A remote CheZ ortholog retains phosphatase function

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Summary

Aspartyl-phosphate phosphatases underlie the rapid responses of bacterial chemotaxis. One such phosphatase, CheZ, was originally proposed to be restricted to proteobacter, suggesting only a small subset of microbes relied on this protein. A putative CheZ phosphatase was identified genetically in the epsilon proteobacter *Helicobacter pylori* (Terry *et al*., 2006, Mol Micro 61:187). *H. pylori* utilizes a chemotaxis system consisting of CheAY, three CheVs, CheW, CheY and the putative CheZ to colonize the host stomach. Here we investigate whether this CheZ has phosphatase activity. We phosphorylated potential targets *in vitro* using either a phosphodonor or the CheAY kinase and [γ-^{32}P] ATP, and found that *H. pylori* CheZ (CheZ$_{HP}$) efficiently dephosphorylates CheY$_{HP}$ and CheAY and has additional weak activity on CheV2. We detected no phosphatase activity toward CheV1 or CheV3. Mutations corresponding to *E. coli* CheZ active site residues or deletion of the C-terminal region inactivate CheZ$_{HP}$ phosphatase activity, suggesting the two CheZ's function similarly. Bioinformatics analysis suggests that CheZ phosphatases are found in all proteobacteria classes, as well as classes Aquificae, Deferribacteres, Nitrospira, and Sphingobacteria, demonstrating that CheZ phosphatases are broadly distributed within Gram-negative bacteria.

Introduction

Chemotaxis is a mechanism that allows microorganisms to sense their chemical environment and respond by moving away from harmful agents or toward attractive ones. Canonical chemotaxis signal transduction involves sensing a chemical signal by a chemoreceptor protein, whose interaction with the coupler protein CheW sends a signal to histidine kinase CheA to undergo ATP-dependent autophosphorylation (Wadhams & Armitage, 2004). The phosphoryl group is then transferred to the response regulator CheY, which binds to the flagellar motor. The level of phosphorylated CheY (CheY-P) effects the rotational direction of the flagellar motor causing the flagella to turn clockwise or counter clockwise. For a microorganism to respond quickly to the external chemical environment, it is necessary for CheY-P to rapidly return to its non-phosphorylated form. CheY-P has an intrinsic dephosphorylation activity that is accelerated by a variety of mechanisms. The first discovered was the CheY phosphatase CheZ, which accelerates the dephosphorylation of CheY-P (Hess *et al*., 1988, Zhao *et al*., 2002, Silversmith, 2010). However, CheZ homologs have not been reported in all microorganisms with chemotaxis systems (Szurmant & Ordal, 2004). Many microorganisms have another family of CheY-P phosphatases, the CheC/FliY/ CheX phosphatases, whose mechanism is similar to CheZ (Muff *et al*., 2007, Wuichet *et al*., 2007, Pazy *et al*., 2010, Silversmith, 2010). Another way to regulate the cellular level of CheY-P is to decrease the flow of phosphoryl groups to CheY using additional CheY-homologous proteins termed phosphate sinks. In the prototype example of this pathway in *Sinorhizobium meliloti*, the phosphate sink is an alternative CheY that accepts phosphoryl

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groups from CheA, but does not interact with the flagellar switch (Sourjik & Schmitt, 1998, Szurmant & Ordal, 2004). By directing phosphate flow to the sink protein, the signaling CheY is able to autodephosphorylate and the signal is terminated.

Helicobacter pylori is a chemotactic ε-proteobacteria that infects 50% of the world's population (Covacci *et al*., 1999). Infection with *H. pylori* can lead to ulcers or gastric cancer (Kandulski *et al*., 2008). *H. pylori* relies on chemotaxis to successfully colonize the gastric mucosa of the host (Foynes *et al*., 2000, Terry *et al*., 2005). The chemotaxis system in *H. pylori* deviates from the *E. coli* model. The major chemotaxis proteins CheAY, CheW, and $CheY_{HP}$ are present. CheAY is a modified CheA with a response regulator receiver domain (REC) fused at its C-terminus (Tomb *et al*., 1997, Foynes *et al*., 2000). There are three CheV proteins called CheV1, CheV2, and CheV3, which are hybrids of CheW and a REC domain. Neither CheZ nor any of the CheC/FliY/CheX phosphatases were annotated in the original genome sequencing of *H. pylori* (Tomb *et al*., 1997). Jiménez-Pearson *et al*. found that *in vitro* CheAY and CheA' (CheAY without its REC domain) transferred phosphoryl groups to both $CheY_{HP}$ and the CheAY REC domain. Because the REC domain of CheAY would not be expected to interact with the flagellar motor, it was suggested that this phosphorylation event might be akin to a phosphate sink and thus that *H. pylori* might employ this method for dephosphorylating $CheY_{HP}$-P (Jiménez-Pearson *et al*., 2005). The lack of known phosphatases and the presence of three CheV proteins also suggested that CheV proteins might act as phosphate sinks in the chemotaxis system of *H. pylori*, but the lower affinity of CheA' for CheV made this an unlikely scenario (Pittman *et al*., 2001, Jiménez-Pearson *et al*., 2005).

Previous work from our lab reported the identification of a remote CheZ homologue, HP0170 (Terry *et al*., 2006). It was identified as a chemotactic (Che+) suppressor of a non-chemotactic *cheW* mutant. Extended incubation of non-chemotactic *cheW* mutants resulted in recovered migration ability in soft-agar media. Proteomic analysis of this Che+ suppressor mutant revealed that it had lost expression of HP0170. Analysis of additional suppressor mutants showed various mutations in the HP0170 gene, including single insertions, multiple base pair insertions, deletions, and missense mutations, that all resulted in loss or mutation of the C-terminal domain of the protein. Using the SAM-T02 program that identifies very remote protein homologs, HP0170 was identified as a remote homologue of CheZ.

Here we investigate the potential phosphatase activity of HP0170 on all *H. pylori* chemotaxis proteins bearing a REC domain: CheAY, the three CheVs, and $CheY_{HP}$. *H. pylori* is different from the well-studied *E. coli* in this respect of having more than two potential phosphatase targets. We show here that HP0170 is a phosphatase for phosphoryl $CheY_{HP}$ ($CheY_{HP}$-P) and phosphoryl CheAY (CheAY-P), as well as a weak phosphatase for phosphoryl CheV2 (CheV2-P). Mutations at residues corresponding to the *E. coli* CheZ active site inactivate HP0170's dephosphorylation activity. The deletion of 12 amino acids at the C-terminus also inactivates HP0170 activity. These findings show that HP0170 functions as a CheZ for *H. pylori*, and thus we refer to it as $CheZ_{HP}$. Using PSI-BLAST to search for $CheZ_{HP}$ orthologs, we found that CheZ is present across several classes of Gram negative bacteria.

Results

HP0170 is a $CheY_{HP}$-P phosphatase

Previous work identified HP0170 as a very remote homolog of the *E. coli* CheZ phosphatase (Terry *et al*., 2006) but it was not known whether this protein retained phosphatase activity. To determine whether HP0170 has phosphatase activity, we first employed the EnzChek ® Phosphate Assay Kit as developed by Silversmith *et al*. 2001 for use with *E. coli* CheY and

CheZ. This method uses the small-molecule phosphodonor monophosphoimidazole (MPI) to phosphorylate CheY, and then measures the steady-state phosphate release due to CheY dephosphorylation. The phosphate release thus depends on both the rates of phosphorylation and dephosphorylation. In the case of *E. coli* CheZ and CheY, CheY's autodephosphorylation activity releases a basal amount of phosphate that is enhanced by CheZ. For our assay, we employed recombinant purified *H. pylori* proteins. We first tested whether *H. pylori* CheY (CheY$_{HP}$) would be phosphorylated by MPI, and varied the concentration of the MPI substrate to determine saturating conditions. Similar to *E. coli* CheY, we found that CheY$_{HP}$ releases phosphate and that this release reaches a saturation point at 2.5 mM MPI, using 5 µM CheY$_{HP}$ (Fig. 1). This behavior suggests that CheY$_{HP}$ can be phosphorylated by MPI and that at concentrations above 2.5 mM MPI, autodephosphorylation is the rate-limiting step because increasing the concentration of phosphodonor does not increase the phosphate release rate. The expected phosphorylation site of CheY$_{HP}$, aspartate 53, is required for the phosphorylation by MPI as a CheY$_{HP}$ D53A mutant does not show any phosphate release in this phosphate release assay (data not show). We also determined that Mg^{2+} is the optimal divalent metal ion for CheY$_{HP}$ phosphorylation/dephosphorylation, similar to other CheY proteins (data not shown). The addition of HP0170 to a reaction containing CheY$_{HP}$ and MPI significantly increases the rate of phosphate release indicating that HP0170 is a CheY$_{HP}$-P phosphatase (Fig. 1). Dephosphorylation of CheY$_{HP}$-P by HP0170 eventually reaches saturation suggesting that this is the maximum dephosphorylation activity by HP0170 under these experimental conditions. These results demonstrate that HP0170 is a CheY$_{HP}$-P phosphatase in *H. pylori* and can be appropriately called CheZ$_{HP}$. Additionally, we expanded the concentrations of CheZ$_{HP}$ tested ranging from 0.1 to 12 µM, while keeping the amount of CheY$_{HP}$ constant at 5 µM. The phosphate release rate increases more-or-less linearly as a function of CheZ$_{HP}$ concentration, with CheZ$_{HP}$ reaching its maximum activity at 1 µM (Fig. 2). This finding is likely due to the autophosphorylation of CheY being the rate-limiting step, such that additional phosphatase cannot increase the release rate. Of note, the presence of CheZ$_{HP}$ alone without CheY$_{HP}$ did not result significant phosphate release (Fig. 1).

Using [γ-^{32}P] ATP to phosphorylate CheY$_{HP}$ via CheAY confirms the phosphatase activity of CheZ$_{HP}$

To confirm that CheZ$_{HP}$ is a CheY$_{HP}$-P phosphatase, we employed a traditional method of phosphorylation using the cognate histidine kinase with radioactive ATP. For this assay, *H. pylori* CheAY was phosphorylated with [γ-^{32}P] ATP which in turn phosphorylated CheY$_{HP}$. We used full length CheAY, as well as a truncated variant with its REC domain removed called CheA' (Jiménez-Pearson *et al.*, 2005). We first phosphorylated CheY$_{HP}$ with either CheAY-P or CheA'-P, under multiple turnover conditions in which ATP was supplied throughout. CheY$_{HP}$ was phosphorylated by both CheAY and CheA' as evident by the appearance of CheY-sized radioactive bands after CheY$_{HP}$ was added to the reaction (Fig. 3A and 3C). This finding is identical to what was found previously by Jiménez-Pearson *et al.* 2005. CheAY-P bands are visible for up to 10 minutes in a reaction containing CheY$_{HP}$ (Fig. 3A). In the presence of CheZ$_{HP}$, CheY-P and CheAY-P bands are visible for much shorter times, presumably as a result of transfer of phosphoryl group to CheY$_{HP}$ and accelerated dephosphorylation of CheY$_{HP}$-P by CheZ$_{HP}$ (Fig. 3B); as discussed in the next section, however, CheZ also targets CheAY-P but does so with less efficiency as compared to CheY-P. Thus multiple reactions are occurring, with CheZ$_{HP}$ targeting CheAY-P and CheY-P, and a further remote possibility that CheZ$_{HP}$ has some contaminating phosphatase that directly targets ATP. We thus simplified this assay by using the truncated variant CheA'. CheA'-P dephosphorylated as soon as CheY$_{HP}$ was added but the phosphorylated CheY$_{HP}$-P remained visible for an extended period of time (Fig. 3C). This rapid movement of phosphate from CheA'-P to CheY$_{HP}$ is presumably due to the absence of the REC

domain in CheA' such that the protein now rapidly transfers phosphoryl groups to $CheY_{HP}$. In this assay, CheA' autophosphorylation and phosphoryl group transfer is continuous. The observation of stable $CheY_{HP}$-P indicates that the rate of phosphoryl transfer from CheA' to $CheY_{HP}$ exceeds the rate of $CheY_{HP}$ autodephosphorylation under these conditions. The addition of $CheZ_{HP}$ quickly dephosphorylates $CheY_{HP}$-P generated by CheA', as $CheY_{HP}$-P bands are barely visible compared to the reaction that lacks $CheZ_{HP}$ (Fig. 3 C and D). We attempted to determine the half-life of $CheY_{HP}$-P by first phosphorylating CheA'-P, removing excess ATP using gel filtration, and then adding this CheA'-P to $CheY_{HP}$. Under these single-turnover conditions, we could not visualize $CheY_{HP}$-P even after only five seconds (data not shown), supporting that $CheY_{HP}$ rapidly dephosphorylates without continuous phosphorylation by CheA'. We estimated the $CheY_{HP}$ autodephosphorylation rate constant, $k_{dephosph}$, by dividing the saturating dephosphorylation rate from Figure 1 (1.42 µM /sec) by the concentration of $CheY_{HP}$ (5 µM) (Silversmith *et al.*, 2001). This rate of *H. pylori* CheY dephosphorylation is 0.28 s^{-1}, approximately 8 times higher than the reported $k_{dephosph}$ of *E. coli* CheY, 0.035 s^{-1} (Silversmith et al., 2001); this calculation indicates that $CheY_{HP}$-P has a half-life of approximately 2.5 seconds. This short half-life is consistent with our inability to detect $CheY_{HP}$-P without repeat phosphorylation. Taken together, these assays results strongly support that $CheZ_{HP}$ is a $CheY_{HP}$-P phosphatase.

$CheZ_{HP}$ has phosphatase activity on CheAY but not on CheA'

We also examined whether $CheZ_{HP}$ has a phosphatase activity on *H. pylori* CheAY and CheA'. We phosphorylated CheAY and CheA' with [γ-^{32}P] ATP, followed by addition of excess cold ATP to follow the dephosphorylation of these proteins. $CheZ_{HP}$ dephosphorylates CheAY-P as seen in Figure 4A and B. The radioactive bands of CheAY-P start to disappear at 25 minutes after the chase. We quantified the band intensity across this gel, and used that information to calculate the half-life of CheAY-P to be approximately 30 minutes, or about 10 times longer than found previously (Jiménez-Pearson *et al.*, 2005). We are not sure of the reason for this discrepancy. In the presence of $CheZ_{HP}$, the radioactive bands disappear by 15 minutes indicating that $CheZ_{HP}$ affects the phosphorylation state of CheAY-P. The calculated half-life of CheAY-P chased with $CheZ_{HP}$ is approximately 12 minutes. CheA'-P, however, was not affected by $CheZ_{HP}$ even with an extended incubation time (Fig. 4C and D). This finding suggests that $CheZ_{HP}$ targets the REC domain of CheAY; however the relatively weak phosphorylation of CheA' as compared to CheAY could indicate that the truncated protein has lost some function. Overall, this set of experiments confirms that $CheZ_{HP}$ is a phosphatase whose activity is not restricted to $CheY_{HP}$-P but also extends to CheAY-P.

$CheZ_{HP}$ uses the same active site residues as does *E. coli* CheZ

To further analyze the activity of $CheZ_{HP}$, we generated and analyzed variants of $CheZ_{HP}$ that changed aspartate 189 to asparagine ($CheZ_{HP}$ D189N), glutamine 193 to arginine ($CheZ_{HP}$ Q193R) and deleted the 12 C-terminal amino acids ($CheZ_{HP}$ ΔC$_{12}$). These residues and sequences are critical for *E. coli* CheZ function. In *E. coli*, mutation of either D143 or Q147 (equivalent to D189 and Q193 in *H. pylori* strain 26695 and D177 and Q181 in *H. pylori* strain SS1, respectively, in $CheZ_{HP}$; see Figure 6) results in proteins that do not dephosphorylate CheY-P (Boesch *et al.*, 2000,Zhao *et al.*, 2002). The C-terminus of *E. coli* CheZ binds to CheY-P to bring it to the CheZ active site (Blat & Eisenbach, 1996,Zhao *et al.*, 2002,Silversmith, 2005). Additionally, one of our original *HP0170* loss-of-function mutants had a point mutation that changed aspartate 189 to asparagine (Terry *et al.*, 2006). Phosphate release assays showed that these point mutations render $CheZ_{HP}$ inactive as a phosphatase of $CheY_{HP}$-P, indicating that D189 and Q193 are important for $CheZ_{HP}$ dephosphorylation of $CheY_{HP}$-P (Fig. 1). The absence of the last 12 amino acids at the C-terminus displays a similar defect to the point mutants (Fig. 1). However, the C-terminus is

located approximately 50 amino acids downstream of the point mutations suggesting that the C-terminus might perform the same function as the C-terminus of *E. coli* CheZ, binding CheY-P, rather than being a part of the active site. Of note, all of these $CheZ_{HP}$ mutant proteins were stable and maintained their length (data not shown), suggesting the mutations did not grossly affect protein folding and stability.

$CheZ_{HP}$ dephosphorylates CheV2 but not CheV1 or CheV3

$CheZ_{HP}$ was also tested for its phosphatase activity on other *H. pylori* chemotaxis proteins that have REC domains: CheV1, CheV2, and CheV3. Under our experimental conditions, phosphate release rates by the CheV proteins are comparatively lower than that of $CheY_{HP}$ (Fig. 5 as compared to Fig. 1; 0.2–0.7 µM /sec versus 1.4 µM /sec), suggesting that while MPI is a phosphodonor for each, it may be a poor substrate. We also determined the optimal Mg^{2+} concentration for phosphorylation and autodephosphorylation of each CheV, and found it to be similar to $CheY_{HP}$ (data not shown). The low phosphate release by CheV proteins could be due either to poor phosphorylation or to slow release, both of which have precedent. The dephosphorylation of *Bacillus subtilis* CheV was shown to be slowed by the presence of the N-terminal CheW domain (Karatan *et al*., 2001), and others have had difficulty detecting phosphorylated *H. pylori* CheV proteins (Jiménez-Pearson *et al*., 2005). The phosphate release rates for CheV1 and CheV3 do not exhibit saturation, as would be expected with Michaelis-Menton kinetics, and suggesting that the affinity of the CheV REC domain for MPI is very weak (Bourret, 2010). This lack of saturation would predict that phosphorylation is the rate limiting step, and thus that phosphatase assays might be quite insensitive. Indeed, we did not detect any enhanced phosphate release when $CheZ_{HP}$ was added to either the CheV1 or CheV3 reactions. CheV2, on the other hand, reaches saturation at 3.5 mM MPI, and has relatively lower phosphate release rate of 0.25 µM /sec compared to 0.5 µM /sec of CheV1 and CheV3 (Fig. 5B). Previous work showed that CheV2 was phosphorylated by acetyl phosphate (Pittman *et al*., 2001,Jiménez-Pearson *et al*., 2005). The addition of $CheZ_{HP}$ to the reaction containing CheV2 increased the phosphate release rate by 1.6 fold, suggesting that $CheZ_{HP}$ has some phosphatase activity on CheV2-P (Fig. 5B). Taken together, these results suggest that $CheZ_{HP}$ is primarily a phosphatase for $CheY_{HP}$-P and CheAY-P, with weak activity on CheV2-P.

Domain analysis of HP0170/$CheZ_{HP}$

Our biochemical analysis strongly supported that $CheZ_{HP}$ is a phosphatase despite its limited homology to *E. coli* CheZ. To search for distant homologs of $CheZ_{HP}$, the amino acid sequence of HP0170 from *H. pylori* strain SS1 was used in a PSI-BLAST query. This analysis identified 268 proteins including *E. coli* CheZ after the 3rd iteration (Table 1). These proteins had been annotated as either hypothetical proteins, CheZ, probable phosphatase, or MCP and were found in the phylum Proteobacteria (classes α, β, γ, δ, and ε), phylum Aquificae (class Aquificae), phylum Deferribacteres (class Deferribacteres), phylum Nitrospirae (class Nitrospira), and phylum Bacteroidetes (class Sphingobacteria). Representative of each genus were used to generate an amino acid sequence alignment (Fig. 6,Table S1). Of note, all CheZ-bearing classes have annotated chemotaxis genes in their genomes, supporting that these CheZ orthologs act in this pathway. The large spaces between groups of conserved residues are result of incorporating sequences that are longer than the majority of the proteins, therefore no conservation was observed (Fig. 6). We found several CheZ regions with high levels of conservation with the most significant corresponding to the *E. coli* CheZ active site and the C-terminal end. The active site residues corresponding to Gln 147 and Asp143 of *E. coli* CheZ are 100% conserved among these proteins. The co-crystal structure of CheZ with CheY-$BeF_3^-$$Mg_2^+$showed residue Asp143 forms a salt bridge with CheY indicating the importance of this residue for CheZ function, and the CheZ active site residue Gln147 is inserted into the CheY active site and to

accelerate the dephosphorylation of CheY-P (Zhao et al., 2002). The C-terminal sequences of CheZ proteins are also conserved supporting the importance of this region in binding to CheY-P, as shown for *E. coli* CheZ (Blat & Eisenbach, 1996). There are a few other moderately conserved regions that correspond to amino acids 43–86 of *E. coli* CheZ. Within this region, residues 67–71 create part of the CheY binding surface located in the middle of the CheZ four-helix bundle (Zhao *et al.*, 2002). Other important regions of *E. coli* CheZ do not seem to be as conserved. For example, the helix-turn-helix hairpin of CheZ that has been shown to be involved in its polar localization via interactions with CheA-short does not show significant conservation, as noted by others (Cantwell *et al.*, 2003). CheA-short is a variant of CheA arising from initiation at an internal methionine, Met97; while sequence alignment of *H. pylori* CheAY with *E. coli* CheA shows that Met97 appears to be conserved (data not shown), we do not yet know if CheA-short exists in *H. pylori* or any bacteria outside of *E. coli* and its close relative *Salmonella enterica* (McNamara & Wolfe, 1997).

Discussion

The primary finding of this study is that the HP0170 protein has phosphatase activity toward $CheY_{HP}$-P, but also acts on two other chemotaxis REC domain proteins, CheAY and CheV2. Furthermore, $CheZ_{HP}$ appears to employ the same mechanism as *E. coli* CheZ, based on the results of a phosphate release assay using various $CheZ_{HP}$ mutants. The co-crystal structure of *E. coli* CheZ with CheY-BeF_3^--Mg^{2+} showed two amino acids to be paramount for phosphatase function. The first residue, Gln147, inserts itself into the active site of CheY-P and orients a water molecule for nucleophilic attack, while the second residue, Asp143, forms a salt bridge with Lys109 of CheY-P to stabilize the interaction between proteins (Zhao *et al.*, 2002). These two residues, as shown in Figure 6, are completely conserved amongst CheZ orthologs. In our original identification of $CheZ_{HP}$ via a genetic screen that found *cheZ* loss-of-function mutations, one of the suppressor mutants had a D189N mutation, corresponding to Asp143 of *E. coli* CheZ, indicating its importance for the function of $CheZ_{HP}$ (Terry *et al.*, 2006). Phosphate release assays with $CheZ_{HP}$ D189N and $CheZ_{HP}$ Q193R resulted in inactivation of the $CheZ_{HP}$ phosphatase activity. The C-terminus of CheZ was proposed to interact directly with CheY-P and bring it to CheZ active site (Silversmith, 2005). Here we found that a $CheZ_{HP}$ mutant lacking these C-terminal amino acids, $CheZ_{HP}\Delta C_{12}$, showed no increase in dephosphorylation of $CheY_{HP}$-P. The results from these three mutants indicate that $CheZ_{HP}$ uses the same mechanism to dephosphorylate $CheY_{HP}$-P as *E. coli* CheZ. Overall, the secondary structure prediction of $CheZ_{HP}$ is alpha helical, similar to *E. coli* CheZ, and thus it seems likely that the common portion folds similarly to *E. coli* CheZ. The N-terminal variable portion may form a separate domain that folds independently, but we have not analyzed this idea further. In $CheZ_{HP}$, this region is highly charged and contains an amino acid repeat of lysines and glutamic acids, as noted previously (Terry *et al.*, 2006).

We found that $CheZ_{HP}$ has additional phosphatase activity on CheAY. The *H. pylori* chemotaxis system has an advantage over that of *E. coli* in that we could test whether $CheZ_{HP}$ can dephosphorylate a range of other REC-containing chemotaxis proteins. Prior to this, CheZ had only been tested on CheY and CheB, and was found to not dephosphorylate CheB (Hess *et al.*, 1988). To our knowledge, there have been no tests of CheZ, or any of the chemotaxis phosphatase, on other REC-containing chemotaxis proteins such as CheV or CheAY. In the case of CheAY, $CheZ_{HP}$ appears to act on the REC domain, as the truncated variant that lacks the REC domain, CheA', was not affected by the presence of $CheZ_{HP}$ (Fig. 4). Many microbes have a REC domain attached to the C terminus of CheA (Wuichet *et al.*, 2007), akin to hybrid histidine kinases (Gao & Stock, 2009). In hybrid histidine kinases, the additional REC domain typically functions to transfer phosphoryl groups to a second unlinked histidine kinase, but this reaction has not been studied in CheAY proteins (Gao &

Stock, 2009). An alternate function suggested by our findings along with those of Dagmar Beier's group, is that the CheAY REC domain functions to funnel phosphates away from the phosphorylated histidine with facilitated removal by a phosphatase such as CheZ, akin to a phosphate sink. Thus a model for phosphate flow in the *H. pylori* chemotaxis system is that CheAY autophosphorylates, and then passes phosphoryl groups to $CheY_{HP}$ or, as a second choice, to its own REC domain (also referred to as CheY2) (Jiménez-Pearson *et al*., 2005). Previous work showed that $CheY_{HP}$ can retrophosphorylate CheAY, and thus phosphoryl groups can flow back from $CheY_{HP}$ to CheAY and finally to the REC domain of CheAY. If $CheZ_{HP}$ targets the CheAY REC domain, phosphoryl groups would be removed from the system and not move to $CheY_{HP}$, thus promoting a phosphate sink-type function.

$CheZ_{HP}$ furthermore has weak activity on CheV2, but we did not detect any activity toward CheV1 and CheV3. The phenotypes associated with loss of *cheV2* are fundamentally different than those associated with *cheV1* and *cheV3* mutants, supporting that there are key differences in these proteins (Lowenthal *et al*., 2009). Specifically, *cheV2* null mutants display a hyper-switching phenotype, while *cheV1* and *cheV3* null mutants are biased toward smooth swimming (Lowenthal *et al*., 2009). It is not yet known if there are different phenotypes associated with phosphorylation mutants of these CheV proteins, and furthermore, whether our observed CheV2-$CheZ_{HP}$ interaction occurs *in vivo*. The CheV slower autodephosphorylation rate and resistance to $CheZ_{HP}$ suggests that a quick turnover rate for most CheVs may not be required for chemotaxis, as suggested by others (Karatan *et al*., 2001, Szurmant & Ordal, 2004). The stability of their phosphorylation state has suggested that their role is more likely to be involved in adaptation (Szurmant & Ordal, 2004). We were unable to identify any significant conservation of known CheZ-interacting residues (Zhu *et al*., 1997, McEvoy *et al*., 1999) on $CheY_{HP}$, CheAY, or CheV2, pointing out that we do not yet have a complete grasp on the molecular basis for protein-protein interactions of the CheZ family.

An additional known *E. coli* CheZ protein-protein interaction is with a CheA variant, CheA-short, to drive CheZ's localization to the chemoreceptor patch (Cantwell *et al*., 2003). The CheZ regions responsible for this localization are located near the apical hairpin turn of the four-helix bundle, residues 80–120 in *E. coli* CheZ (Cantwell & Manson, 2009). This region is not well conserved amongst various $CheZ_{HP}$ orthologs including $CheZ_{HP}$, suggesting that the mechanism for cellular localization of $CheZ_{HP}$ might be different. Our preliminary observations show that $CheZ_{HP}$ localizes to the pole and can be observed in both soluble and membrane fractions independent of chemoreceptors, CheAY or CheW. (P. Lertsethtakarn, Jenny Draper, and K.M. Ottemann, unpublished). Additionally, CheA-short has not been shown to exist in *H. pylori*, therefore, it is possible that $CheZ_{HP}$ might localize via different mechanism or with different chemotaxis proteins. The $CheZ_{HP}$ N-terminus is the most variable region of the protein and is about 40 amino acids longer than *E. coli* CheZ; indeed, it seems like the C-terminal portion forms a CheZ domain that is consistent between orthologs, with an additional domain or extension on the N-terminal end (Fig. 6). In *E. coli* CheZ, the N-terminus forms the majority of the extended coiled-coil structure (Zhao *et al*., 2002), but limited activity has been ascribed to it. There are, however, several point mutations in this region that enhance CheZ phosphatase activity, suggesting this region contributes to phosphatase function (Huang & Stewart, 1993, Sanna & Simon, 1996). The low conservation level in this region, however, suggests that its function might differ between bacteria, or that it simply is not under positive selection. The N-terminal region might be responsible for CheZ's ability to have several substrates in addition to the proposed function of the C-terminus as well as functioning as an interface for its cellular localization.

The identification of $CheZ_{HP}$ expands the number of classes of bacteria that harbor the CheZ phosphatase. A database search for $CheZ_{HP}$ orthologs using $CheZ_{HP}$ from *H. pylori* SS1

recovered a large number of Gram-negative bacteria with $CheZ_{HP}$ orthologs. This analysis strongly suggests that CheZ-type phosphatases are not restricted to the proteobacteria, as previously hypothesized (Wuichet *et al.*, 2007). We found CheZ-orthologs in several non-proteobacter classes, but not in the Gram-positive organisms or archaea, indicating that CheZ is not as widely distributed as CheC-type phosphatases (Muff & Ordal, 2008). Interestingly, some of the identified proteins were annotated as methyl-accepting chemoreceptors (MCP) suggesting the CheZ domain might be fused to other chemotaxis proteins, although this awaits experimental analysis. The high level of conservation of the active site and the CheY-P binding domain (Fig.6) suggests that CheZ function is well conserved among the orthologs. There is little conservation outside of these regions, however, making identification of CheZ orthologs difficult. In general, CheZ and CheC-family phosphatase proteins have been noted to display very low amounts of conservation from organism to organism (Muff & Ordal, 2008). The basis for this low sequence conservation in this family is not yet known. Our work adds the knowledge, however, that remote CheZ proteins can retain function, and thus that CheZ family members play significant roles in chemotaxis systems of a wide variety of bacteria.

Experimental procedures

Molecular Biology Manipulations

H. pylori strain SS1 (Lee *et al.*, 1997) genomic DNA (Promega Wizard DNA Preparation Kit) was used as a template for amplification of *cheV1* (*hp0019*), *cheV2* (*hp0616*), *cheV3* (*hp0393*), *cheY_{HP}* (*hp1067*), and *cheZ_{HP}* (*hp0170*). Gene numbers are from *H. pylori* strain 26695 (Tomb *et al.*, 1997). All primers are listed in Supplemental Table 2. These genes were cloned into pGEX-6p-2 for overexpression of GST-tagged proteins to facilitate protein purification. The plasmids are pGEX-6p-2-cheV1, pGEX-6p-2-cheV2, pGEX-6p-2-cheV3, pGEX-6p-2-cheY_{HP}, and pGEX-6p-2-cheZ_{HP}. pTrc-CheAY and pTrc-CheA' (Jiménez-Pearson *et al.*, 2005) were generous gifts from Dagmar Beier (University of Würzburg, Germany).

pGEX-6p-2-cheY_{HP}D53A, pGEX-6p-2-cheZ_{HP}D189N, and pGEX-6p-2-cheZ_{HP}Q193R were generated by site directed mutagenesis using *PfuTurbo* DNA polymerase following the manufacturer's protocol (Stratagene). pGEX-6p-2-cheY and pGEX-6p-2-cheZ_{HP} were used as templates and primers are listed in Supplemental Table 2. To generate the variant of $CheZ_{HP}$ with 12 amino acids removed from the C-terminus, $CheZ_{HP}\Delta C_{12}$, pGEX-6p-2-cheZ_{HP} was used as a template for inverse PCR (iPCR) using primers hp0170iPCRfor2 and hp0170iPCRrev2.

Protein purification

All proteins were over-expressed in *E. coli* BL21 (Promega). Cultures were grown in LB or 2XYT plus ampicillin to OD_{600} 0.5–0.8 and induced with 0.1–1 mM of IPTG for 4–16 hours at 37°C or 30°C. Cells were harvested by centrifugation, lysed by sonication and then centrifuged at 206,020g for 45 minutes to remove membranes and unlysed cells. The supernatant was applied to a GST Prep 16/10 column (GE Healthcare) for purification of GST-tagged proteins or a His Prep FF16/10 (GE Healthcare) for purification of His-tagged CheAY or CheA'. GST-tagged proteins were treated with Prescission protease® to cleave GST, followed by repurification on a GST Prep 16/10 column to remove GST. Proteins were dialyzed in dialysis buffer (50 mM Hepes, pH 7.6, 50 mM KCl, and 20–30% glycerol) and stored in −80°C.

Phosphate release assay

Phosphate release was monitored using the EnzChek ® Phosphate Assay Kit (Invitrogen) as described by Silversmith *et al*. (Silversmith *et al*., 2001). To determine the autodephosphorylation rate of $CheY_{HP}$, CheV1, CheV2, CheV3, and $CheZ_{HP}$ we utilized 450µl reaction containing buffer (100 mM Hepes, pH 7 and 20 mM $MgCl_2$), 1 mM MESG (2-amino-6-mercapto-7-methyl-purine riboside), 0.5 U PNP (purine nucleoside phosphorylase), and various concentration of phosphodonor monophosphoimidazole (MPI), a kind gift of Ruth Silversmith (University of North Carolina, Chapel Hill). The reaction was allowed to equilibrate at room temperature until the OD_{360} remained stable, after which 5 µM of each protein (except $CheZ_{HP}$ in which 1 µM was used) was mixed into the reaction. The change in absorbance at OD_{360} was monitored for 10 minutes at room temperature. To monitor dephosphorylation activity of $CheZ_{HP}$ on $CheY_{HP}$-P and CheV-P, the reaction was set up as above but equilibrated in the presence of 1 µM $CheZ_{HP}$. $CheY_{HP}$ or one of the CheV proteins was added as above and the reaction was monitored for 10 minutes. To determine the optimal metal concentrations for $CheY_{HP}$ and the CheV proteins the reaction was performed as above with the replacement of 20 mM $MgCl_2$ with several concentrations either $MgCl_2$, KCl, $MnCl_2$, or no added metal (data not shown). To determine phosphatase release rates, we calculated the amount of phosphate released at each time point using the extinction coefficient at 360nm of 0.0091 μM^{-1} cm^{-1} at pH 7 (Silversmith *et al*., 2001). The release rate was determined by performing a linear fit (Excel, Microsoft), to calculate a slope of plotted phosphate release as a function of time. The first two time points were excluded from this analysis due to slight fluctuation of OD_{360} after protein addition.

Phosphorylation assays with [γ-^{32}P]ATP

A labeled ATP solution was created by mixing 2 mM unlabelled ATP with 11 µM [γ-^{32}P] ATP (10Ci/mMole) (PerkinElmer LAS Inc), to yield a final ATP solution of 2.01 mM. Reactions containing either 2 µM CheAY or 6 µM CheA', 43 or 114 µM labeled ATP solution, 20 mM $MgCl_2$, 50 mM KCl, 50 mM Tris/HCl pH 7.5, were incubated for 10 minutes at room temperature. $CheY_{HP}$ or $CheY_{HP}$+$CheZ_{HP}$ was then added to the reaction at 2:4 or 2:4:1 ratio of CheA:CheY:CheZ. Samples were collected and stopped in 2X SDS sample buffer (Scopes & Smith, 2006).

To determine if $CheZ_{HP}$ affected CheAY, a mixture of 200 µM unlabeled ATP plus 3 µM [γ-^{32}P] ATP (10Ci/mMol) was used to phosphorylate CheAY for 10 minutes at room temperature, followed by a "chase" with 10 mM unlabelled ATP in the presence or absence of $CheZ_{HP}$. For CheA', 12 µM CheA' was phosphorylated with a mixture of 400 µM unlabeled ATP plus 6 µM [γ-^{32}P]ATP (10Ci/mMol) at room temperature for 45 minutes, followed by ATP removal using a PD SpinTrap G-25 column (GE Healthcare). Purified CheA'-P was allowed to sit at room temperature for five minutes and the reactions were chased with unlabeled ATP as described above. Samples were loaded onto 12% or 15% SDS-PAGE to separate the protein mixture. Gels were dried and exposed to X-ray film (KODAK BioMax XAR Film) to visualize the radioactive bands or exposed to a phosphoroimager cassette and scanned to obtain the image with Typhoon TRIO (Amersham Biosicences). ImageQuant 5.2 (GE Healthcare) was used to quantify radioactive bands to calculate phosphorylated half life of each protein.

Sequence alignment and analysis

Amino acid sequence from HP0170 *H. pylori* strain SS1 (GenBank accession number GU223223) was used for PSI-BLAST analysis. The 2nd iteration yielded sequences annotated as CheZ under 'Sequences with E-values worse than threshold.' Three proteins, annotated as CheZ or putative CheZ, were selected for the 3rd iteration as shown in Table 1. This resulted in 268 proteins identified in the next iteration. A representative from each

genus with the highest E-value was selected for multiple sequence alignment with ClustalW and the alignment file was input into Jalview to generate graphical representation of the level of amino acids conservation (Chenna *et al*., 2003,Waterhouse *et al*., 2009). A 20 amino acid region surrounding the active site and the area corresponding to the C-terminus 14 amino acids were the most highly conserved, and were selected from the alignment and input into WebLogo (Crooks *et al*., 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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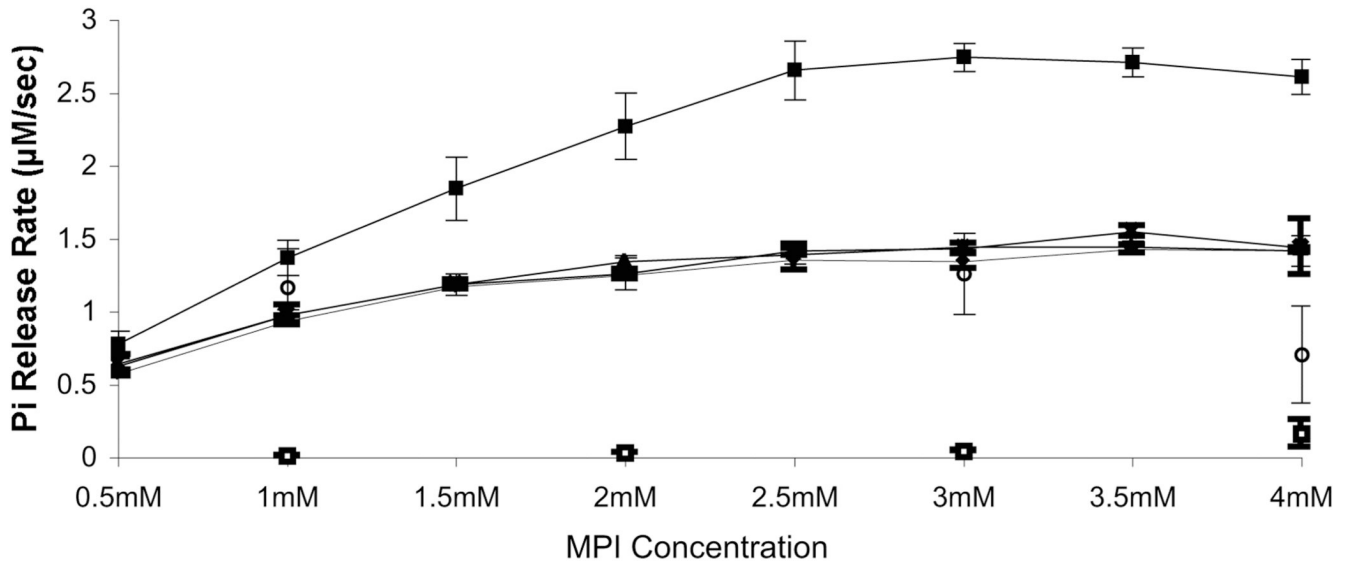
Figure 1. CheY$_{HP}$-P autodephosphorylation and dephosphorylation in the presence of CheZ$_{HP}$ and its variants

Shown is the amount of phosphate released using MPI as a phosphodonor. Lines are marked as follows: ♦, CheY alone; ■, CheY$_{HP}$ plus CheZ$_{HP}$; ▲, CheY$_{HP}$ plus CheZ$_{HP}$ D189N; x, CheY$_{HP}$ plus CheZ$_{HP}$ Q193R; ○, CheY$_{HP}$ plus CheZ$_{HP}$ΔC$_{12}$; □, CheZ$_{HP}$ alone. Each reaction contains 5 µM CheY and 1 µM CheZ$_{HP}$ and the indicated concentration of MPI. The data represents an average of two to four experiments and error bars show standard error. The lines overlap for CheY alone, CheY$_{HP}$ plus CheZ$_{HP}$ D189N, CheY$_{HP}$ plus CheZ$_{HP}$ Q193R and CheY$_{HP}$ plus CheZ$_{HP}$ΔC$_{12}$.
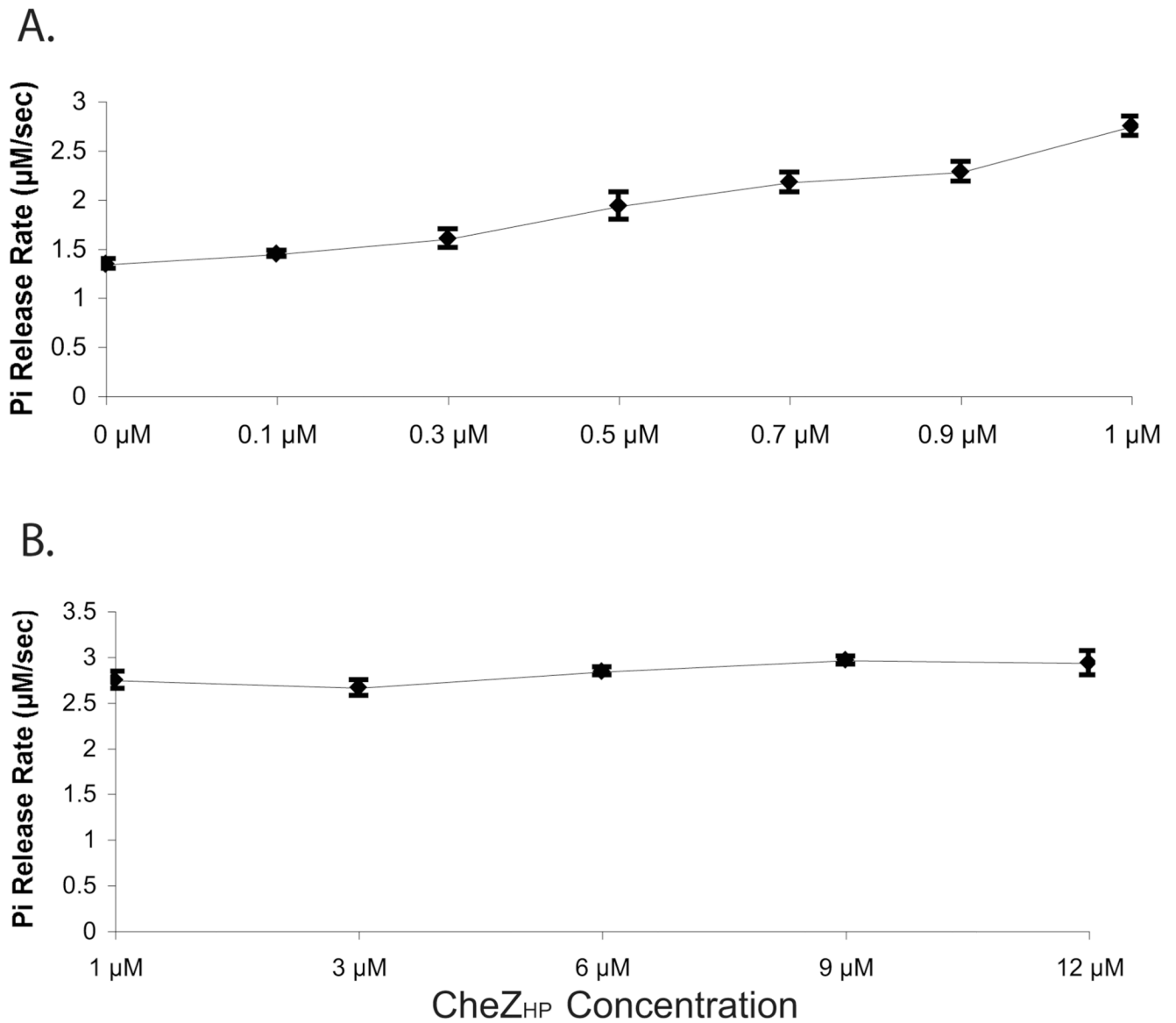
A.

B.

Figure 2. CheY$_{HP}$–P dephosphorylation saturates at 1µM CheZ$_{HP}$
Shown is the amount of phosphate released using MPI as a phosphodonor. The reaction includes 5 µM CheY$_{HP}$, 3 mM MPI, and CheZ$_{HP}$ at varying concentrations. (A) CheZ$_{HP}$ at lower concentration ranging from $0 - 1$ µM. (B) CheZ$_{HP}$ concentration ranging from 1–12 µM. The data represents the average of three experiments, and error bars show standard error.
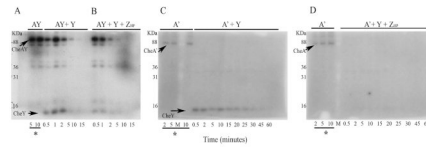
Figure 3. Phosphorylation assay of CheY$_{HP}$ using CheAY-P and CheA'-P
The amount of phosphor-CheY, phosphor-CheAY, and phosphor-CheA' were monitored using autoradiography of SDS-PAGE gels. CheAY and CheA' were phosphorylated *in vitro* for 10 minutes before the addition of CheY with or without CheZ$_{HP}$. 2 µM of CheAY, 12 µM CheA', 4 µM of CheY$_{HP}$ and 1 µM of CheZ$_{HP}$ were used unless specified. (A) CheAY phosphorylated with 43 µM ATP with addition of CheY$_{HP}$. (B) Same set up as (A) with the addition of CheZ$_{HP}$. (C) CheA' phosphorylated with 114 µM ATP with the addition of 12 µM CheY$_{HP}$. (D) Same as (C) with the addition of 3 µM CheZ$_{HP}$. Underlined time points marked with asterisks indicate phosphorylation of CheAY or CheA' before addition of CheY$_{HP}$ and CheZ$_{HP}$. kDa indicates the molecular weight marker lane, with values indicating kilodaltons. The data is a representative of three experiments. The extra bands in are likely to be partially degraded CheAY-P that was also observed shortly after purification of CheAY.
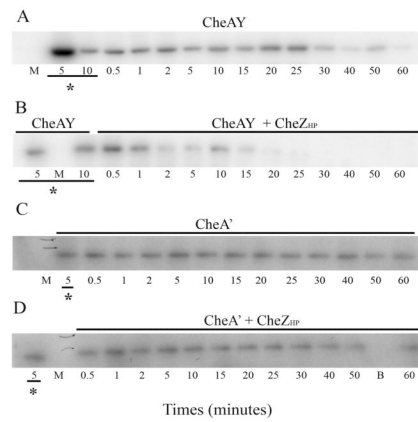
Figure 4. CheZ_{HP} has phosphatase activity on CheAY but not on CheA'

Pulse-chase phosphorylation assays were done as described in the text, using CheAY and CheA' phosphorylated with 203 µM ATP as described in the methods, followed by addition of 50-fold excess unlabelled ATP and CheZ$_{HP}$. Aliquots were removed at the indicated time points. (A) 2 µM CheAY was phosphorylated for 10 minutes followed by addition of 10 mM of unlabelled ATP. (B) Same as (A) with the addition of 1 µM CheZ$_{HP}$ at the same time as the unlabelled ATP. (C) 12 µM of purified CheA'-P was chased with 10mM unlabeled ATP. (D) Same as (C) with 6 µM CheZ$_{HP}$ added along with the unlabeled ATP. Underlined time points with asterisks indicate the absence of unlabeled ATP and CheZ$_{HP}$. The bands show phosphorylated CheAY in (A) and (B), and phosphorylated CheA' in (C) and (D). This data is a representative of two experiments. M and B indicated marker lane and blank lane, respectively. Gels were exposed to phosphoroimager cassette for 3 hours to overnight, or to X-ray film for 24–48 hours. CheA' bands always appear weaker likely due to some loss of activity associated with this truncation.
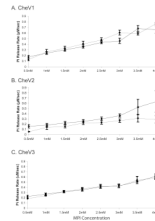
Figure 5. Dephosphorylation of the CheV proteins

Shown is the amount of phosphate released using MPI as a phosphodonor. ♦ CheV alone, ■ CheV with CheZ$_{HP}$. Each reaction contains 5 µM of the indicated CheV proteins s with and without 1 µM CheZ$_{HP}$. (A) CheV1, (B) CheV2, and (C) CheV3. The data represents an average of three experiments, and error bars show standard error.
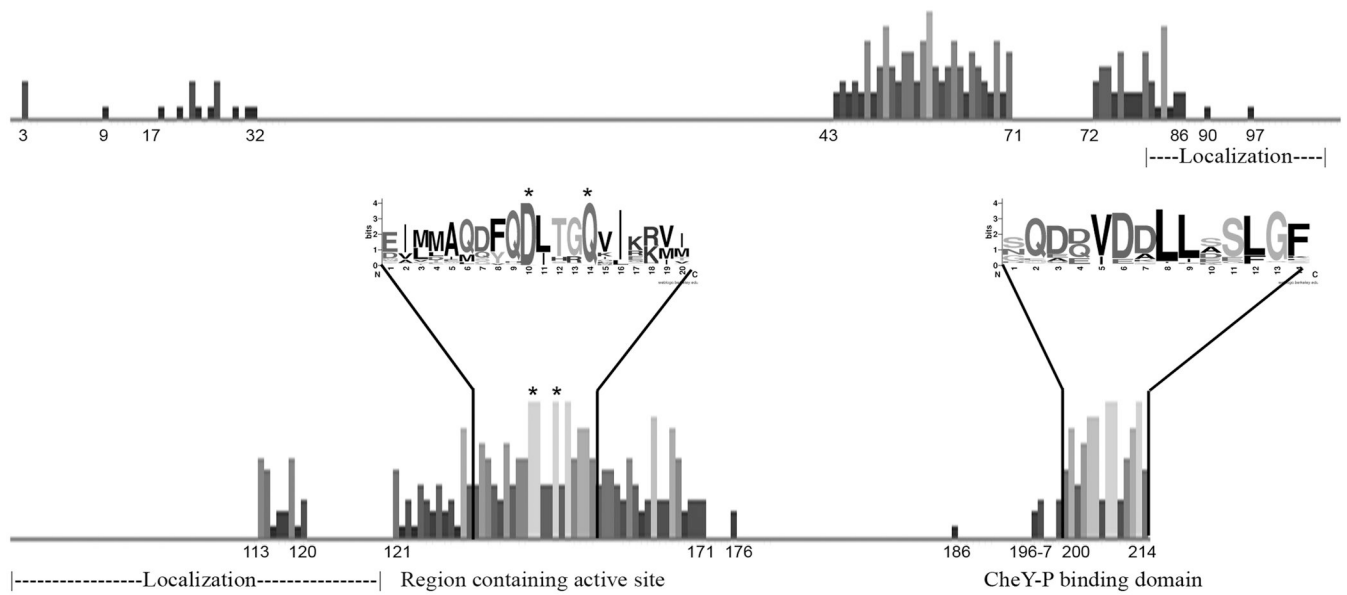
Figure 6. Amino acids conservation among CheZ$_{HP}$ orthologs
Shown is the output of a multiple sequence alignment displayed with degree of conservation as height of the bar at each position. Inset sequence logo format shows sequences of highly conserved regions. The numbering corresponds to *E. coli* CheZ sequence. The conserved Asp 143 and Gln 147 in *E. coli* CheZ are indicated by asterisks.

Table 1

Selected proteins for PSI-BLAST 3rd iteration.

Annotation	Bacterial strain	Score (Bits)	E-value
Chemotaxis phosphatase, CheZ	*Desulfatibaciillum alkenivorans*	44.7	0.009
Putative chemotaxis phosphatase, CheZ	*Hydrogenobaculum Sp.* YO4AAS1	40.8	0.13
Chemotaxis phosphatase, CheZ	*Shewanella sediminis* HAW-EB3	38.5	0.6