Phosphate flow in the chemotactic response system of *Helicobacter pylori*

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It is well established that motility is an essential virulence trait of the human gastric pathogen *Helicobacter pylori*. Accordingly, chemotaxis contributes to the ability of *H. pylori* to colonize animal infection models. Chemotactic signal transduction in *H. pylori* differs from the enterobacterial paradigm in several respects. In addition to a separate CheY response regulator protein (CheY1), *H. pylori* contains a CheY-like receiver domain (CheY2) which is C-terminally fused to the histidine kinase CheA. Furthermore, the genome of *H. pylori* encodes three CheV proteins consisting of an N-terminal CheW-like domain and a C-terminal receiver domain, while there are no orthologues of the chemotaxis genes *cheB*, *cheR* and *cheZ*. To obtain insight into the mechanisms controlling the chemotactic response of *H. pylori*, we investigated the phosphotransfer reactions between the purified two-component signalling modules *in vitro*. We demonstrate that both CheY1 and CheY2 are phosphorylated by CheA ~ P and that the three CheV proteins mediate the dephosphorylation of CheA ~ P, but with a clearly reduced efficiency as compared to CheY1 and CheY2. Furthermore, our data indicate retrophosphorylation of CheAY2 by CheY1 ~ P, suggesting a role of CheY2 as a phosphate sink to modulate the half-life of CheY1 ~ P.

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INTRODUCTION

Helicobacter pylori is the causative agent of chronic type B gastritis and gastric or duodenal ulcers (Blaser, 1992; Peterson, 1991). Moreover, infection with *H. pylori* increases the risk of developing gastric malignancies like adenocarcinoma or mucosa-associated lymphoid tissue (MALT) lymphoma (Uemura *et al.*, 2001). Flagella-based motility is an important virulence trait of this pathogen since non-motile mutants have been shown to be unable to colonize mice and piglets (Eaton *et al.*, 1992, 1996; Ottemann & Lowenthal, 2002). Accordingly it has been demonstrated that non-chemotactic mutants of *H. pylori* are attenuated in animal infection models (Ottemann & Lowenthal, 2002; McGee *et al.*, 2005; Terry *et al.*, 2005).

Bacterial chemotaxis is a bias of movement towards beneficial or away from harmful chemicals. It has been reported that *in vitro*, *H. pylori* exhibits chemotaxis towards urea and various amino acids, while bile acids act as chemorepellents (Yoshiyama *et al.*, 1999; Cerda *et al.*, 2003; Worku *et al.*, 2004). The biochemical processes governing chemotactic behaviour have been extensively studied in *Escherichia coli* (reviewed by Szurmant & Ordal, 2004;

Wadhams & Armitage, 2004). Briefly, binding of a ligand to the extracytoplasmic domain of a methyl-accepting chemotaxis protein (MCP) induces a conformational change of its cytoplasmic signalling domain which interacts with the adaptor protein CheW and the histidine kinase CheA. Changes in MCP conformation alter the autokinase activity of CheA and phosphorylated CheA transfers the phosphoryl group from the conserved histidine residue of its P1 domain to the response regulator CheY. The phosphorylated CheY (CheY ~ P) interacts directly with the protein FliM in the flagellar motor switch complex to change the direction of flagellar rotation. The response is terminated by CheZ which accelerates the hydrolysis of CheY ~ P.

From the genome sequence, which contains nine orthologues to known chemotaxis genes (Alm *et al.*, 1999; Tomb *et al.*, 1997), it can be predicted that chemotactic signalling in *H. pylori* differs from the enterobacterial paradigm. *H. pylori* encodes three classical MCPs (HP0082, HP0099, HP0103), which are likely to sense ligands external to the cell, a soluble MCP orthologue (HP0599) which might respond to internal physiological signals, a coupling protein CheW (HP0391), a histidine kinase CheA containing a C-terminal CheY-like receiver domain (CheAY2/HP0392) and a separate CheY response regulator (CheY1/HP1067). Orthologues of the genes *cheR* encoding a methyltransferase

Abbreviation: MCP, methyl-accepting chemotaxis protein.

and *cheB* encoding a response regulator with methylesterase activity are not present. In *E. coli* CheR and CheB are part of a stimulus-adaptation system which acts by modulating the ability of the MCPs to induce CheA autophosphorylation through the reversible methylation of glutamic acid residues in the cytoplasmic methylation region of the MCPs. Interestingly, the *H. pylori* MCPs contain several of the conserved glutamate residues of the methylation domain and the closely related organisms *Helicobacter hepaticus*, *Campylobacter jejuni* and *Wolinella succinogenes* encode orthologues of CheR and CheB; however, these CheB-like proteins do not contain a receiver domain (Parkhill *et al.*, 2000; Baar *et al.*, 2003; Suerbaum *et al.*, 2003). *H. pylori* contains three orthologues of the CheV protein which was first described in *Bacillus subtilis* and was shown to be involved in the adaptation to attractants in this organism. CheV consists of an N-terminal CheW-like domain and a C-terminal receiver domain. Phosphorylation of the receiver domain of CheV is required for efficient adaptation (Karatan *et al.*, 2001). Inactivation of *cheV1* (HP0019) significantly reduced swarming of *H. pylori* on semi-solid agar plates, while swarming was unchanged in insertion mutants of *cheV2* (HP0616) and *cheV3* (HP0393), respectively, and in a *cheV2/cheV3* double mutant (Pittman *et al.*, 2001). Therefore, the mechanisms of adaptation adopted by *H. pylori* remain unclear.

An orthologue of the CheY ~ P-specific phosphatase CheZ, which is involved in signal termination in β - and γ -*Proteobacteria*, is missing in *H. pylori*. However, the presence of a CheY protein (CheY2) fused to the C terminus of the histidine kinase CheA as well as of a separate CheY response regulator (CheY1) is reminiscent of the chemotaxis system of *Sinorhizobium meliloti*. This organism encodes two separate CheY proteins which are both rapidly phosphorylated by CheA. However, only one CheY protein (CheY2) interacts with the flagellar motor when phosphorylated and, therefore, controls flagellar rotation directly, while the second CheY protein (CheY1) functions as a modulator of the chemotactic response by acting as a phosphate sink which drains away the phosphoryl group from CheY2 ~ P via retro-phosphorylation of the histidine kinase CheA. Since CheY1 is present in *S. meliloti* in a tenfold excess over CheA, retro-phosphorylation in the signal transduction chain is an efficient mechanism for dephosphorylation of CheY2 ~ P (Schmitt, 2002).

Since virtually nothing is known about the molecular mechanisms controlling chemotaxis, which is an important virulence trait, in *H. pylori*, we purified the recombinant two-component chemotaxis proteins to investigate the phosphotransfer reactions between the different signalling modules *in vitro*. We show that CheY1, CheY2 and the three CheV proteins dephosphorylate CheA ~ P efficiently, but exhibit different affinities towards CheA. Furthermore, our data indicate that CheAY2 is retrophosphorylated by CheY1 ~ P, suggesting a role of the C-terminal CheY2 domain of CheAY2 as a phosphate sink to modulate the half-life of CheY1 ~ P.

METHODS

Bacterial strains and growth conditions. *H. pylori* G27 is a clinical isolate (Xiang *et al.*, 1995) and was cultivated as described previously (Beier *et al.*, 1997). *Campylobacter jejuni* 4344 is a clinical isolate which was obtained from the German *Campylobacter* and *Helicobacter* Reference Laboratory (Freiburg, Germany). The *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani broth. When necessary antibiotics were added to the following final concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$, and kanamycin, 25 $\mu\text{g ml}^{-1}$.

Construction of plasmids expressing *H. pylori* chemotaxis proteins fused to His₆ or GST affinity tags. Chromosomal DNA of *H. pylori* G27 was used as template DNA to PCR-amplify the *H. pylori* chemotaxis genes. The PCR primers used are listed in Table 2. All cloned PCR products were sequenced to ensure proper PCR amplification. The full-length *cheA* gene (HP0392/*cheAY2*) was amplified with primers *cheF* and *cheR*. The resulting PCR product which contains an internal *NheI* site was partially digested with *NheI* and *BamHI* and the 2412 bp fragment was subsequently cloned into pTrcHisA vector DNA, generating pTrc-*cheAY2*. The truncated *cheA'* allele encoding amino acids 1–667 of CheAY2 was amplified with primers *cheF* and CA'-R, digested with *NheI* and *BamHI* and cloned into pTrcHisA to yield plasmid pTrc-*cheA'*. The *cheY1* gene (HP1067) was amplified with primer pair *cheY1-5/cheY1-3* and the resulting PCR fragment was cloned into pGEX-3X vector DNA after restriction with *BamHI/EcoRI*, generating pGEX-*cheY1*. The *BamHI/EcoRI* *cheY1* fragment was also cloned into pSL1180 vector DNA and the resulting plasmid was used as template for site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), creating *cheY1-D53N*. The *cheY1-D53N* allele was subsequently cloned into pGEX-3X. To construct pTrc-*cheY2* expressing the receiver domain of the bifunctional histidine kinase CheAY2, a 384 bp DNA fragment encoding amino acids 678–803 of CheAY2 was amplified with primers CARR-F and *cheR* and cloned into *NheI*- and *BamHI*-digested pTrcHisA vector DNA. The genes *cheV1* (HP0019), *cheV2* (HP0616) and *cheV3* (HP0393) were amplified with primer pairs *cheV1-5/cheV1-3*, *cheV2-5/cheV2-3* and *cheV3-5/cheV3-3*, respectively, and the resulting PCR fragments were subsequently cloned into pQE30 vector DNA. The *cheY1* orthologue of *C. jejuni* was amplified from chromosomal DNA of *C. jejuni* 4344 with primer pair Cj*cheY1-5/CjcheY1-3* and the resulting DNA fragment of 390 bp was cloned into pGEX-3X vector DNA after digestion with *BamHI* and *EcoRI*, yielding plasmid pGEX-Cj*cheY1*.

Expression and purification of fusion proteins. The His₆- and GST-fusion proteins derived from the pTrc and pGEX constructs, respectively, were produced in *E. coli* DH5 α . pQE plasmids with inserts encoding His₆-fusion proteins were propagated in *E. coli* M15. Expression and purification of GST- and His₆-fusion proteins was performed as described previously (Perraud *et al.*, 1998; Beier & Frank, 2000).

In vitro phosphorylation assays. *In vitro* phosphorylation assays were carried out at 30 °C in a final volume of 25 μl reaction buffer (50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.2 mM ATP) containing CheAY2 or CheA' and, where appropriate, the respective response regulator proteins. The concentration of proteins and the reaction times applied are specified in the figure legends. Autophosphorylation of CheAY2 and CheA', as well as transphosphorylation of CheY1, CheY1-D53N, CheY2, CheV1, CheV2 and CheV3 in the presence of CheAY2 and CheA', respectively, under multiple turnover conditions, was initiated by the addition of 50 nM [γ -³²P]ATP (5000 Ci mmol⁻¹). Phosphotransfer reactions from CheA' ~ P to CheY1, CheY1-D53N, CheY2, CheV1, CheV2 and CheV3, respectively, were initiated by the addition of the respective response regulator protein to CheA' which had been incubated

Table 1. Strains and plasmids

Bacterial strain or plasmid	Relevant features	Reference or source
Strains		
<i>H. pylori</i> G27	Clinical isolate	Xiang <i>et al.</i> (1995)
<i>C. jejuni</i> 4344	Clinical isolate	German Reference Laboratory for <i>Helicobacter</i> and <i>Campylobacter</i> , Freiburg, Germany
<i>E. coli</i> DH5 α	Strain used for high-efficiency transformation	Gibco
<i>E. coli</i> M15	Strain used for overproducing His ₆ -tagged proteins	Qiagen
Plasmids		
pSL1180	Cloning vector	Amersham Biosciences
pTrcHisA	Expression vector for N-terminal His ₆ tag cloning	Invitrogen
pQE30	Expression vector for N-terminal His ₆ tag cloning	Qiagen
pGEX-3X	GST gene fusion and expression vector	Amersham Biosciences
pTrc-cheAY2	pTrcHisA expressing the bifunctional CheAY2 protein of <i>H. pylori</i>	This study
pTrc-cheA'	pTrcHisA expressing a truncated derivative of CheAY2 comprising amino acids 1–676 and lacking the C-terminal CheY2 domain	This study
pGEX-cheY1	pGEX-3X expressing CheY1 of <i>H. pylori</i>	This study
pSL-cheY1/D53N	pSL1180 containing a 373 bp <i>Bam</i> HI– <i>Eco</i> RI fragment encoding a mutated derivative of CheY1 of <i>H. pylori</i> with a D53N substitution	This study
pGEX-cheY1/D53N	pGEX-3X expressing a mutated derivative of <i>H. pylori</i> CheY1 with a D53N substitution	This study
pTrc-cheY2	pTrcHisA expressing the C-terminal CheY2 domain (amino acids 677–803) of the bifunctional CheAY2 protein of <i>H. pylori</i>	This study
pQE-cheV1	pQE30 expressing CheV1 of <i>H. pylori</i>	This study
pQE-cheV2	pQE30 expressing CheV2 of <i>H. pylori</i>	This study
pQE-cheV3	pQE30 expressing CheV3 of <i>H. pylori</i>	This study
pGEX-CjcheY1	pGEX-3X expressing CheY1 of <i>C. jejuni</i>	This study

Table 2. Oligonucleotides used in this study

Name	Sequence (5'–3')*	Site†	Strand	Position‡
cheF	attcaggctagcATGGATGATTTGCAAGAAATAATG	<i>Nhe</i> I	–	402 934–402 957
cheR	agcttggatccTCACGATTGGTCTCCTTCTAA	<i>Bam</i> HI	+	400 546–400 566
CA'-R	agcttggatccctcaATCGCTAGGGGAATTTTGCTC	<i>Bam</i> HI	+	400 929–400 951
CARR-F	attcaggctagcTATATTGTCTTAGCGATTGATG	<i>Nhe</i> I	–	400 908–400 929
cheY1-5	catcacggatccGAAACTACTGGTAGTAGATGA	<i>Bam</i> HI	+	1 126 269–1 126 291
cheY1-3	tttcacgaattcTTAATCGTTGTCCCTAAACAACC	<i>Eco</i> RI	–	1 126 620–1 126 642
cheY1-D53N1	GTGCTTATTACAAATTGGAACATGC		+	1 126 413–1 126 437
cheY1-D53N2	GCATGTTCCAATTTGTAATAAGCAC		–	1 126 413–1 126 437
cheV1-5	catcacggatccGCTGATAGTTTAGCGGGCATTG	<i>Bam</i> HI	+	18 377–18 399
cheV1-3	tttcttggtaccTTATGCTAATTCCAAAAATTGCTTAAC	<i>Kpn</i> I	–	19 321–19 345
cheV2-5	catcacggatccGTAAGAGATATTGACAAAACGAC	<i>Bam</i> HI	+	661 114–661 136
cheV2-3	tttcttctgcagTTATGAAAAGCGTTTTTTTAAGCATTTC	<i>Pst</i> I	–	662 031–662 058
cheV3-5	catcacggatccGCAGAAAAACAGCTAACGATTTA	<i>Bam</i> HI	+	403 922–403 946
cheV3-3	tttcttctgcagTTACGCATTCTTGTCTAAAATCTTAGA	<i>Pst</i> I	–	403 014–403 037
CjcheY1-5	catcacggatccTGAAATTGTTAGTTGTTGATGACAG	<i>Bam</i> HI	–	1 050 999–1 051 023
CjcheY1-3	tttcacgaattcTACTCAGCTGCACCTTCTC	<i>Eco</i> RI	+	1 050 632–1 050 651

*Sequences in upper-case letters are derived from the genome sequence of *H. pylori* 26695 (Tomb *et al.*, 1997). Sequences introduced for cloning purposes are given in lower-case letters; restriction recognition sequences are underlined.

†Restriction recognition sites.

‡Nucleotide positions refer to the genome sequence of *H. pylori* 26695 (Tomb *et al.*, 1997) or *C. jejuni* (Parkhill *et al.*, 2000) in the case of CjcheY1-5 and CjcheY1-3.

for 15 min in the presence of 50 nM [γ - 32 P]ATP (5000 Ci mmol $^{-1}$) in reaction buffer. To determine the dephosphorylation rates of CheAY2 ~ P and CheA' ~ P, either 2 μ M CheAY2 or 2 μ M CheA' were phosphorylated in the presence of 50 nM [γ - 32 P]ATP at 30 °C for 10 and 15 min, respectively. Subsequently, both reactions were chased by the addition of unlabelled ATP to a final concentration of 10 mM. To analyse the dephosphorylation rate of CheY2 in the presence of other chemotaxis response regulator proteins, CheA' and CheY2 were incubated with 50 nM [γ - 32 P]ATP for 15 min at 30 °C. Then unlabelled ATP and/or the respective response regulator protein was added. The reactions were stopped at the indicated time points by the addition of sample buffer (60 mM Tris/HCl, pH 7.5, 50 mM Na $_2$ EDTA, 10 % glycerol, 2 % SDS, 5 % β -mercaptoethanol, 0.05 % bromophenol blue) and the reaction mixtures were separated by electrophoresis on SDS-15 % polyacrylamide gels (1.5 mm). Quantification of the signals was performed using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences) and ImageMaster TotalLab Software (Amersham Biosciences). All experiments were performed at least three times independently.

In vitro phosphorylation of the purified response regulator proteins with radioactively labelled acetyl phosphate was performed by incubating CheY1, CheY2, CheV1, CheV2 and CheV3, respectively, for 10 min at room temperature in a final volume of 25 μ l in reaction buffer (50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM MgCl $_2$) containing 5 μ Ci 32 P-labelled acetyl phosphate (80 Ci mmol $^{-1}$; Hartmann Analytic). The dephosphorylation rates of CheY1 ~ P and CheY2 ~ P in the absence of CheA' were determined by phosphorylating the response regulators in the presence of 5 μ Ci 32 P-labelled acetyl phosphate (80 Ci mmol $^{-1}$) for 10 min at room temperature and subsequently chasing the reactions by the addition of unlabelled acetyl phosphate to a final concentration of 10 mM. Retrophosphorylation experiments were performed in a final volume of 50 μ l reaction buffer in the presence of 5 μ Ci 32 P-labelled acetyl phosphate (80 Ci mmol $^{-1}$). The analysis of samples and signal quantification was performed as described above.

RESULTS

Purification of recombinant chemotaxis two-component proteins of *H. pylori* and *C. jejuni*

The *H. pylori* chemotaxis genes *cheAY2*, *cheY*, *cheV1*, *cheV2* and *cheV3* were cloned into appropriate vectors creating N-terminal fusions to a His $_6$ or glutathione S-transferase affinity tag, allowing the overexpression of the recombinant proteins in *E. coli* and their purification by affinity chromatography. CheAY2, as well as a derivative of CheAY2 lacking the C-terminal CheY-like domain (CheA'), were fused with an N-terminal His $_6$ affinity tag. In addition, a construct was established which expresses the C-terminal CheY-like domain of CheAY2 as a separate protein (CheY2) with an N-terminal His $_6$ -tag. CheY1 and a derivative of CheY1 with a substitution of the putative phosphate-accepting aspartic acid residue at position 53 by asparagine (CheY1-D53N) were fused to glutathione S-transferase. The recombinant CheV proteins, CheV1, CheV2 and CheV3, were overexpressed in *E. coli* with an N-terminal His $_6$ -tag. Furthermore, the orthologous gene of *cheY1* from *C. jejuni* (Cj118c) was cloned and was overexpressed with an N-terminal glutathione S-transferase domain. A schematic representation of the purified recombinant proteins is shown in Fig. 1(a).

Kinetics of autophosphorylation of CheAY2 and its C-terminally truncated derivative CheA'

When CheAY2 and CheA' were incubated in the presence of [γ - 32 P]ATP autophosphorylation of the histidine kinases could be detected (Fig. 2). The ATP-dependent autophosphorylation of CheA' followed a simple exponential time

Fig. 1. Schematic representation of the chemotaxis two-component proteins of *H. pylori*. (a) Representation of the recombinant chemotaxis two-component proteins used in this study. The phosphorylated histidine residue in the P1 domain of the histidine kinase (H) and the phosphate-accepting aspartic acid residues in the response regulator domains (D) are highlighted. The molecular masses of the proteins are given on the right. (b) Representation of the phosphotransfer reactions between the CheA histidine kinase and the chemotaxis response regulator proteins CheY1, CheY2 and CheV1-CheV3. Arrows indicate the direction of phosphorylation.

Fig. 2. Kinetics of ATP-dependent autophosphorylation of CheAY2 and CheA' and dephosphorylation of CheAY2~P and CheA'~P. (a) Time-course of CheAY2 (◆) and CheA' (■) autophosphorylation. Purified CheAY2 and CheA' (2 μM each) were incubated in the presence of 50 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. At the indicated time points the reactions were stopped by the addition of sample buffer containing 50 mM Na_2EDTA and the samples were analysed by SDS-PAGE. The relative amount of phosphorylated CheAY2 and CheA' was determined by Phosphor-Image analysis of the respective gels. The maximal phosphorylation level obtained for the respective histidine kinase was arbitrarily set as 100 %. Data points represent the means of three independent experiments. On the right phosphor images of representative SDS-15 % polyacrylamide gels are shown. (b) Time-course of the dephosphorylation of CheAY2~P (◆) and CheA'~P (■). CheAY2 and CheA' (2 μM each) were incubated in the presence of 50 nM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 10 and 15 min, respectively. Then the reactions were chased by the addition of unlabelled ATP to a final concentration of 10 mM. At the indicated time points the reactions were stopped and the samples were analysed as described above. Data points represent the means of three independent experiments.

course, reaching the maximum level of phosphorylation of CheA' after 15 min which remained constant for the rest of the incubation period (60 min; Fig. 2a). In contrast, the full-length protein, CheAY2, reached the maximum level of phosphorylation after 8 min and then the relative level of CheAY2~P continuously declined within 60 min of incubation to about 40 % of the maximum level. This time course suggested that the C-terminal CheY-like domain of CheAY2 (CheY2) accelerates the dephosphorylation of the CheAY2 P1 domain containing the highly conserved histidine residue. To determine the half-life of CheAY2~P and CheA'~P, the phosphorylation reactions were chased after 10 and 15 min, respectively, by the addition of an excess of unlabelled ATP. CheAY2~P hydrolysed with a half-life of 3 min and hydrolysis was almost complete after 15 min of chase (Fig. 2b). In the case of CheA'~P, approximately 40 % of the amount of phosphorylated CheA' obtained after 15 min incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ could still be detected after 30 min of chase (Fig. 2b). When the phosphorylation reaction was chased for 60 min, 35 % of the initial amount of CheA'~P remained detectable (data not

shown). Therefore, the chase experiments corroborate the conclusion that the presence of CheY2 in the bifunctional CheAY2 protein significantly decreases the half-life of the phosphorylated histidine residue in the P1 domain of the CheA kinase.

CheA'-dependent phosphorylation of CheY2 and CheY1, and dephosphorylation of CheA'~P by the CheV proteins

To study the phosphotransfer reactions from the CheA histidine kinase to the receiver modules involved in chemotactic signalling in *H. pylori*, CheAY2 and CheA' were independently combined with the respective response regulator proteins in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Incubation of either CheAY2 or CheA' with CheY2 under multiple turnover conditions resulted in efficient phosphorylation of CheY2 (Fig. 3a, lanes 2 and 5, respectively). When the phosphotransfer to CheY1 was analysed under the same experimental conditions, dephosphorylation of the respective histidine kinase was observed; however, phosphorylated

CheY1 did not accumulate (Fig. 3a, lanes 3 and 6, respectively). Dephosphorylation of CheA'~P did not occur when CheA' and CheY1-D53N were combined in the presence of [γ-³²P]ATP (data not shown). These data indicated that the dephosphorylation of CheA'~P in the presence of CheY1 was due to the transfer of the phosphoryl group from the histidine kinase to D53 of CheY1. When CheY1 was added to a mixture of CheA' and [γ-³²P]ATP which had been preincubated for 15 min, CheY1~P could be detected within the first 10 s of incubation, but not at later time points (Fig. 3b). A similar fast phosphotransfer was observed upon addition of CheY2 to the reaction mixture (Fig. 3b). In the case of both CheY proteins, dephosphorylation of CheA'~P was complete 5 s after the addition of the response regulator protein. Neither CheY1

Fig. 3. Analysis of phosphoryl group transfer between the CheA histidine kinase and CheY1, CheY2 and the CheV proteins under multiple turnover conditions. (a) CheA' (2 μM) was incubated in the presence of [γ-³²P]ATP (lane 1) and upon addition of 4 μM CheY2 (lane 2) and 4 μM CheY1, respectively, (lane 3) for 10 min at 30 °C. Similarly, CheA' (2 μM) was incubated in the presence of [γ-³²P]ATP (lane 4) and upon addition of 4 μM CheY2 (lane 5), 4 μM CheY1 (lane 6), 4 μM CheV1 (lane 7), 4 μM CheV2 (lane 8) and 4 μM CheV3 (lane 9), respectively, for 15 min at 30 °C. The reactions were stopped by the addition of sample buffer containing 50 mM Na₂EDTA. (b) CheA' (2 μM) was phosphorylated in the presence of [γ-³²P]ATP for 15 min (lane 1) and then 4 μM CheY2 (lanes 2 and 3) and 4 μM CheY1 (lanes 4 and 5), respectively, were added. After 5 (lanes 2 and 4) and 10 s (lanes 3 and 5) the reactions were stopped by the addition of sample buffer containing 50 mM Na₂EDTA. The reaction mixtures were analysed by electrophoresis on a SDS-15 % polyacrylamide gel. Signal detection was performed by phosphorimaging.

nor CheY2 was phosphorylated in the presence of [γ-³²P]ATP alone (data not shown). Interestingly, the purified recombinant CheY protein of *E. coli*, which was used as a control, also caused the rapid dephosphorylation of CheA'~P, but, as observed with CheY1 from *H. pylori*, *E. coli* CheY~P did not accumulate under our experimental conditions (data not shown), while efficient phosphorylation of *E. coli* CheY by its cognate *E. coli* histidine kinase CheA has been reported under steady-state conditions (Bourret *et al.*, 1990). When the CheV proteins were incubated in the presence of CheA' and [γ-³²P]ATP under multiple turnover conditions, moderate dephosphorylation of CheA'~P was observed in the case of CheV1 and CheV2, while CheA'~P was almost completely dephosphorylated in the presence of CheV3, indicating transfer of the phosphoryl group from CheA' to the receiver domain of the CheV proteins. As in the case of CheY1, the phosphorylated

Fig. 4. CheA'-dependent phosphorylation of CheY2 and dephosphorylation of CheY2~P in the presence of other chemotaxis response regulator proteins. (a) CheA'-dependent phosphorylation of CheY2 in the presence of CheY1, CheY1-D53N, CheV1, CheV2 and CheV3, respectively. Reaction mixtures containing 2 μM CheA' (lane 1), 2 μM CheA' and 4 μM CheY2 (lanes 2 and 11), and 2 μM CheA', 4 μM CheY2 and 1 or 4 μM of the indicated response regulator protein (CheY1, lanes 3 and 4; CheY1-D53N, lanes 5 and 6; CheV3, lanes 7 and 8; CheV1, lanes 9 and 10; CheV2, lanes 12 and 13) were incubated in the presence of [γ-³²P]ATP for 15 min. The reactions were stopped by the addition of sample buffer containing 50 mM Na₂EDTA and the reaction mixtures were analysed on an SDS-15% polyacrylamide gel. (b) Kinetics of dephosphorylation of CheY2~P alone (◆) or in the presence of CheY1 (□), CheY1-D53N (○), CheV1 (△), CheV2 (×) and CheV3 (*). CheA' and CheY2 (2 μM each) were incubated in the presence of [γ-³²P]ATP for 15 min. Then the respective response regulator protein was added to a final concentration of 2 μM (*t*₀). At the indicated time points the reactions were stopped by the addition of sample buffer containing 50 mM Na₂EDTA and the samples were analysed by SDS-PAGE. The relative amount of phosphorylated CheY2 was determined by phosphorimaging of the respective gels. The amount of phosphorylated CheY2 present after 15 min of incubation in the presence of CheA' and [γ-³²P]ATP and before the addition of the additional response regulator protein was arbitrarily set as 100% (*t*₀). Data points represent the means of three independent experiments. On the right, phosphorimages of representative SDS-15 % polyacrylamide gels are shown. (c) Kinetics of dephosphorylation of CheY2~P in the presence of an excess of unlabelled ATP. CheA' and CheY2 (2 μM each) were incubated in the presence of [γ-³²P]ATP for 15 min. Then unlabelled ATP (◇, final concn 10 mM) or both unlabelled ATP and CheY1 (■, final concn 10 mM and 2 μM, respectively) were added to the reaction mixture (*t*₀). At the indicated time points the reactions were stopped and analysed by SDS-PAGE. Signal quantification was performed as described above. On the right, phosphorimages of representative SDS-15 % polyacrylamide gels are shown.

CheV proteins could not be detected (Fig. 3a, lanes 7–9). Similar results were obtained when the phosphotransfer between CheAY2 and the CheV proteins was analysed under multiple turnover conditions (data not shown). When the CheV proteins were added to CheA' which was auto-phosphorylated in the presence of [γ - 32 P]ATP for 15 min, complete dephosphorylation of CheA'~P was observed after 15 s incubation in the case of CheV3, while in the case of CheV1 and CheV2, 45 and 60 %, respectively, of the initial amount of CheA'~P was still detectable after 60 s incubation (data not shown). As observed for CheY1 and CheY2, the CheV proteins were not phosphorylated by [γ - 32 P]ATP (data not shown).

CheY1, but not CheV1, CheV2 and CheV3, interferes with the CheA'-dependent phosphorylation of CheY2

To study the effect of the various chemotaxis response regulator proteins on the CheA'-dependent phosphorylation of CheY2, CheA' and CheY2 were incubated in the presence of [γ - 32 P]ATP and each of the response regulators independently under multiple turnover conditions. While the CheV proteins had no effect on the phosphorylation of CheY2 (compare Fig. 4a, lane 2 with lanes 7–10, and lane 11 with lanes 12–13), CheY2~P could no longer be detected when CheY1 and CheY2 were present simultaneously in

equimolar concentrations (compare Fig. 4a, lane 2 with lane 4). When CheY2 was present in the reaction mixture in a fourfold excess relative to CheY1, phosphorylation of CheY2 could be observed, but was clearly reduced as compared to the control reaction containing only CheY2 (compare Fig. 4a, lane 2 with lane 3). When the recombinant CheY1-D53N protein lacking the highly conserved phosphate-accepting aspartic acid residue was added to the reaction, no effect on the phosphorylation of CheY2 was observed (Fig. 4a, lanes 5 and 6). These results suggested that CheY1 strongly prevails over CheY2 in competition for the phosphoryl group donor CheA' ~ P.

Next we analysed the effect of the chemotaxis response regulator proteins on CheY2 ~ P which was generated by preincubation with CheA' in the presence of [γ - 32 P]ATP for 15 min. When CheY1 was added to the reaction mixture containing CheA', CheY2 and [γ - 32 P]ATP, the relative amount of CheY2 ~ P declined rapidly and within 30 min of incubation to about 10 % of the initial amount observed when CheY1 was added (t_0). In contrast, about 45 % of the amount of CheY2 ~ P present at t_0 remained detectable after 30 min when the decay of CheY2 ~ P was analysed in the absence of an additional response regulator protein (Fig. 4b). As expected from the results presented in Fig. 4(a), CheY1-D53N had no effect on the decay of CheY2 ~ P in the time-course experiment. A similar kinetic curve as monitored in the presence of CheY1 was also obtained when the heterologous recombinant CheY1 protein from *C. jejuni* was used, indicating a productive interaction of *H. pylori* CheA' with this response regulator (data not shown). Addition of the CheV proteins somewhat reduced the amount of CheY2 ~ P within the first 15 min of incubation; however, the relative amount of CheY2 ~ P still present after 30 min was almost unchanged as compared to the reaction containing no additional response regulator protein.

We then performed a chase experiment to measure the decay of CheY2 ~ P in the absence of multiple phosphorylation turnover. When an excess of unlabelled ATP was added to CheA' and CheY2 which were preincubated in the presence of [γ - 32 P]ATP for 15 min, the kinetics of CheY2 ~ P hydrolysis was very similar to the time course obtained after addition of CheY1 to the reaction mixture (compare Fig. 4b and 4c). Under these experimental conditions the half-life of CheY2 ~ P was determined to be 3.3 min. The simultaneous addition of CheY1 and unlabelled ATP had no pronounced effect on the half-life of CheY2 ~ P (3.0 min; Fig. 4c). The difference in the kinetics of dephosphorylation of CheY2 ~ P observed in the absence or presence of an excess of unlabelled ATP indicated that, in the unchased reaction, phosphorylation of CheA' and phosphotransfer to CheY2 continuously occurred in the investigated time period, resulting in the presence of a higher relative amount of CheY2 ~ P after 30 min incubation. It should be noted that CheA' ~ P was hardly detectable in the time-course experiments (data not shown). Therefore, we conclude that the effect on the half-life of CheY2 ~ P observed after

addition of CheY1 to a mixture of CheA' and CheY2 in the presence of [γ - 32 P]ATP is due to the almost exclusive phosphorylation of CheY1 by newly formed CheA' ~ P as a consequence of a more efficient interaction with CheA' ~ P. Consequently, the prevalence of CheY1 in the competition for the phosphoryl group donor CheA' ~ P almost completely blocks any further phosphorylation of CheY2. These data suggest that CheY1 interacts more efficiently with CheA' ~ P, possibly through a higher affinity for the histidine kinase than CheY2. This is also supported by the observation that CheY1 caused the complete dephosphorylation of CheA' ~ P within 10 s at 0 °C when it was added to CheA' phosphorylated with [γ - 32 P]ATP for 15 min, while about 20 % of the initial amount of CheA' ~ P was still detectable when CheY2 was investigated under the same experimental conditions (data not shown).

Phosphorylation of CheY1 and CheY2 by acetyl phosphate and retrophosphorylation of CheY2 by CheY1 ~ P

Several response regulator proteins including CheY of *E. coli* are phosphorylated *in vitro* in the presence of low-molecular-mass phosphate donors like acetyl phosphate (McCleary & Stock, 1994; Lukat *et al.*, 1992). When the recombinant CheY1 and CheY2 proteins of *H. pylori* were incubated with 32 P-labelled acetyl phosphate, phosphorylation of both response regulators was observed (Fig. 5).

Fig. 5. Phosphorylation of CheY1, CheY2 and the CheV proteins by acetyl phosphate. CheY2 (24 μ M, lane 1), CheY1 (7 μ M, lane 2), CheV1 (4 μ M, lane 3), CheV2 (20 μ M, lane 4) and CheV3 (6 μ M, lane 5) were incubated in the presence of 5 μ Ci 32 P-labelled acetyl phosphate for 10 min. The reactions were stopped by the addition of sample buffer containing 50 mM Na₂EDTA, and the reaction mixtures were analysed by electrophoresis on a SDS-15 % polyacrylamide gel. Interestingly, phosphorylation signals from proteins of higher molecular mass than the monomeric CheV2 could also be detected which might suggest phosphorylation-induced oligomerization of CheV2, since contaminating amounts of similar sizes could not be detected in significant amounts when the purified CheV2 was analysed by SDS-PAGE and Coomassie staining (data not shown).

However, the efficiency of *in vitro* phosphorylation differed significantly since CheY2 had to be present at a concentration of 24 μM to detect CheY2~P (Fig. 5, lane 1), while a strong phosphorylation signal was obtained when CheY1 was included in the reaction mixture at a concentration of 7 μM (Fig. 5, lane 2). *In vitro* phosphorylation of CheV2 by acetyl phosphate could also be detected (Fig. 5, lane 4), while CheV1 and CheV3 were not phosphorylated under these conditions (Fig. 5, lanes 3 and 5, respectively). However, the presence of rather low concentrations of CheV1 and CheV3 in the reaction mixtures, which was due to the lower solubility of the recombinant proteins, might account for the lack of detectable phosphorylation. Chase experiments with unlabelled acetyl phosphate revealed that CheY1~P was hydrolysed completely within 30 min of incubation, displaying a half-life of 20 s. In contrast, CheY2~P exhibited increased stability under these conditions since about 55 % of the initial amount of CheY2~P was still detectable after a 30 min chase (data not shown). As expected, the CheY-D53N protein could not be phosphorylated when incubated with ^{32}P -labelled acetyl phosphate (data not shown), suggesting the phosphorylation of the response regulators on the conserved aspartic acid residue by the low-molecular-mass phosphate donor molecule.

To investigate whether retrotransfer of the phosphoryl group from one of the CheY proteins to the conserved histidine residue in the P1 domain of CheA is occurring in the chemotaxis system of *H. pylori*, we incubated either CheY1 or CheY2 in the presence of ^{32}P -labelled acetyl phosphate and the CheA histidine kinase. When the truncated protein CheA' was included in the reaction mixture, phosphorylated CheA' could not be detected (data not shown). However, when CheAY2 was combined with CheY1, phosphorylation of the bifunctional protein was observed, while at the same time CheY1~P was hydrolysed, suggesting retrotransfer of the phosphoryl group to CheAY2 (Fig. 6a, lane 2). CheY2~P was not significantly hydrolysed in the presence of CheAY2 and phosphorylation of CheAY2 could not be detected (Fig. 6a, lane 4). As a control CheAY2 and CheA' were incubated with ^{32}P -labelled acetyl phosphate at the same protein concentration as used in the retrophosphorylation experiments. As expected, no phosphorylation of the histidine kinases was observed (Fig. 6a, lane 5 and Fig. 6b, lane 1). Although *in vitro* phosphorylation of the C-terminal receiver module of CheAY2 by acetyl phosphate could in principle occur, due to the low efficiency of phosphorylation exhibited by the separated CheY2 module CheAY2 was apparently present at too low a concentration to create a detectable signal.

Since retrophosphorylation from CheY1~P should primarily target the histidine residue in the P1 domain of CheAY2, we investigated the effect of the truncated CheA' protein on the phosphorylation state of the CheY proteins when the three chemotaxis proteins were present simultaneously. Incubation of a mixture of CheY1 and CheY2 with ^{32}P -labelled acetyl phosphate resulted in the

Fig. 6. Phosphorylation of CheY1 and CheY2 by acetyl phosphate in the presence of the histidine kinases CheAY2 and CheA'. (a) Retrophosphorylation of CheAY2 by CheY1~P. CheY1 (7 μM , lanes 1 and 2) or CheY2 (50 μM , lanes 3 and 4) were incubated for 10 min with ^{32}P -labelled acetyl phosphate in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 8 μM CheAY2. As a control, 13 μM CheAY2 was incubated with ^{32}P -labelled acetyl phosphate (lane 5). (b) Phosphotransfer from CheY1~P to CheY2 via CheA'. CheY1 (7 μM , lane 2), CheY2 (50 μM , lane 3) and a mixture of either 7 μM CheY1 and 50 μM CheY2 (lane 4) or 7 μM CheY1, 50 μM CheY2 and 3.5 μM CheA' (lane 5) were incubated with ^{32}P -labelled acetyl phosphate. As a control, 3.5 μM CheA' was also incubated with ^{32}P -labelled acetyl phosphate (lane 1). (c) Phosphotransfer from CheY1~P of *C. jejuni* to CheY2 via CheA'. Purified recombinant CheY1cj (7 μM) was incubated with ^{32}P -labelled acetyl phosphate (lane 1) in the presence of 3.5 μM CheA' (lane 2), 50 μM CheY2 (lane 3) and 50 μM CheY2 and 3.5 μM CheA' (lane 4) for 10 min. The reactions were stopped by the addition of sample buffer containing 50 mM Na₂EDTA and the reaction mixtures were analysed by electrophoresis on an SDS-15 % polyacrylamide gel.

phosphorylation of both CheY proteins (Fig. 6b, lane 4). When CheA' was included in the reaction mixture, the amount of CheY1 ~ P was clearly reduced while the amount of CheY2 ~ P increased, suggesting retrophosphorylation of CheA' by CheY1 ~ P and the subsequent phosphorylation of CheY2 by CheA' ~ P (Fig. 6b, lane 5). The same result was obtained when CheY1 was replaced by the recombinant orthologous protein from *C. jejuni* (Fig. 6c). However, in both experiments phosphorylated CheA' could not be detected directly. From these experiments we conclude that phosphorylated CheY1 is able to efficiently transfer the phosphoryl group back to the CheAY2 histidine kinase, whereas efficient phosphotransfer from CheY2 ~ P to the P1 domain of CheAY2 does not occur. A summary of the phosphotransfer model based on the results of these experiments is shown in Fig. 1(b).

DISCUSSION

The chemotaxis system of *H. pylori* contains multiple signal transduction proteins which interact in a complex way. The chemotaxis histidine kinase CheAY2 is a bifunctional protein containing also a C-terminal CheY-like receiver domain. Such bifunctional CheAY proteins are also present in the other members of the ϵ -Proteobacteria whose genomes have been sequenced so far (Parkhill *et al.*, 2000; Baar *et al.*, 2003; Suerbaum *et al.*, 2003). To investigate the phosphotransfer reactions between the CheA histidine kinase and the various chemotaxis response regulator proteins of *H. pylori*, we constructed two derivatives of CheAY2, one lacking the C-terminal CheY2 domain (CheA'), the other comprising the separate CheY2 receiver domain (Fig. 1a). We have shown that the N-terminal CheA domain of the histidine kinase protein can be autophosphorylated, presumably on the histidine of the P1 domain, and that the presence of the CheY2 receiver domain influences the stability of the phosphorylated bifunctional protein (Fig. 2). The reduced stability of CheAY2 ~ P compared to CheA' ~ P is probably due to an intramolecular phosphoryl group transfer from the histidine residue in the P1 domain to the phosphate-accepting aspartic acid residue in the C-terminal CheY2 domain and the subsequent rapid hydrolysis of CheY2 ~ P. In accordance with this conclusion, rapid dephosphorylation of CheA' ~ P was also observed when the separated CheY2 domain was added (Fig. 3b). A similar effect of the receiver domain on the autophosphorylation kinetics of an unorthodox histidine kinase has been reported for the redox sensor ArcB of *E. coli* which is composed of a kinase domain, a receiver module and a C-terminal Hpt-domain (Georgellis *et al.*, 1997). Because in chase experiments the phosphorylated CheY2 protein exhibited a higher stability in the absence of CheA' than in its presence (data not shown and Fig. 4c), we hypothesize that the interaction with the CheA' histidine kinase stimulates the hydrolysis of CheY2 ~ P. This effect might be even more pronounced in an intramolecular interaction in the bifunctional CheAY2 protein.

Both CheY1 and CheY2 caused the efficient dephosphorylation of CheAY2 ~ P and CheA' ~ P (Fig. 3), while the phosphorylation state of the *H. pylori* histidine kinases HP244 and HP165 used as a control was unaffected by the addition of the chemotaxis response regulators (data not shown), demonstrating that CheAY2 constitutes the cognate histidine kinase for both CheY1 and CheY2. Unexpectedly, phosphorylated CheY1 did not accumulate in multiple turnover phosphotransfer reactions with CheAY2 or CheA', but this may possibly be explained by a relatively slow autophosphorylation of CheA *in vitro* when compared to the comparatively rapid hydrolysis of CheY1 ~ P. For the intrinsic dephosphorylation of CheY1 ~ P a half-life of 20 s was determined, which is in the same range as the half-life of CheY ~ P from *E. coli* (Lukat *et al.*, 1991) but approximately twice as long as the half-lives of CheY1 and CheY2 from *S. meliloti* (Sourjik & Schmitt, 1998). As was observed with the CheY proteins of *S. meliloti* (Sourjik & Schmitt, 1998), CheY1 and CheY2 of *H. pylori* did not exhibit phosphatase activity towards each other (Fig. 6b). The results of three component phosphorylation assays suggested clearly that CheY1 prevails in the competition for CheA' ~ P over CheY2 (Fig. 4) and we interpret this as a higher affinity of CheY1 for CheA'; however, it is possible that the intramolecular phosphotransfer reaction within the bifunctional CheAY2 protein may be favoured *in vivo*.

In *H. pylori* no homologues of proteins with a known CheY ~ P-specific phosphatase activity like CheZ, CheC and CheX are present. CheC was identified in *B. subtilis* and works in concert with another chemotaxis protein CheD (Szurmant *et al.*, 2004). So far only the function of CheX from *Thermotoga maritima* was analysed in some detail, although orthologues of the *cheX* gene are currently found in more than 20 sequenced bacterial genomes (Park *et al.*, 2004). CheC and CheX share a sequence signature which is present also in the flagellar switch protein FliY from both organisms and, consequently, FliY was also demonstrated to act as a phosphatase on CheY ~ P (Szurmant *et al.*, 2003). Therefore, the observation that *H. pylori* contains a CheY1 protein and the bifunctional CheAY2 histidine kinase suggested a similar mechanism of termination of chemotactic signalling as originally observed in the chemotaxis system of *S. meliloti*. This organism harbours two CheY molecules one of which interacts with the flagellar motor (CheY2) while the other acts as a phosphate sink (CheY1) which modulates the half-life of CheY2 ~ P. This occurs via retro-transfer of the phosphoryl group from CheY2 ~ P to CheA and the subsequent CheA-dependent phosphorylation of CheY1 (Sourjik & Schmitt, 1996; 1998). Our data presented here are consistent with efficient retro-transfer of the phosphoryl group from CheY1 ~ P to CheAY2 and suggests a role of CheY2 as a phosphate sink involved in signal termination (Fig. 6a). In fact, in a three-component reaction where CheY1 is present predominantly in its phosphorylated form, while the P1 domain of CheA' is initially unphosphorylated, the phosphotransfer from CheA' ~ P is clearly biased towards CheY2 despite the

Fig. 7. Alignment of the amino acid sequences of the CheY proteins of *H. pylori* (Hp), *C. jejuni* (Cj), *E. coli* (Ec) and *S. meliloti* (Sm). Gaps introduced to maximize the alignments are indicated by dots. The similarity between the homologous proteins is highlighted by different shading: black, all amino acids in a column are identical; light grey, more than half of the amino acids in a column are identical or belong to a strong similarity group; dark grey, more than half of the amino acids in a column belong to a weak similarity group or the amino acid marked in dark grey could be grouped into a weak similarity group with every amino acid of the same column belonging to a strong similarity group marked in light grey. Positions which are different in the CheY proteins interacting with the flagellar motor and acting as phosphate sink, respectively, are marked by arrows.

higher affinity of CheY1 for CheA (Fig. 6b and c). From the fact that in the retrophosphorylation experiments we could only detect CheAY2~P, but not CheA'~P, we conclude that the phosphorylation signal generated by CheAY2 derives mainly from the phosphorylated C-terminal CheY2 domain and not from the P1 domain. This observation further supports the idea of a rapid flow of the phosphoryl group from CheY1~P to CheY2 via the P1 domain of the CheA histidine kinase. Interestingly, an alignment of the CheY proteins of *E. coli*, *S. meliloti*, *H. pylori* and *C. jejuni* revealed two positions which might discriminate the CheY proteins interacting with the flagellar motor (CheY-Ec; CheY2-Sm; CheY1-Hp; CheY1-Cj) from those acting as a phosphate sink (CheY1-Sm; CheY2-Hp; CheY2-Cj): at the position corresponding to A74 in CheY of *E. coli* there is lysine in the 'phosphate sink' proteins and the position corresponding to I95 of *E. coli* CheY (V in CheY1-Hp and CheY2-Sm) is substituted by lysine or arginine in the 'phosphate sink' proteins (Fig. 7). However, these amino acids are probably not involved in the interaction with the FliM protein (McEvoy *et al.*, 1999; Shukla *et al.*, 1998).

H. pylori contains three orthologues of the chemotaxis protein CheV, consisting of an N-terminal CheW-like domain and a C-terminal receiver domain. CheV was first described in *B. subtilis* (Fredrick & Helmann, 1994) and it was shown that phosphorylated CheV is involved in the adaptation to attractants (Karatan *et al.*, 2001). To date only one of these proteins in *H. pylori*, CheV1, has been shown to be required for normal chemotactic motility in swarming assays (Pittman *et al.*, 2001). In accordance with the observation that binding of acetyl phosphate to CheV2 was detected by fluorescence spectroscopy (Pittman *et al.*, 2001), we could directly show the phosphorylation of CheV2 in the presence of 32 P-labelled acetyl phosphate. Here we show that the

CheV proteins are able to dephosphorylate CheA', indicating the transfer of the phosphoryl group to the C-terminal receiver domain of the CheV proteins. Therefore, it is likely that the function of the CheV proteins is connected to their ability to be phosphorylated. Due to the low affinity of the CheV proteins for the CheA histidine kinase a role of the CheV proteins as a phosphate sink in terminating the chemotactic signalling seems rather unlikely.

We cannot, however, rule out the possibility that in *H. pylori* chemotaxis, a so far unknown CheY1~P- or CheY2~P-specific phosphatase is involved in signal termination. Furthermore, the apparent slow half-life of CheY2~P which, according to our results should act as a 'phosphate sink', may suggest that there could be a phosphatase specific for this response regulator. *H. pylori* contains an orthologue of FliY; however, the sequence similarity to the regions conserved between FliY, CheC and CheX from *B. subtilis* and *T. maritima* which act in CheY~P hydrolysis is rather low.

Following the *S. meliloti* model, the CheY1 protein of *H. pylori*, which is freely diffusible in the cell, would interact with the flagellar motor. It was shown that the MCPs together with CheW and CheA form higher order clusters which are localized at the cell pole in *E. coli* and *Rhodospirillum rubrum* (Maddock & Shapiro, 1993; Sourjik & Berg, 2000; Martin *et al.*, 2003; Wadhams *et al.*, 2000). In swarmer cells of *Caulobacter crescentus* the chemoreceptor McpA has been demonstrated to be localized to the flagellated cell pole (Alley *et al.*, 1992). Assuming a similar localization of the chemoreceptor complex in the monotrichously flagellated bacterium *H. pylori*, direct interaction of CheAY2 with the flagellar motor is conceivable, but seems unlikely due to the complex stoichiometry of the MCP-CheW-CheA complexes which is a prerequisite for proper signal amplification (Levit *et al.*, 2002; Shimizu *et al.*, 2000).

Computerized tracking of the movement of the *H. pylori* strain N6 revealed a swimming pattern consisting of short straight runs with frequent changes of direction, whereas mutants of *H. pylori* N6 with inactivated histidine kinase CheAY2 moved in long straight runs (Foynes *et al.*, 2000), suggesting that phosphorylation of CheAY2 induces tumbling. Furthermore, in these assays a mutant with an insertional inactivation of *cheY1* exhibited excessive tumbling. This observation is not consistent with the putative interaction of CheY1 with the flagellar motor proposed by our data, but would rather suggest a role of CheY1 in the termination of chemotactic signalling. For the moment, this apparent contradiction remains unexplained.

In conclusion, through reconstitution of the *H. pylori* chemotaxis proteins implicated in the signalling pathway, we have evidence of a complex phosphorelay *in vitro* which has similarities to other systems with multiple CheY proteins. The data presented here indicate that the receiver domain present in the bifunctional CheAY2 protein acts as a phosphate sink, fine tuning the activity of the freely diffusible CheY1 protein which is thought to interact with the flagellar motor. The role of the CheV proteins remains unclear at the moment, but they may be engaged in a further fine regulation of the phosphate flow in this complex chemotaxis system.

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