundamental differences in the mechanisms utilized by CheR for MCP methylation. We anticipate that future studies in organisms more suitable for *in vivo* studies will provide further insight into the more prevalent pentapeptide-independent methylation systems.

Experimental Procedures

Bacterial Strains and Plasmids

Construction of plasmids pEP01 encoding *T. maritima* CheR, pTM1428 encoding full-length *T. maritima* receptor TM1428, and pTM1143c encoding the C-terminal cytoplasmic domain of *T. maritima* receptor TM1143 (residues 225–530) was described previously (Perez *et al.*, 2006). *S. enterica* CheR was expressed in an *E. coli* salt-inducible strain GJ1158 (Bhandari and Gowrishankar, 1997) from pT7CheR, generated by PCR amplification of the *cheR* gene from pME43 (Simms *et al.*, 1987) and insertion of an *Nde*I-*Hin*dIII *cheR* fragment into complementary sites in vector PJES307 (Tabor and Richardson, 1985). Plasmid pME98 (Simms *et al.*, 1985), encoding the *S. enterica tar* gene, was used as a vector to express *tar* in *E. coli* HCB437 (Δ(*tsr*)7021Δ(*trg*)100 *zbd*::Tn5Δ(*cheA*-*cheZ*)2209 *met*F159(Am)) (Wolfe *et al.*, 1987).

Mutagenesis of tm1428 and tar

TM1428 C-terminal truncation (TM1428Δ5) was constructed using a 3′ primer designed to anneal to GTG (V561) of pTM1428 and introduce a termination codon directly following nucleotides encoding V561, truncating the last five residues (ERFKI) of TM1428. Following amplification of the truncated gene flanked by restriction sites *Sal*I and *Not*I, the *Sal*I-*Not*I *tm1428* fragment was inserted into complementary sites in the pET28b vector (Novagen), generating plasmid pTM1428Δ5. The TarΔpp construct (residues 1–560) was generated using the QuikChange Site-directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, using *tar* containing pME98 plasmid DNA as template, a stop codon was introduced at the fifth to last codon by altering AAC to TAA, yielding *tarΔpp*, which was transformed into HCB437 for expression. Mutations for both constructs were confirmed by DNA sequencing (Genewiz, Inc., North Brunswick, NJ).

Expression and Purification of CheR Proteins and Receptors

Expression and purification of *S. enterica* and *T. maritima* CheR were performed as previously described (Perez *et al.*, 2004; Perez *et al.*, 2006). Salt-washed membrane fractions containing full-length chemoreceptors (WT Tar, TarΔpp, TM1428, and TM1428Δ5) were prepared as described previously (Perez *et al.*, 2004), as was preparation and purification of TM1143c (Perez *et al.*, 2006). TM1143c was estimated to be ~90% pure and CheR proteins ~70% pure. Concentrations of CheR and TM1143 were estimated by comparison to protein standards on Coomassie Blue-stained SDS polyacrylamide gel electrophoretograms.

Receptor Methylation Assays

Receptor methylation assays to determine initial rates of methylation were performed as described previously (Perez *et al.*, 2006). Each 100-μl reaction mixture contained 40–50 μl of either salt-washed membranes or receptor cytoplasmic domains (each containing ~6 μM receptor), and CheR protein preparation in 100 mM potassium phosphate, 50 μM [^{3}H]-*S*-adenosylmethionine at 162 Ci/mol (specific activity, 15 Ci/mmol; NEN Life Science Products, Inc.), pH 7.0. Samples were pre-equilibrated for 10 min at either 30°C or 37°C, and reactions were initiated by addition of 10 μl of CheR (ranging from 0.94 to 37.7 pmol of *S. enterica* CheR and 1.95 to 4.97 pmol of *T. maritima* CheR to achieve linear rates). For typical assays, five to six time points were taken at 3–6 min intervals, and initial rates were estimated using linear regression analysis. Each CheR-receptor pair was assayed at two different CheR concentrations to confirm linearity with respect to CheR and all methylation rates reported were derived from assays repeated three times.

T. maritima TM1143c-CheR Pulldown Assays

Pulldown assays were performed according to the manufacturer's protocol utilizing Ni Sepharose 6 fast flow beads (Amersham Biosciences). Briefly, assays were performed by incubating 30 μM or 60 μM CheR at 30°C for 1 h with 30 μM or 60 μM His-tagged TM1143c bound to 20 μl of Ni beads previously equilibrated in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). Wash steps for the beads were bypassed to avoid dissociation of a potentially weak CheR-TM1143c complex. Beads were mixed with 4X SDS loading buffer and heated at 100°C for 10 min prior to analysis by SDS-PAGE electrophoresis. Controls without TM1143c were performed to estimate the limits of detection in these assays. Gel bands were quantified by Coomassie blue staining, followed by densitometry with ImageJ software.

Data Base Search and Protein Sequence and Structure Analysis

Utilizing the census data, available at http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html (Galperin, 2005), C-terminal ends of MCP sequences were screened for

the presence of putative pentapeptide-binding motifs. MCPs containing this motif were selected based on the following criteria: MCPs were required to possess the xZxxZ putative binding motif (Shiomi *et al.*, 2002a) (where Z represents either F, W, or Y at positions 2 and 5 of the pentapeptide) and contain a C-terminal tail of at least ten residues in length extending beyond the alignment of C-terminal ends of receptors lacking pentapeptides and preceding the xZxxZ motif. Secondary structure predictions were performed using Jpred (Cuff *et al.*, 1998). The TIGR database (www.tigr.org) was used to select homologous CheR proteins from all MCP-containing bacterial genomes included in the M. Y. Galperin census. The β-subdomain for each CheR homolog was identified and multiple sequence alignments (LxxxYxF sequence downstream of the β-subdomain was used as an anchor) were generated using ClustalW (Thompson *et al.*, 1994). Buried surface areas were calculated using CNS 1.1 (Brünger *et al.*, 1998) using a probe radius of 1.4 Å. Interface contacts were examined using iMoltalk (Diemand and Scheib, 2004) and Pymol (Delano, 2002).

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A

TM1143	ENVQMLKEIV **ARYKI**
TM1428	KLSDELKEDV **ERFKI**
TM1146	ELAQELQRRI **EFFKI**
TM0429	ELSEQLSTLV **QKFKV**
TM0918	ELSEQLSTLV **QKFKV**
TM0023	ELSEQLSTLV **QKFKV**

B

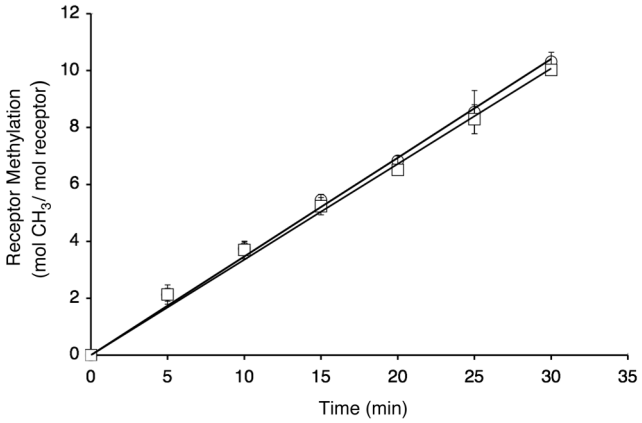

FIG. 1. Analysis of *T. maritima* receptor C-terminal ends and their role in methylation

A. Sequence alignment of *T. maritima* transmembrane receptor C-terminal ends. A multiple sequence alignment was generated with ClustalW (Thompson *et al.*, 1994) using the highly conserved domain (HCD) core (Zhulin, 2001) as the anchor for alignment. The last fifteen residues of each of receptor are shown, with the last five residues in bold. B. Methylation of TM1428 and TM1428Δ5. Methyltransferase activity was determined as described in Experimental Procedures using TM1428 (O) and TM1428Δ5 (□) as substrates. Assays were performed with 1.95 and 3.90 pmol of *T. maritima* CheR and the data shown are the average values from three independent experiments with standard errors.

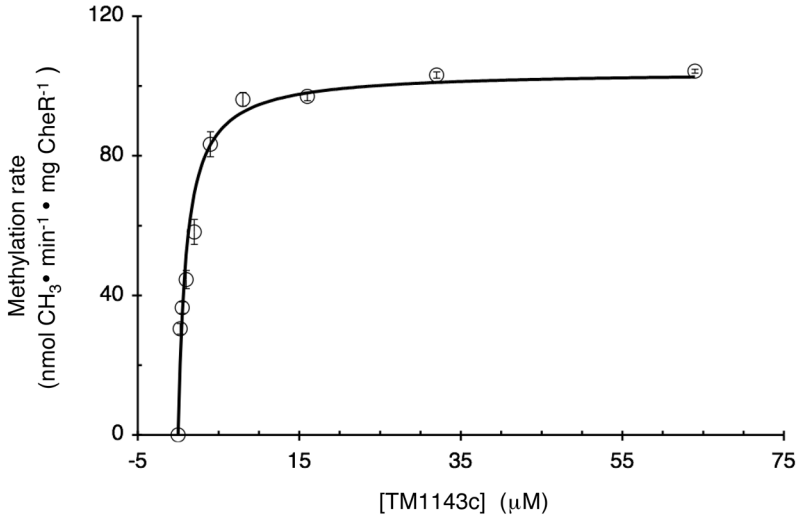

FIG. 2. Kinetics of methylation of TM1143c catalyzed by *T. maritima* CheR

Methyltransferase activity was determined as described in Experimental Procedures by incubating different concentrations of TM1143c (0 to 64 μM) with 0.20 μM methyltransferase CheR for 25 min at 30°C. K_m and K_{cat} values of 1.14 ± 0.20 μM and 3.60 ± 0.40 mol CH_3·min-1·mg CheR-1 were estimated by fitting data to the Michaelis-Menton equation using SigmaPlot 8.0. The data shown are the average values of initial velocities from three experiments with standard errors and the fitted curve used for estimation of K_m and K_{cat}.

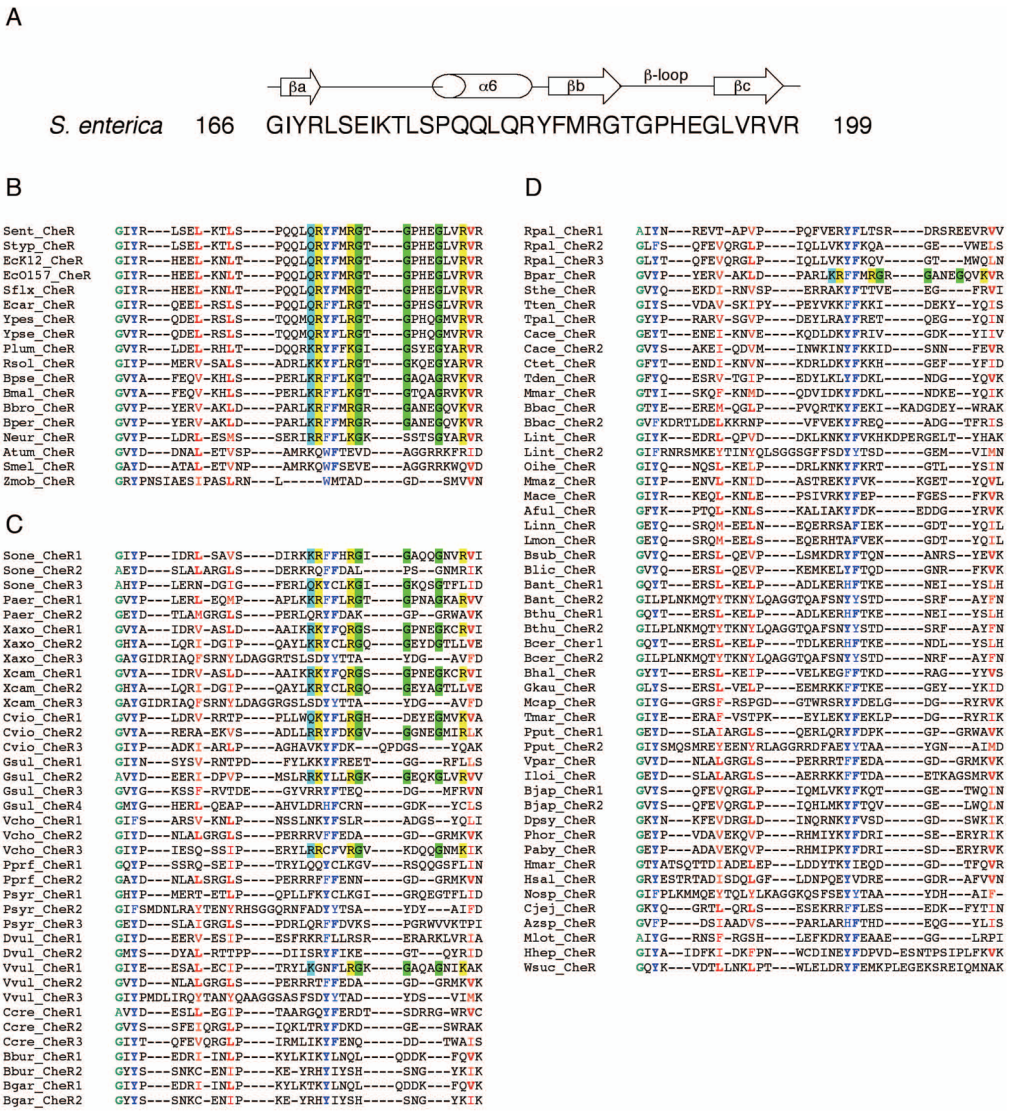

FIG. 3. Multiple sequence alignments of CheR β-subdomains

A. Secondary structure diagram of the *S. enterica* CheR β-subdomain derived from crystal structures (Djordjevic and Stock, 1997, Djordjevic, 1998 #1644) with the amino acid sequence and residue numbers indicated. A multiple sequence alignment for all β-subdomain sequences was generated with ClustalW (Thompson *et al.*, 1994) and the β-subdomain sequences were categorized as described in the text. B. Group I: β-subdomains from organisms containing only 1 CheR protein and ≥1 MCP with a putative pentapeptide sequence. C. Group II: β-subdomains from organisms containing ≥2 CheR proteins and ≥1 MCP with a putative pentapeptide sequence. D. Group III: β-subdomains from organisms containing ≥1 CheR and only MCPs that lacked putative pentapeptide sequences. Conserved amino acid residues in all β-subdomain sequences (≥85%) are colored: small (A, G), green; hydrophobic (L, V, I, M, F, Y, W, H), red; and aromatic (Y, F, W, H), blue; with the most common residue at each position in bold. Amino acid residues that are conserved and that are proposed to be important for CheR-pentapeptide interactions are highlighted: small (G), green; positively charged (R, K), yellow; and side chain amine/amide containing residues (Q, K, R), cyan. The organism abbreviation for each CheR homolog and the corresponding TIGR identification number are: Aful, *Archaeoglobus fulgidus* (AF1037); Atum,

Agrobacterium tumefaciens (NT02AT0609); Azsp, *Azoarcus sp.* EbN1 (NT01AE1293); Bant, *Bacillus anthracis* Ames(BA1665, BA0995); Bbac, *Bdellovibrio bacteriovorus* (NT02BB3177, NT02BB2588); Bbro, *Bordetella bronchiseptica* (NT01BB2503); Bbur, *Borrelia burgdorferi* (BB0040, BB0414); Bcer, *Bacillus cereus* (NT01BC1535, NT01BC0909); Bgar, *Borrelia garinii* (NT01BG0039, NT01BG0418); Bhal, *Bacillus halodurans* (NT01BH1876); Bjap, *Bradyrhizobium japonicum* (NT01BJ0473, NT01BJ2738); Blic, *Bacillus licheniformis* (NT03BL2556); Bmal, *Burkholderia mallei* (BMA2856); Bpar, Bordetella parapertussis (NT02BP1517); Bper, Bordetella pertussis (NT03BP1055); Bpse, Burkholderia pseudomallei (ntbp3311); Bsub, Bacillus subtilis (NT01BS2884); Bthu, Bacillus thuringiensis (NT02BT1675, NT02BT1053); Cace, Clostridium acetobutylicum (NT01CA2443, NT01CA0131); Ccre, *Caulobacter crescentus* (CC0435, CC0598, CC3472); Cjej, *Campylobacter jejuni* (NT01CJ0929); Ctet, *Clostridium tetani* (NT02CT1848); Cvio, *Chromobacterium violaceum* (NT01CV2459, NT01CV3397, NT01CV3658); Dpsy, *Desulfotalea psychrophila* (NT01DP3085); Dvul, *Desulfovibrio vulgaris* (DVU1595, DVU2076); Ecar, *Erwinia carotovora* (NT06EC1756); EcK12, *Escherichia coli* K12 (NT01EC2290); Ec0157, *Escherichia coli* O157:H7 (NT02EC2821); Gkau, *Geobacter kaustophilus* (NT01GK2440); Gsul, *Geobacter sulfurreducens* (GSU0295, GSU1143, GSU2215, GSU3195); Hhep, Helicobacter hepaticus (NT02HP0476); Hmar, Haloarcula marismortui (NT01HMA2142); Hsal, Halobacterium salinarum (NT01HS0757); Iloi, Idiomarina loihiensis (NT01IL1191); Linn, Listeria innocua (NT01LI0715); Lint, Leptospira interrogans (NT02LI2313, NT02LI1981); Lmon, Listeria monocytogenes (NT01LM0742); Mace, *Methanosarcina acetivorans* (NT02MA3813); Mcap, *Methylococcus capsulatus* (MCA0829); Mmar, *Methanococcus maripaludis* (NT04MM0987); Mlot, *Mesorhizobium loti* (NT02MLB0021); Mmaz, *Methanosarcina mazei* (NT01MM1835); Neur, *Nitrosomonas europaea* (NT01NE2059); Nosp, *Nostoc sp.* PCC7120 (NT01NS2346); Oihe, *Oceanobacillus iheyensis* (NT01OI1946); Paby, *Pyrococcus abyssi* (NT01PA1746); Paer, *Pseudomonas aeruginosa* (NT03PA0199, NT03PA3856); Phor, *Pyrococcus horikoshii* (NT01PH0504); Plum, *Photorhabdus luminescens* (NT01PL2011); Pprf, *Photobacterium profundum* (NT01PP0812, NT01PP0945); Pput, *Pseudomonas putida* (PP4392, PP3760); Psyr, *Pseudomonas syringae* (PSPPH0802, PSPH2602, PSPPH3412); Rpal, *Rhodopseudomonas palustris* (NT02RP0142, NT02RP1682, NT02RP1731); Rsol, *Ralstonia solanacearum* (NT01RSA1413); Sent, *Salmonella enterica* serovar *Typhimurium* CT18 (NT03ST2156); Sflx, *Shigella flexneri* (NT02SF2178); Smel, *Sinorhizobium meliloti* (NT01SM0883); Sone, *Shewanella oneidensis* (SO2124, SO3251, SO2325); Sthe, *Symbiobacterium thermophilum* (NT07ST1815); Styp, *Salmonella Typhimurium* LT2 (NT05SE0993); Tden, *Treponema denticola* (TDE0647); Tmar, *Thermotoga maritima* (TM0464); Tpal, *Treponema pallidum* (TP0630); Tten, *Thermoanaerobacter tengcongens* (NT01TT1507);Vcho; *Vibrio cholerae* (VC1399, VC2201, VCA1091); Vpar, *Vibrio parahaemolyticus* (NT01VP0730); Vvul, *Vibrio vulnificus* (NT01VVA1160, NT01VV0231, NT01VVA0376); Wsuc, *Wolinella succinogenes* (NT01WS1194); Xaxo, *Xanthomonas axonopodis* (NT01XA2561, NT01XA3795, NT01XA1741); Xcam, *Xanthomonas campestris* (NT01XC2547, NT01XC3635, NT01XC1630); Ypes, *Yersinia pestis* (NT02YP2177); Ypse, *Yersinia pseudotuberculosis* (NT05YP2660); Zmob, *Zymomonas mobilis* (NT01ZM0078).

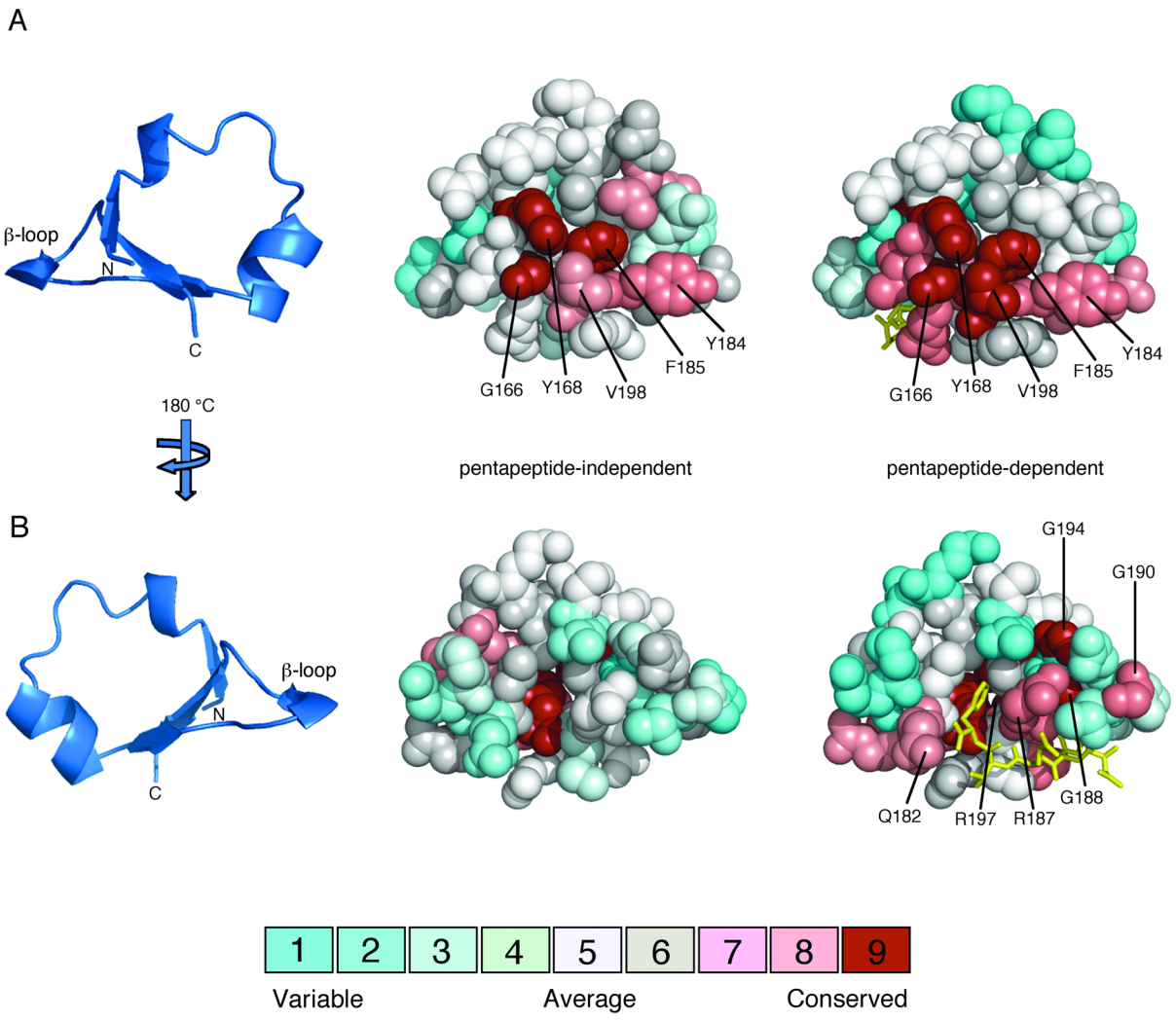

FIG. 4.

Amino acid conservation on the surfaces of pentapeptide-independent and pentapeptide-dependent β-subdomains. Multiple sequence alignments of pentapeptide-independent and pentapeptide-dependent CheR β-subdomains, classified in Figure 3, were generated using ClustalW (Thompson *et al.*, 1994). Amino acid conservation scores for each residue were calculated using the Consurf maximum likelihood method (Glaser *et al.*, 2003) (http://consurf.tau.ac.il) and were mapped onto space-filling models of the CheR β-subdomain (PDB accession code 1BC5) (Djordjevic and Stock, 1998) using Pymol (Delano, 2002). The coloring scheme for conservation ranges from cyan (variable) to red (conserved) and the pentapeptide (gold) is shown in stick representation. A. β-subdomain interior face. The ribbon diagram of the β-subdomain (left) indicates the orientation of the space-filling models, viewed toward the surface that packs against the catalytic domain of CheR. Residues that are highly conserved in both pentapeptide-independent (center) and pentapeptide-dependent (right) β-subdomains are labeled. B. β-subdomain exterior face. The orientation corresponds to a 180° rotation around a vertical axis relative to the view in A. Residues that are highly conserved only in pentapeptide-dependent β-subdomains (right) are labeled.

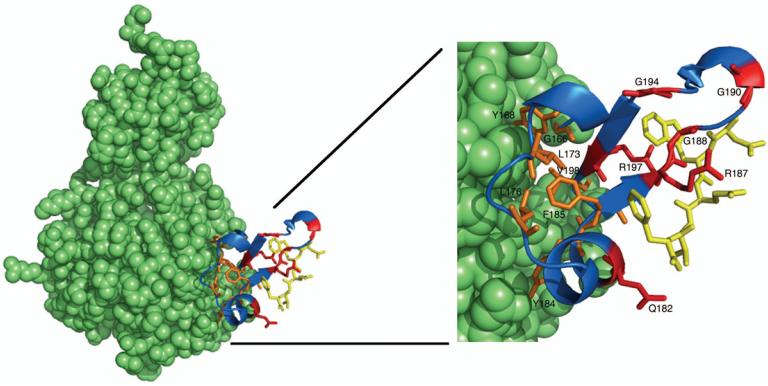

FIG. 5. Conservation of residues within the β-subdomain of CheR

The CheR-pentapeptide structure (left) is displayed as a space-filling model (green), except for the β-subdomain (blue) and pentapeptide (gold), which are shown as a ribbon diagram and in stick representation, respectively. An enlarged view of the β-subdomain (right) shows conserved residues in stick representation. Universally conserved β-subdomain residues are shown in orange and residues that are conserved exclusively in pentapeptide-dependent β-subdomains are shown in red. Images were generated using Pymol (Delano, 2002).

TABLE 1

Cross species methylation rates [a]

Receptor	Methyltransferase	30°C	37°C
WT Tar	*S. enterica* CheR	0.66 ± 0.07	0.86 ± 0.06
	T. maritima CheR	0.21 ± 0.03	0.32 ± 0.04
TM1428fl	*S. enterica* CheR	0.016 ± 0.001	0.018 ± 0.002
	T. maritima CheR	0.35 ± 0.02[b]	0.50 ± 0.04[b]
TarΔpp	*S. enterica* CheR	0.018 ± 0.002	0.020 ± 0.002
	T. maritima CheR	0.20 ± 0.04	0.31 ± 0.05

[a] Methyltransferase activities are expressed as mol CH_3· mol $CheR^{-1}$·min^{-1} and are averages of three independent experiments.

[b] Values are from Perez *et al.* {Perez, 2006 #3187}.

TABLE 2

MCPs containing putative pentapeptide sequences

Phylum/Organism[a]	MCPs[b]	MCPs w/pp	CheR
α-proteobacteria			
Agrobacterium tumefaciens	20	9	1
Caulobacter crescentus	18	6	3
Sinorhizobium meliloti	9	4	1
Zymomonas mobilis	3	1	1
β-proteobacteria			
Bordetella bronchiseptica	8	1	1
Bordetella pertussis	5	1	1
Burkholderia mallei	17	3	1
Burkholderia pseudomallei	21	5	1
Chromobacterium violaceum	42	3	3
Nitrosomonas europaea	3	2	1
Ralstonia solanacearum	22	4	1
γ-proteobacteria			
Erwinia carotovora	36	19	1
Escherichia coli K12	5	2	1
Escherichia coli O157:H7	5	2	1
Photobacterium profundu m	39	1	2
Photorhabdus luminescens	2	1	1
Pseudomonas aeruginosa	26	1	2
Pseudomonas syringae	48	1	3
Salmonella Typhimurium LT2	9	4	1
S. enterica serovar Typhi CT18	6	3	1
Shewanella oneidensis	26	2	3
Shigella flexneri	4	1	1
Vibrio cholerae	45	2	3
Vibrio vulnificus	52	1	3
Xanthomonas axonopodis	21	12	3
Xanthomona s campestris	20	8	3
Yersinia pestis	6	2	1
Yersinia pseudotuberculosis	7	3	1
δ-proteobacteria			
Desulfovibrio vulgaris	28	2	2
Geobacter sulfurreducens	33	5	4
Spirochetes			
Borrelia burgdorferi	5	1	2
Borrelia garinii	5	1	2

[a] Each specific phylum or class is underlined.

[b] Total number of MCPs were obtain from the signal transduction

census {Galperin, 2005 #3139}.