Chemotaxis in the human gastric pathogen *Helicobacter pylori*: different roles for CheW and the three CheV paralogues, and evidence for CheV2 phosphorylation

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The complete genome sequence of *Helicobacter pylori* has revealed the presence of a novel set of chemotaxis genes including three *cheV* paralogues. CheV is a bi-functional protein, the N-terminal domain being homologous to the signalling-complex linker protein CheW, while the C-terminal domain is homologous to the response-regulator CheY, but its precise function in chemotaxis is unknown. In this study, each of the three *cheV* paralogues were insertionally inactivated in strain 26695 to determine their importance in the chemotactic signal-transduction pathway of *H. pylori*. Mutation of HP0019 (*cheV1*) had a severe inhibitory effect on chemotaxis, as determined by a swarm-plate assay. In contrast, strains carrying single mutations in either *cheV2* (HP0616) or *cheV3* (HP0393) displayed wild-type swarming behaviour, as did a *cheV2/cheV3* double mutant. However, expression of the *cheV2* or *cheV3* genes in *Escherichia coli* resulted in an inhibition of chemotaxis in a wild-type strain, indicating their role in chemotaxis, although these genes were unable to complement isogenic *E. coli* *cheW* or *cheY* mutants. The product of *cheV2*/HP0616 was overexpressed in *E. coli* and purified to homogeneity. Protein fluorescence quenching experiments showed that CheV2 was capable of binding acetyl phosphate, a small-molecule phosphodonator. The measured K_m for acetyl phosphate was 21 mM. It is concluded that in the absence of a *cheZ* gene, the CheV proteins could act as phosphate sinks to control the cellular level of phospho-CheY in *H. pylori*. However, only CheV1 was critical for chemotaxis, indicating a specific role distinct from the other paralogues in the signal-transduction pathway. Significantly, none of the CheV proteins could substitute for the loss of CheW, as an *H. pylori* *cheW* null mutant was non-chemotactic.

Keywords: *cheY*, *cheW*, *cheV*, motility, fluorescence, acetyl phosphate, phosphorylation

INTRODUCTION

The human gastric pathogen *Helicobacter pylori* is the aetiological agent of chronic active type B gastritis (Marshall *et al.*, 1985) and is strongly associated with gastric and duodenal ulceration (Moss & Calam, 1992; Wyatt *et al.*, 1987). Long-term infection with *H. pylori* is also a risk factor for gastric cancer (Forman *et al.*, 1991). The importance of motility as a virulence factor in this organism has been firmly established, and several studies have shown that aflagellate, non-motile mutants are unable to colonize animal models (Eaton *et*

al., 1992, 1996). More recently, it has been established that chemotaxis, the behavioural process which allows the cell to move towards and away from attractants and repellents respectively, is also an essential virulence factor (Foyes *et al.*, 2000).

The paradigm of the bacterial chemotactic response is the system found in enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium*, and has been extensively reviewed (Bourret *et al.*, 1991; Blair, 1995; Stock & Surette, 1996). It has become clear, however, that many bacteria have more complex chemotaxis

systems than the enterics, with additional or duplicated genes (Armitage & Schmitt, 1997). The complete genome sequencing of two strains of *H. pylori* (Tomb *et al.*, 1997; Alm *et al.*, 1999) revealed the presence of six *che* gene homologues. These are: the response regulator gene *cheY* (HP1067/JHP358), a histidine kinase gene *cheA* (HP0392/JHP989), *cheW*, encoding the ternary-complex-forming protein CheW (HP0391/JHP990) and three unlinked copies of *cheV*. The latter form a paralogous family and have been designated *cheV1* (HP0019/JHP17), *cheV2* (HP0616/JHP559) and *cheV3* (HP0393/JHP988) by Doig *et al.* (1999). Each *cheV* gene encodes a protein consisting of an N-terminal domain homologous to CheW and a C-terminal domain homologous to CheY. At least three genes are present that encode transducer-like receptor proteins, which possess several conserved glutamate residues that could be methylated as part of a stimulus-adaptation system. However, there is no evidence of potential methyltransferase (*cheR*) or methylesterase (*cheB*) genes, and so the details of adaptation are unclear. The *cheA*, *cheW* and *cheV3* genes are arranged in an operon while *cheY* and the two further copies of *cheV* are located at unlinked sites on the chromosome. Beier *et al.* (1997) characterized the response regulator gene *cheY* and showed that it was part of a larger operon containing chemotaxis-unrelated stress-induced genes. The deduced histidine kinase CheA is actually a bi-functional protein in *H. pylori*, as it is fused to an N-terminal CheY domain (Jackson *et al.*, 1995; Kelly, 1998) and has been redesignated CheAY, encoded by the *cheAY* gene (Foyne *et al.*, 2000). Thus, *H. pylori* contains five CheY-like domains on separate proteins, but there is no evidence for a *cheZ* gene (encoding a protein which enhances the intrinsic CheY phosphatase activity) in this bacterium. Mutants in either *cheY* or *cheAY* have been shown to be non-chemotactic (Beier *et al.*, 1997; Foyne *et al.*, 2000).

One of the unusual features of the *H. pylori* chemotaxis machinery is the presence of the three paralogues of CheV. CheV was first identified as a novel chemotaxis protein in *Bacillus subtilis* (Frederick & Helmann, 1994) and partial redundancy with CheW was reported (Rosario *et al.*, 1994). However, the actual function of CheV in chemotaxis has not been elucidated. To begin to address this problem in *H. pylori*, we have determined, by insertional mutagenesis, the essentiality for chemotaxis of each of the three *cheV* paralogues in this bacterium. These genes have also been expressed in *E. coli* wild-type and isogenic $\Delta cheY$ and $\Delta cheW$ mutants to investigate the interaction of the *H. pylori* proteins with the enteric chemotaxis system. In *E. coli* CheY, aspartate residue D57 is the site of phosphorylation via CheA or small-molecule phosphodonors such as acetyl phosphate (Lukat *et al.*, 1992; McCleary & Stock, 1994; Da Re *et al.*, 1999). In *H. pylori*, the CheY domains of each of the CheV paralogues contain this conserved aspartate, raising the possibility that in the absence of a CheZ homologue, these domains could act as 'phosphate sinks' to control the level of phospho-

CheY. In support of this, we have now shown by fluorescence studies that at least one of the *H. pylori* CheV proteins (CheV2) is capable of binding acetyl phosphate.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *H. pylori* was routinely grown in brain heart infusion (BHI) medium (Oxoid) with 1.5% (w/v) Bacto agar (Difco), supplemented with 7% (v/v) lysed horse blood. A selective antibiotic supplement of vancomycin, polymyxin B and amphotericin B, each at a final concentration of 10 µg ml⁻¹, was added to all *H. pylori* growth media. *H. pylori* was cultured at 37 °C for 48 h in a Variable Atmosphere Incubator (VAIN; Don Whitley Ltd) in an atmosphere of 5% (v/v) CO₂, 87% (v/v) N₂ and 8% (v/v) O₂. The medium for swarm analysis of *H. pylori* was BHI base with 0.32% (w/v) Bacto agar (Difco), supplemented with 7% (v/v) sterile calf serum. Excess moisture was carefully dried from the agar surface before use. The plates were incubated at 37 °C for up to 5 d in the VAIN cabinet.

E. coli strains for plasmid subcloning and overexpression were grown on Luria-Bertani (LB) agar or in LB broth. Supplements of 100 µg ampicillin ml⁻¹, 30 µg chloramphenicol ml⁻¹ and 30 µg kanamycin ml⁻¹ were used as appropriate for the selection and maintenance of plasmids. *E. coli* swarm assays were carried out over a 6–8 h period at 30 °C on LB plates with 0.3% (w/v) Bacto agar (Difco), using inocula from stationary-phase LB broth cultures. Graded expression of pTM30 constructs was achieved by incorporation of IPTG in log increments over the range 10⁰–10³ µM in the swarm agar plates.

Electron microscopy. Cell suspensions were deposited onto Formvar-coated grids, blotted off and negatively stained by the addition of 1% (w/v) phosphotungstic acid to the grids for 3 min. After blotting and air-drying, the grids were viewed and photographed using a Philips CM10 transmission electron microscope.

Preparation and sequencing of DNA. Plasmid DNA for screening clones, sequencing and gene disruption experiments was routinely isolated using anion-exchange resin spin-columns (QIAGEN), according to the manufacturer's instructions. Double-stranded sequencing of plasmid DNA was carried out using Applied Biosystems Taq DyeDeoxy terminator cycle sequencing reactions analysed on an ABI model 373A automated DNA sequencer. Synthesis of custom primers was carried out on an Applied Biosystems model 392 DNA synthesizer. *H. pylori* total chromosomal DNA was extracted using a modified SDS lysis procedure (Marmur, 1961). Restriction endonucleases, T4 DNA ligase and Taq polymerases were purchased from Promega, and Pfu polymerase from Stratagene, and were used according to the manufacturer's instructions.

Construction and characterization of *H. pylori* isogenic insertion mutants. A set of PCR primers was designed to amplify the entire coding regions of the *cheW*, *cheV1*, *cheV2* and *cheV3* genes, both for insertional activation after cloning of the products into pGEM-3Zf(–) (Promega) and for expression after subcloning into pTM30 (Morrison & Parkinson, 1994). The primers used were: CheW-F (5'-TCGAGGATCCAAGCAACCAATTAAAGAT-3'),

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source or reference
<i>E. coli</i>		
DH5 α	ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 <i>supE4 hsdR17 recA1 endA1 gyrA96 thi-1 relA deoR</i>	Stratagene
BL21 (λ DE3)	<i>hsdS gal (λclts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</i>	Studier & Moffat (1986)
RP437	<i>thr(Am)-1 leuB6 his-4 metF(Am)159 eda-50 rpsL136 [thi-1 ara-14 mtl-1 xyl-5 tonA31 tsx-78]</i>	P. Hamblin, University of Oxford
RP1616	<i>(cheZ)DEm67-25 thr(Am)-1 leuB6 his-4 metF(Am)159 eda-50 rpsL136 [thi-1 ara-14 mtl-1 xyl-5 tonA31 tsx-78]</i>	P. Hamblin, University of Oxford
RP4606	<i>cheW(Am)113 thr(Am)-1 leuB6 his-4 rpsL136 [thi-1 (gal-att-L)DE99 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78]</i>	P. Hamblin, University of Oxford
RP5232	<i>(cheY)DEm60-21 thr(Am)-1 leuB6 his-4 metF(Am)159 rpsL136 [thi-1 ara-14 lacY1 mtl-1 xyl-5 tonA31 tsx-78]</i>	P. Hamblin, University of Oxford
<i>H. pylori</i>		
26695	Wild-type	Akopyants <i>et al.</i> (1995)
MSP100	26695 <i>cheV1::cat</i>	This study
MSP200	26695 <i>cheV2::cat</i>	This study
MSP300	26695 <i>cheV3::cat</i>	This study
MSP400	26695 <i>cheV2::aph/cheV3::cat</i>	This study
MSP500	26695 <i>cheW::cat</i>	This study
Plasmids		
pTM30	Expression vector	Morrison & Parkinson (1994)
pGEM-3Zf(-)	Cloning vector	Promega Ltd
pUCAT	pUC18 containing <i>cat</i> cassette from <i>C. coli</i>	Wang & Taylor (1990)
pUKAN	pUC18 containing <i>aphAIII</i> from <i>C. coli</i>	Wang & Taylor (1990)
pMSP1	pGEM-3Zf(-) containing <i>H. pylori cheW</i> inserted into unique <i>HincII</i> site	This study
pMSP4	pGEM-3Zf(-) containing <i>H. pylori cheV1</i> inserted into unique <i>HincII</i> site	This study
pMSP5	pGEM-3Zf(-) containing <i>H. pylori cheV3</i> inserted into unique <i>HincII</i> site	This study
pMSP6	pGEM-3Zf(-) containing <i>H. pylori cheV2</i> inserted into unique <i>HincII</i> site	This study
pMSP8	pTM30 with <i>cheV1</i> inserted at the <i>Bam</i> HI and <i>Kpn</i> I sites	This study
pMSP9	pTM30 containing <i>cheW</i> inserted at the <i>Bam</i> HI and <i>Kpn</i> I sites	This study
pMSP10	pTM30 containing <i>cheV3</i> inserted at the <i>Bam</i> HI and <i>Kpn</i> I sites	This study
pMSP11	pTM30 containing <i>cheV2</i> inserted at the <i>Bam</i> HI and <i>Kpn</i> I sites	This study
pMSP12	pMSP1 with <i>cheW</i> inactivated by insertion of <i>cat</i> cassette at unique <i>Bsr</i> BR1 site	This study
pMSP15	pMSP4 with <i>cheV1</i> inactivated by insertion of <i>cat</i> cassette at unique <i>Nco</i> I site	This study
pMSP16	pMSP5 with <i>cheV3</i> inactivated by insertion of <i>cat</i> cassette at unique <i>Bsa</i> BI site	This study
pMSP17	pMSP6 with <i>cheV2</i> inactivated by insertion of <i>cat</i> cassette at unique <i>Bcl</i> I site	This study
pMSP18	pMSP6 with <i>cheV2</i> inactivated by insertion of <i>aphAIII</i> cassette at unique <i>Bcl</i> I site	This study

CheW-R (5'-TCGAGG**TACCGAAGTCTTTTTT**AAGATTTC-3'), CheV1-F (5'-TCGAGG**ATCCAGCTGATAGTT**-TAGCGGGC-3'), CheV1-R (5'-TCGAGG**TACCTGCTAA**-TTCCAAAAATTG-3'), CheV2-F (5'-TCGAGG**ATCCA**-GTAAGAGAGATATTGACAAA-3'), CheV2-R (5'-TCGAGG**TACCTTATGAAAGCGTTTTTTT**-3'), CheV3-F (5'-TCGAGG**ATCCAGCAGAAAAACAGCTA**-3') and CheV3-R (5'-TCGAGG**TACCCGCATTCTTGCTAAAA**-T-3'). These primers introduced *Bam*HI or *Kpn*I restriction sites (shown in bold italics) for the in-frame subcloning of these genes into the pTM30 polylinker (see below). The second codon (forward primers) or final codon (reverse primers) of the gene is underlined. PCR reactions were performed with *Pfu* polymerase using genomic DNA from strain 26695 as template.

pGEM-3Zf(-) was linearized with *HincII* and blunt-end ligated with the products resulting from PCR with the above primers, generating plasmids pMSP1, pMSP4, pMSP5 and pMSP6 (Table 1). These plasmids were linearized at unique restriction sites within the *H. pylori* gene inserts, as detailed in Table 1. Protruding 5' termini were in-filled using the Klenow fragment of the *E. coli* DNA polymerase I where necessary. For insertional inactivation, a chloramphenicol acetyltransferase (*cat*) cassette derived from *Campylobacter coli* (Wang & Taylor, 1990) was used. The 900 bp *cat* cassette excised from pUCAT by *HincII* digestion was used to inactivate *cheW* and *cheV2*. However, it possesses *rho*-independent transcription terminators at the 3' terminus. A non-polar version of this cassette was used to inactivate *cheV1* and *cheV3*. PCR primers Cat-F (5'-TCCGTCGTCGGTAT-

CGTATGG-3') and Cat-R (5'-TTATCAGTGCACAAAC-TGGG-3') were designed to amplify an 829 bp product from pUCAT containing the promoter region of the *cat* gene but lacking the 3' transcriptional terminators. Both cassettes were purified using a Gel Extraction Kit (QIAGEN) and blunt-end ligated into the appropriate plasmids. Recombinant clones were selected by growth on LB agar containing 30 µg chloramphenicol ml⁻¹ and the orientation of the cassettes determined by restriction analysis. The following plasmids were generated: pMSP12, pMSP15, pMSP16 and pMSP17 (see Table 1). To construct a plasmid suitable for generating a *H. pylori* *cheV2/cheV3* double mutant, the *C. coli*-derived *aphAIII* cassette (Wang & Taylor 1990) was amplified from pUKAN with *Pfu* polymerase, using primers Kan-F (5'-ACTGAGATCTACTCTATGAAGCGCCATATT-3') and Kan-R (5'-CAATAGATCTTTTACATCATCTAAATC-TAGG-3'). Plasmid pMSP17 was linearized at the unique *BclI* site within the *cheV2* gene and blunt-end ligated with the *aphAIII* cassette. *E. coli* transformants were selected by growth on LB agar containing 30 µg kanamycin sulphate ml⁻¹. The resulting plasmid was designated pMSP18.

Transformation of *H. pylori* 26695 with pMSP12, pMSP15, pMSP16 and pMSP17, and of strain MSP300 with pMSP18, was carried out as previously described (Ferrero *et al.*, 1992). Transformants were selected by addition of 30 µg chloramphenicol ml⁻¹ or 30 µg kanamycin sulphate ml⁻¹ to the plating medium as appropriate. Genomic DNA was extracted from putative recombinants, and correct insertion of the resistance cassettes evaluated by PCR using the above primers.

Cloning and expression of *H. pylori* *cheW* and *cheV* genes in pTM30 and transformation of *E. coli* chemotaxis mutants. Complementation and expression experiments with the *H. pylori* chemotaxis genes were done by subcloning the *cheW*, *cheV1*, *cheV2* and *cheV3* genes in-frame into the *Bam*HI and *Kpn*I sites of the expression vector pTM30, and transforming the resulting constructs (Table 1) into the *E. coli* wild-type strain RP437, and chemotaxis mutants RP5232 (Δ *cheY*) and RP4606 (Δ *cheW*). The fidelity of the constructs was checked by DNA sequencing. Expression of proteins was induced with IPTG and monitored by SDS-PAGE on 10% (w/v) polyacrylamide gels. Swarming and motility phenotypes were determined for the complemented wild-type and mutant strains on LB plus 0.3% (w/v) Difco agar, containing IPTG in the range 0–1 mM.

Purification of the CheV2 protein. pMSP11 was grown in 6 × 1 l batches of LB plus 100 µg ampicillin ml⁻¹ until an OD₆₀₀ of 0.5 was reached. IPTG was added to 1 mM final concentration and growth continued for 5 h, after which the cells were harvested by centrifugation (8000 g, 4 °C, 20 min), resuspended in 20 ml 10 mM HEPES buffer pH 7.5 (buffer A) and disrupted in a French press at 12000 p.s.i. (82.8 MPa). The broken cells were centrifuged (150000 g, 4 °C, 2 h) and the supernatant (cell-free extract) removed. A series of ammonium sulphate precipitations were carried out on the supernatant and the precipitates forming at 30% and 40% saturation were combined and dissolved in 3 ml buffer A. The solution was applied to a DEAE-Sepharose column connected to a BioLogic HP system equilibrated in buffer A. Proteins were eluted by applying the following salt gradient: (i) 0–0.2 M NaCl for 15 min, (ii) 0.2 M NaCl for 15 min, (iii) 0.2–0.6 M NaCl for 180 min, (iv) 0.6–1.0 M NaCl for 15 min. CheV2-enriched fractions eluted at 0.25–0.35 M NaCl. These were pooled, concentrated to about 4 ml and applied to a Superdex 75 gel-filtration column equilibrated in 10 mM HEPES pH 7.5 containing 0.2 M NaCl (buffer B) connected to a Pharmacia FPLC system. Proteins were eluted in 2 ml fractions at 2 ml

min⁻¹ in buffer B for 60 min. Purification was monitored by SDS-PAGE and fractions containing pure CheV2 were pooled and concentrated to 1–2 ml. N-terminal sequencing by the automated Edman degradation method (carried out by Dr A. J. G. Moir in the authors' Department) was used to confirm the identity of the purified protein.

Fluorescence spectroscopy. This was performed using a Hitachi F-2500 spectrofluorimeter with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. The assay mixture consisted of 0.1–0.2 µM CheV2 in 1.5 ml 10 mM HEPES pH 7.5/1 mM EDTA/1 mM magnesium chloride (buffer C). The sample cuvette was maintained at 25 °C in the spectrofluorimeter housing. Fluorescence changes upon the addition of acetyl phosphate (0.5–50 mM final concentration) were monitored until the fluorescence change stabilized. Fluorescence values were corrected for dilution.

Protein sequence and phylogenetic analyses. Multiple sequence analyses were performed using the UWGCG program PILEUP (Devereux *et al.*, 1984). For phylogenetic analyses, sequences were aligned in CLUSTAL X (Thompson *et al.*, 1997) and the output file used in PHYLIP (Felsenstein, 1993) to produce a distance matrix tree with the confidence level of the tree topology determined by bootstrapping (100 datasets). The final trees were viewed in TREEVIEW (Page, 1996).

RESULTS

Mutagenesis of *cheV* and *cheW* genes in *H. pylori*

In order to determine the importance of the predicted *cheV* genes in *H. pylori* chemotaxis, a set of isogenic mutants in strain 26695 was constructed. Fig. 1 shows the chromosomal arrangement of the genes analysed in this study, and the strategy used for insertional mutagenesis. PCR-amplified genes cloned into pGEM-3Zf(–) were insertionally inactivated at unique restriction sites using antibiotic-resistance cassettes, and transferred into *H. pylori* 26695 by natural transformation. The strongly polar *cat* cassette (Wang & Taylor, 1990) could only be used to inactivate HP0391 (*cheW*) and HP0616 (*cheV2*) as in these cases any downstream genes are either known not to be involved in chemotaxis or are transcribed with opposite polarity. Downstream of HP0019 (*cheV1*) is a gene (*nspC*) encoding a putative carboxynorspermidine decarboxylase, which has recently been shown to be essential in *H. pylori* (Chalker *et al.*, 2001). Attempts to obtain chromosomal mutations in HP0019 by transformation with plasmids containing the standard (polar) *cat* cassette, in either orientation, were not successful. However, transformation with pMSP15, containing a non-polar *cat* cassette (constructed as described in Methods) in the correct orientation resulted in successful transformation of *H. pylori*, presumably as a result of readthrough into the *nspC* gene. The resulting *cheV1* mutant showed the same growth rate as the parent strain. This non-polar cassette was also used to inactivate HP0393 (*cheV3*), which appears to be part of the same operon as the *cheAY* gene, HP0392 and *cheW* (HP0391; Fig. 1). In addition to these single mutants, a *cheV2/cheV3* double mutant (see below) was also constructed by transformation of strain MSP300 (26695 *cheV3::cat*) with pMSP18, which contains the *C. coli* *aphAIII* kanamycin-resistance gene

Fig. 1. Organization of *cheV* and *cheW* genes in *H. pylori* 26695, mutagenesis strategy and verification of mutant strains. Mutants were constructed by cloning *cat* or *aphAIII* cassettes (inverted triangles) into unique restriction sites in the insert DNA of the relevant plasmids shown (see also Table 1). The distance (bp) from the translation start of the gene to the cassette insertion site is also shown. For *cheW* (a) and *cheV2* (c), the standard polar *cat* cassette (Wang & Taylor, 1990) was used for inactivation, while for *cheV1* (b) and *cheV3* (a), a cassette lacking the 3' transcriptional termination signals was inserted with the same polarity as the target gene to allow readthrough into the downstream genes (indicated by the arrow above the cassette insertion site). (d) 0.8% agarose gel of PCR products obtained with the parental strain 26695 genomic DNA as template (lanes 1, 3, 5 and 7) or with DNA from *cheV1* (lane 2), *cheW* (lane 4), *cheV3* (lane 6) or *cheV2* (lane 8) mutants, using primers *cheV1*-F and *cheV1*-R (lanes 1 and 2), *cheW*-F and *cheW*-R (lanes 3 and 4), *cheV3*-F and *cheV3*-R (lanes 5 and 6) or *cheV2*-F and *cheV2*-R (lanes 7 and 8). Lane M contains size markers (sizes indicated). (e) 0.6% agarose gel of PCR products obtained with the parental 26695 genomic DNA as template (lanes 1 and 3) or with DNA from the *cheV2::aphAIII::cat* strain MSP400 (lanes 2 and 4) using primers *cheV2*-F and *cheV2*-R (lanes 1 and 2) or *cheV3*-F and *cheV3*-R (lanes 3 and 4). In (d) and (e) the increase in size of the single PCR products obtained when mutant DNA is used as the template indicates the correct insertion of the *cat* or *aphAIII* cassettes.

(Wang & Taylor, 1990) inserted at the unique *BclI* restriction site in HP0616. For each mutant, verification that correct chromosomal insertion of the cassettes had occurred by a double homologous recombination event was obtained by PCR analysis of parental and mutant

genomic DNA (Fig. 1). In each case, a single PCR product of the predicted molecular size was obtained.

Electron microscopy of negatively stained cells showed that each of the mutants constructed had a normal

Fig. 2. Chemotaxis of *H. pylori* wild-type 26695 (left of each panel) and mutant strains (right of each panel) indicated by swarming ability on soft agar. Overnight BHI broth cultures (10 µl) of *H. pylori* strains were spotted onto the surface of dried BHI plates containing 0.32% (w/v) agar. The plates were incubated microaerobically for 5 d at 37 °C and then photographed. The establishment of a nutrient gradient during growth results in chemotactic swarming of the cells outward from the inoculum site, forming characteristic concentric rings. Cells which are deficient in chemotaxis do not swarm and remain close to the site of inoculation.

pattern and number of flagella, indicating that there was no perturbation of flagellar biosynthesis or assembly. Moreover, the motility of strain 26695 and the mutants examined under phase-contrast microscopy was similar in terms of the percentage of motile cells, and the overall level of swimming activity (data not shown).

The chemotactic ability of the *H. pylori* mutants was determined by swarm-plate assays on semi-solid BHI medium (Fig. 2). The formation of a visible swarm surrounding the site of inoculation results from the production of a nutrient gradient during growth, to

which the cells respond chemotactically by moving outwards, forming characteristic concentric rings. Non-chemotactic cells will remain near the site of inoculation. The parental strain 26695 consistently formed large swarms after 3–4 d growth (Fig. 2). However swarming was abolished in the HP0391 (*cheW*) mutant (Fig. 2); the cells only grew at the site of inoculation and did not move outwards to any significant extent. This gene is therefore essential for *H. pylori* chemotaxis. Swarming was also severely reduced in the HP0019 (*cheV1*) mutant in comparison to the parental strain (Fig. 2), but was not completely abolished. In contrast, mutations in HP0616 (*cheV2*) or HP0393 (*cheV3*) did not result in any decrease in swarming ability compared to the isogenic wild-type parent (Fig. 2). A *cheV2/cheV3* double mutant was constructed to test whether these genes can replace each other's function. However, this strain also clearly exhibited normal swarming behaviour (Fig. 2). The restriction sites used for insertion of the *cat* cassette into *cheV2* and *cheV3* are located towards the 3' end of the genes, which might result in the synthesis of partially functional truncated and/or fusion proteins containing the CheW domains. In order to rule out this possibility, the expression in *E. coli* of CheV from the IPTG-inducible *ptac* promoter in pMSP10 and pMSP11 was compared to that of the same plasmids containing the *cat*-inactivated genes. Whereas IPTG-inducible proteins of the predicted size for CheV (33 kDa) were expressed in the case of the wild-type genes, these were clearly absent in the mutant constructs and no truncated or fusion proteins were observed (data not shown). Overall, the mutant phenotypes suggest that *cheV1* plays an important role in chemotaxis and that its function cannot be substituted for by either *cheV2* or *cheV3*.

Effect of expression of *H. pylori* chemotaxis genes in *E. coli* wild-type and chemotaxis mutants

To further characterize the function of the *cheV* and *cheW* genes in *H. pylori*, attempts were made to complement *E. coli* chemotaxis mutants by expression of the heterologous genes and determine the effect of expression on chemotaxis in the wild-type. The following *E. coli* strains were used: the parental strain RP437, which showed normal chemotaxis and swarming motility; RP5232 ($\Delta cheY$) and RP4606 ($\Delta cheW$), which both had a smooth-swimming phenotype. These mutants were confirmed to be non-chemotactic by swarm plate assays. The *H. pylori cheW*, *cheV1*, *cheV2* and *cheV3* genes were amplified by PCR and cloned into the pTM30 expression vector (Morrison & Parkinson, 1994), which allows controlled expression from a *ptac* promoter using IPTG concentrations in the range 0–1000 µM. The constructs (see Table 1) were transformed into RP437. IPTG-dependent expression of the CheW, CheV2 and CheV3 proteins was confirmed by SDS-PAGE analysis, but CheV1 expression from pMSP8 was weak and IPTG independent (data not shown), and so this plasmid was not used further. RP5232 ($\Delta cheY$) and RP4606 ($\Delta cheW$) transformed with pMSP10 or pMSP11, bearing the *H. pylori cheV3* and *cheV2* genes

Fig. 3. Effect of heterologous expression of *H. pylori* chemotaxis genes on *E. coli* chemotaxis. RP437 was transformed with pTM30 (black bars) or with pTM30-containing *H. pylori* *cheV3* (pMSP10; diagonal striped bars), *cheV2* (pMSP11; dashed bars) or *cheW* (pMSP9; clear bars). Overnight LB cultures of the transformants (10 μl) were spotted onto the surface of LB plates containing 0.3% (w/v) agar and the concentrations of IPTG indicated, and incubated at 30 °C for 8 h, after which time the diameters of the swarms were measured. The experiment was repeated several times with similar results; the results of a representative experiment are shown.

respectively, did not show restoration of swarming at any level of IPTG induction from 0 to 1 mM. Similarly, expression of CheW from pMSP9 in RP4606 ($\Delta cheW$) also did not complement the swarming phenotype of this strain at any IPTG concentration up to 1 mM. However, expression of *cheW*, *cheV2* and *cheV3* in the parental strain RP437 had clear IPTG concentration dependent inhibitory effects on chemotaxis (Fig. 3). The most dramatic effects were observed with *cheW* and *cheV2*, where swarming was severely reduced at high levels of induction (Fig. 3), but expression of *cheV3* also resulted in significant inhibition of swarming in *E. coli* (Fig. 3).

Phylogenetic and sequence analysis of CheV paralogues: conservation of active-site residues in the CheY-like N-terminal domain

CheV proteins are present in only some Gram-positive and Gram-negative bacteria but not in Archaea. Fig. 4(a) shows the phylogenetic relationships between the three CheV paralogues in *H. pylori* 26695 and all of the CheV protein sequences present in the databases to date. *Bacillus halodurans*, *Vibrio cholerae* and *H. pylori* all contain more than one *cheV* gene and in these bacteria the encoded paralogues are generally quite divergent from each other. This is particularly apparent in the case of *V. cholerae*. In *H. pylori*, the three paralogues share less than 30% sequence identity and appear to have diverged differently. HP0393 is most closely related to the single CheV protein in *C. jejuni*, which is also part of a *cheV-cheAY-cheW* operon. HP0019 and HP0616 are more similar to each other than either is to HP0393 although they have also diverged significantly from each other during evolution.

Of particular interest for the function of CheV is the presence of a C-terminal CheY-like domain in this protein. Fig. 4(b) shows a sequence alignment of the C-terminal domains of the *H. pylori* paralogues with the *E. coli* and *H. pylori* CheY proteins. The highly conserved residues that form the active site of the *E. coli* CheY are present or are replaced with conservative substitutions in all three *H. pylori* CheV proteins. These include D57, the site of phosphorylation (Sanders *et al.*, 1989a), D12 and D13 (D12 is replaced by a glutamate in CheV2), which are involved in the co-ordination of a Mg^{2+} ion which is essential for phosphorylation and dephosphorylation to occur (Volz, 1993), and K109, involved in the phosphorylation-induced conformational change (Volz, 1993). The position corresponding to T87 in *H. pylori* and *E. coli* CheY molecules is occupied by a hydroxy amino acid (threonine or serine) in virtually all known response regulators (Volz, 1993). In the CheY domains of the three CheV's, the corresponding T87 residue is replaced by serine. It is clear, therefore, that the CheY domains of each of the CheV proteins in *H. pylori* could potentially accept phosphoryl groups.

Evidence for phosphorylation of CheV2 by acetyl phosphate

In order to gain evidence for phosphorylation of the CheY domains of the CheV proteins, *E. coli* cultures harbouring pMSP8, pMSP10 or pMSP11 were induced with 1 mM IPTG, harvested, and attempts made to purify to homogeneity the overexpressed CheV proteins. Insufficient amounts of CheV1 and CheV3 were expressed from pMSP8 and pMSP10, respectively, for successful purification, but CheV2 was optimally expressed to about 20% of the soluble protein (Fig. 5a) and was successfully purified by a combination of ion-exchange and gel-filtration chromatography after an initial ammonium sulphate fractionation of crude cell-free extracts (Fig. 5b). The N-terminal sequence of the purified protein was determined to be MLQDPVRDI-DKTT. Residues 6–13 are identical to residues 2–9 of the deduced CheV2 protein (residues 1–5 arise from the in-frame cloning into the pTM30 vector; see Methods). In view of the presence of the appropriate conserved residues in the CheY-like domains of each of the proteins, CheV2 was considered to be a suitable representative paralogue for biochemical studies.

The phosphorylation of response-regulators such as CheY and PhoB by small-molecule phosphodonors has been successfully studied by fluorescence spectroscopy, exploiting the tryptophan residue adjacent to the phospho-accepting active-site aspartate residue in these proteins (Lukat *et al.*, 1992; McCleary, 1996; Fig. 4b). The corresponding residue in CheV2 is a leucine (Fig. 4b); there are two Trp residues in the protein, but both are in the N-terminal CheW domain. However, F255 may be a suitable reporter of phosphorylation; the corresponding region in the *E. coli* CheY undergoes a large change of environment upon phosphorylation (Stock & Surette, 1996). The fluorescence emission

Fig. 4. Phylogenetic relationships of CheV proteins and sequence conservation in the C-terminal CheY-like domains of the *H. pylori* proteins. In (a) a CLUSTAL X multiple sequence alignment of all of the CheV homologues was used as the input to PHYLIP to generate a bootstrapped distance matrix tree (nodal bootstrap values indicated). The bar represents 1 inferred substitution per 10 residues. PSAER, *Pseudomonas aeruginosa*; VPARA, *Vibrio parahaemolyticus*; VCHOL, *Vibrio cholerae*; BACSU, *Bacillus subtilis*; BHALO, *Bacillus halodurans*; CJEJUNI, *Campylobacter jejuni*; HPYLORI, *Helicobacter pylori*. (b) A PILEUP alignment of the CheY domains of the three *H. pylori* CheV paralogues (HP0019, HP0393 and HP0616), the CheY domain of the CheAY protein (HpCheAY), the *H. pylori* CheY protein itself (HpCheY) and the *E. coli* CheY (EcCheY) is shown. Identical residues are boxed in black and similar residues are boxed in grey. Key *E. coli* CheY active-site residues D12, D13 (asterisks), D57 (the site of phosphorylation; filled circle) and K109 (filled diamond) are conserved in all of the *H. pylori* CheY domains. X indicates the position of the tryptophan residue in the *E. coli* CheY which reports conformational changes upon phosphorylation by fluorescence quenching (Lukat *et al.*, 1992). Although this residue is conserved in *H. pylori* CheY, it is replaced by aliphatic hydrophobic amino acids in all of the CheV proteins. A nearby phenylalanine residue in HP0616 (Z) may be responsible for the fluorescence changes observed in the purified protein (see Fig. 6).

Fig. 5. Overexpression and purification of the *H. pylori* CheV2 protein (HP0616). In (a), two *E. coli* DH5 α (pMSP11) cultures were grown aerobically in LB to an OD₆₀₀ of 0.5. IPTG (1 mM) was then added to one culture and incubation continued for 5 h. Cells were then harvested and subjected to SDS-PAGE as described in Methods. The Coomassie-blue-stained gel shows the IPTG-dependent expression of the 33 kDa CheV2 protein (arrow). Molecular mass markers (sizes indicated) were run in lane M. (b) Purification of CheV2. Lane M contains molecular mass markers. Lane 1, cell-free extract from DH5 α (pMSP11) after 5 h induction with IPTG. Lane 2, 30–40% saturation ammonium sulphate precipitate. Lane 3, fractions from DEAE-Sepharose chromatography eluting at 0.25–0.35 M NaCl. Lane 4, fractions from Superdex-75 gel filtration chromatography. The arrow indicates the 33 kDa CheV2 protein.

spectrum of CheV2 is shown in Fig. 6(a). The fluorescence properties of CheV2 were characterized by excitation and emission maxima of 280 nm and 340 nm, respectively. The excitation maximum reflects the predominance of Tyr and Phe over Trp residues in the protein. Addition of acetyl phosphate to the protein in the presence of Mg²⁺ ions caused a significant quench in fluorescence, with no change in the emission maximum (Fig. 6a). The dependence of the fluorescence change on acetyl phosphate concentration was hyperbolic (Fig. 6b) and the maximum fluorescence quench at 50 mM acetyl phosphate was about 5%. CheV was considered to be an acetyl-phosphatase with phospho-CheV as an intermediate, in an analogous manner to *E. coli* CheY (Lukat *et al.*, 1992):

Thus the K_m of CheV2 for acetyl phosphate is equal to $K_s k_3 / k_2$ in this scheme and can be determined from the fluorescence change assuming that the observed quenching is a direct effect of the reduced quantum yield of phospho-CheV2 relative to CheV2 (Lukat *et al.*, 1992). The reciprocal of the slope of the line in Fig. 6(c) gave a K_m value of 21 mM.

DISCUSSION

H. pylori contains a complex chemotaxis system, with a unique combination of proteins including the fused CheAY histidine-kinase/response-regulator and three CheV paralogues (Kelly, 1998; Foynes *et al.*, 2000). In this study we have focussed on determining the essentiality and possible function of the latter proteins.

The presence of the N-terminal CheW domain in all CheV proteins may suggest a function in the early stages of signal transduction, from the receptors to CheA. Indeed, Rosario *et al.* (1994) showed that partial functional redundancy exists between the *B. subtilis* CheV and CheW proteins, as expression of the N-terminal domain of CheV was sufficient to rescue the chemotactic defect of a *cheW* mutant of this organism. In *H. pylori*, we have shown that *cheW* is an essential chemotaxis gene, most likely because CheW links the receptors to activation of CheA, as in *E. coli*, but the role of CheW cannot apparently be replaced by any of the three CheV proteins in a *cheW* mutant background. Overexpression of *H. pylori* CheW in *E. coli* inhibited swarming and induced a smooth-swimming phenotype in the wild-type strain RP437. This is the same effect seen upon overexpression of the homologous *E. coli* CheW (Sanders *et al.*, 1989b). The production of a molar excess of CheW may result in inhibition of the interaction required to catalyse CheA autophosphorylation, possibly by excess CheW displacing the CheW–CheA complex from the receptor (Hamblin *et al.*, 1997). The result would be the formation of an inactive complex that cannot initiate the flow of phosphate to CheY. This would reduce the levels of phospho-CheY, resulting in tumble suppression. Hamblin *et al.* (1997) successfully complemented an *E. coli* $\Delta cheW$ mutant (RP4606) with *cheW* from *Rhodobacter sphaeroides*, which is a member of the α -subgroup of Proteobacteria. In contrast, the *H. pylori* *cheW* gene was apparently unable to complement RP4606. Lack of complementation may be due to insufficient functional similarity, or to an unsuitable level of CheW overexpression

Fig. 6. Fluorescence changes in CheV2 upon addition of acetyl phosphate. (a) Fluorescence emission spectrum of 0.2 μ M CheV2, at an excitation wavelength of 280 nm. Dashed line, protein fluorescence in the absence of acetyl phosphate; solid line, fluorescence in the presence of 50 mM acetyl phosphate. (b) Titration of fluorescence change with acetyl phosphate. The CheV2 concentration was 0.1 μ M in buffer C. (c) Determination of K_m for acetyl phosphate. The ratio of the acetyl phosphate-induced fluorescence quench ($I_0 - I$, where I is the fluorescence intensity at a given acetyl phosphate concentration, and I_0 is the fluorescence intensity of the CheV-Mg²⁺ complex in the absence of acetyl phosphate) and the difference between the fluorescence intensity at saturating acetyl phosphate and I (i.e. $I - I_\infty$) is equal to $[\text{acetyl phosphate}]/K_m$ by analogy with the reaction scheme for CheY proposed by Lukat *et al.* (1992) and described in the text for CheV. Thus the reciprocal of the slope of the line relating this ratio to the phosphodonator concentration gave a K_m value of 21 mM acetyl phosphate.

(Sanders *et al.*, 1989b). Protein stoichiometry is important in chemotaxis, and although the pTM30 vector gives graded expression with IPTG, the lowest concentration of IPTG used (1 μ M) may still have induced a molar excess of this protein over the other chemotaxis proteins in *E. coli*.

The results from insertional inactivation of each of the three *cheV* genes in *H. pylori* revealed that CheV1 is critical for chemotaxis, as a *cheV1* null mutant was severely affected in its ability to swarm on soft agar, although swarming was not completely abolished. The expression of the two other *cheV* genes may be responsible for this residual level of swarming. However, the single *cheV2* and *cheV3* mutants were fully chemotactic and formed swarms on semi-solid agar indistinguishable from those of the parental strain. These genes cannot simply substitute for each other's function, as a *cheV2/cheV3* double mutant strain was also fully chemotactic. In the double mutant it seems likely that CheV1 can substitute for the roles of both CheV2 and CheV3. The results suggest a dominant and specialized function for CheV1 in *H. pylori* chemotaxis. The expression of *H. pylori cheV2* or *cheV3* in the *E. coli* Δ *cheW* or Δ *cheY* mutants did not restore a swarming phenotype. However, as with *cheW*, when expressed in the isogenic *E. coli* wild-type strain these genes caused a clear inhibition of swarming. Therefore, CheV2 and CheV3 can interact with the *E. coli* chemotaxis system but as they consist of both a CheW and a CheY domain, the mechanism of interaction may be complex. The CheW domains could bind to the chemoreceptors and inhibit chemotaxis in the same manner as that described for the *H. pylori* CheW. However, the CheY domains could also compete with the native *E. coli* CheY for phosphate from CheA. The overall effect of this would be to sequester phosphoryl groups from the native *E. coli* CheY, thus causing a build-up of unphosphorylated *E. coli* CheY which would be unable to bind to the motor switch. The net effect would be that the cells would tumble rarely, giving a smooth-swimming phenotype and an inability to swarm. The ability of CheV2 and CheV3 to perturb chemotaxis in *E. coli* demonstrates that these proteins do have a role in chemotaxis, despite the fact that normal swarming behaviour was still observed for *H. pylori cheV2* and *cheV3* mutants. However, in this study we have not examined the swimming patterns of the *H. pylori* mutants in detail, and it would be informative to study their behaviour by video tracking, to reveal any motility defects not apparent in simple swarm assays.

Thus, the most likely roles for the CheV proteins in *H. pylori* could involve the ability of their CheY domains to be phosphorylated. All of the proteins contain the necessary active-site residues, and we have shown using fluorescence spectroscopy that CheV2, as a representative of the CheV family, is able to bind the small-molecule phosphodonator acetyl phosphate in a similar manner to that which is characteristic of CheY and other response-regulators (Lukat *et al.*, 1992; McCleary, 1996). This is the first evidence for phosphorylation of any CheV protein. The low affinity observed indicates that acetyl phosphate itself is not physiologically important as a phosphodonator to CheV *in vivo*; the current view of *E. coli* CheY from stopped-flow kinetic studies of acetyl phosphate binding is that CheA provides a high local concentration of phosphate groups (Da Re *et al.*,

1999). Phosphorylation of CheV proteins may function in controlling the flow of phosphate to CheY itself, by the CheV proteins acting as 'phosphate sinks'. The N-terminal CheW domain of CheV may interact with a site on the cytoplasmic signalling domain of the receptors and catalyse phosphoryl transfer from CheA onto the C-terminal CheY domain of the protein. The receptor-binding site may be the same as used by the CheW protein, thus inhibiting the flow of phosphate to the CheY protein itself by a process of competitive binding. The need for a phosphate sink may be related to the absence of a *cheZ* homologue in *H. pylori*, as is the case with other non-enteric bacteria. For example, there are no *cheZ* homologues in *Rb. sphaeroides* and *Sinorhizobium meliloti* but these organisms do possess several copies of *cheY* (Armitage & Schmitt, 1997; Sourjik & Schmitt, 1996). A phosphate sink function to control phospho-CheY levels has been suggested for these additional CheY proteins (Armitage & Schmitt, 1997) and for the CheY domain of CheAY in *H. pylori* (Foynes *et al.*, 2000). Our results suggest that the CheY domains of the CheV proteins also have an important role as phospho-acceptors.

The purification of all of the *H. pylori* chemotaxis proteins, and the reconstitution of the signal-transduction cascade *in vitro* to demonstrate how intramolecular and intermolecular phosphotransfer from CheAY to the other CheY domains occurs, will be needed to fully explain the role and mechanism of the individual CheV proteins, and to further our understanding of this novel chemotaxis system.

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