A fixed-time diffusion analysis method determines that the three *cheV* genes of *Helicobacter pylori* differentially affect motility

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Helicobacter pylori is a chemotactic bacterium that has three CheV proteins in its predicted chemotaxis signal transduction system. CheV proteins contain both CheW- and response-regulator-like domains. To determine the function of these proteins, we developed a fixed-time diffusion method that would quantify bacterial direction change without needing to define particular behaviours, to deal with the many behaviours that swimming *H. pylori* exhibit. We then analysed mutants that had each *cheV* gene deleted individually and found that the behaviour of each mutant differed substantially from wild-type and the other mutants. *cheV1* and *cheV2* mutants displayed smooth swimming behaviour, consistent with decreased cellular CheY-P, similar to a *cheW* mutant. In contrast, the *cheV3* mutation had the opposite effect and the mutant cells appeared to change direction frequently. Additional analysis showed that the *cheV* mutants displayed aberrant behaviour as compared to the wild-type in the soft-agar chemotaxis assay. The soft-agar assay phenotype was less extreme compared to that seen in the fixed-time diffusion model, suggesting that the *cheV* mutants are able to partially compensate for their defects under some conditions. Each *cheV* mutant furthermore had defects in mouse colonization that ranged from severe to modest, consistent with a role in chemotaxis. These studies thus show that the *H. pylori* CheV proteins each differently affect swimming behaviour.

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INTRODUCTION

Many micro-organisms move in a directed fashion in response to their environment. A common mechanism for movement is via rotary motor organelles called flagella. These motors are regulated by the chemotaxis signal transduction system, which transduces environmental cues into a swimming response. The core of this signal transduction system consists of chemoreceptors, a kinase (CheA), a receptor-kinase coupler (CheW) and a phosphorylatable response regulator (CheY) that controls flagellar rotation (Blair, 1995; Szurmant & Ordal, 2004).

There are additional proteins that modulate the amount of phosphorylated CheY (CheY-P). Accessory proteins

responsible for adaptation and other functions abound, such as methylation of the chemoreceptors by CheR. As more and more prokaryotic genomes have been sequenced, it is becoming clear that motile microbes each have the core signal transduction proteins with a somewhat unique set of these modulator proteins, although it is not yet apparent why particular microbes have particular sets (Blair, 1995; Szurmant & Ordal, 2004). One such microbe is *Helicobacter pylori*, a bacterium that uses flagellar motility and chemotaxis to fully colonize animal stomachs (Eaton *et al.*, 1992, 1996; Foyne *et al.*, 2000; Ottemann & Lowenthal, 2002; Terry *et al.*, 2005). Understanding some of the less-usual attributes of this microbe's chemotactic signal transduction system was the goal of this study.

H. pylori contains homologues of many of the core chemotaxis genes found in other organisms, including *cheA* (actually a hybrid *cheA-Y*: contains homologous domains to both *Escherichia coli cheA* and *cheY*), *cheW* and

Abbreviation: FBS, fetal bovine serum.

A supplementary movie showing an example of direction changes in the *H. pylori cheV1* mutant is available with the online version of this paper.

cheY. The *cheA*, *cheY* and *cheW* genes are necessary for chemotaxis in soft agar, as expected (Beier *et al.*, 1997; Foynes *et al.*, 2000; Pittman *et al.*, 2001; Terry *et al.*, 2005). *H. pylori* lacks genes coding for the CheR and CheB chemotaxis methylation proteins, and has three *cheV* genes. We were particularly curious about the role of the CheV proteins, as previous studies in *Bacillus subtilis* had suggested that these proteins can function in adaptation (Karatan *et al.*, 2001).

CheV proteins are hybrids of CheW and a response regulator domain similar to CheY. The functions of the *H. pylori* CheV proteins are not well understood. The first study of these proteins created null mutants of each and analysed them in a chemotaxis soft-agar assay (Pittman *et al.*, 2001). In this assay, bacteria navigate through agar channels to form expanded colonies. Formation of the expanded colonies requires growth, chemotaxis and motility. In this assay, Pittman *et al.* (2001) found that loss of *cheV1* (HP0019) resulted in a strong defect, whereas neither null mutations in *cheV2* (HP0616) or *cheV3* (HP0393), nor a double mutant of both *cheV2* and *cheV3*, had a detectable phenotype in the same assay. Both *cheV2* and *cheV3*, however, did interfere with chemotaxis when expressed in *E. coli*, suggesting that the proteins encoded by these genes can interact in the chemotaxis pathway (Pittman *et al.*, 2001). CheV2 has been shown to be phosphorylated *in vitro* by acetyl phosphate (Pittman *et al.*, 2001), and the isolated CheY domain of all three CheV proteins can accelerate the dephosphorylation of CheA *in vitro*, supporting the notion that all CheVs can interact with the CheA chemotaxis protein (Jimenez-Pearson *et al.*, 2005). These studies thus show that CheV1 plays an important but ill-defined role in *H. pylori* chemotaxis, and that the roles of CheV2 and CheV3 in chemotaxis are unclear.

Many other microbes have CheVs, but the best studied is that of *B. subtilis*. *B. subtilis* has one *cheV* and one *cheW* in its genome. Deletion mutants of either *cheW* or *cheV* display reduced ability relative to wild-type to migrate up a concentration gradient of attractant (Rosario *et al.*, 1994). Both *cheW* and *cheV* null mutations have an altered basal flagellar bias skewed toward counter-clockwise rotation and thus the smooth swimming/high CheY-P state, although both mutants can still switch flagellar rotation (Karatan *et al.*, 2001; Rosario *et al.*, 1994). A double deletion of *cheV* and *cheW*, on the other hand, resulted in bacteria phenotypically null for chemotaxis, and with a strong bias towards clockwise rotation (low CheY-P) (Rosario *et al.*, 1994). A *cheV* truncation that retained only the *cheW* domain could partially complement either a *cheW* deletion or a *cheW cheV* double deletion to restore the response to attractant. However this mutant was not able to adapt (Karatan *et al.*, 2001). Likewise, bacteria with a point mutation in *cheV*, leading to a protein unable to accept phosphates on the CheY domain, also demonstrated reduced ability to adapt (Karatan *et al.*, 2001). Taken together, these studies show two things about CheV's

function in *B. subtilis*. The first is that CheV, along with CheW, is required for full chemoreceptor–CheA coupling. The second is that CheV's CheY domain is needed for adaptation, most likely through deactivating the coupling function of the CheW domain upon acceptance of phosphate (Karatan *et al.*, 2001; Rosario *et al.*, 1994). It is of note that *B. subtilis* does have the CheR/B proteins, and it has been suggested that CheV forms an alternative adaptation mechanism (Rao *et al.*, 2008).

Many other bacteria, including *Salmonella typhimurium* serovar Enterica, have at least one CheV. The *cheV* of *S. typhimurium* serovar Enterica is part of the flagellar regulon (Frye *et al.*, 2006; McClelland *et al.*, 2001; Wang *et al.*, 2006). *cheV* deletions in a Δ *cheBR* background cause a chemotaxis defect (Wang *et al.*, 2006), while *cheV* deletions in the wild-type background have no phenotype (Frye *et al.*, 2006).

One challenge for determining the function of the CheV proteins is the lack of assays for chemotaxis in *H. pylori*. Historically, much has been learned from observing bacterial swimming behaviour (Berg & Brown, 1972). We found this approach challenging as *H. pylori* exhibits many types of swimming behaviours, including stops, curves, reversals and speed changes, and so it is hard to determine whether any particular behaviour is most important, and also how to quantify these various behaviours. To overcome this challenge, we developed a method to describe bacterial swimming behaviour mathematically, employing a simple diffusion model. Our model does not require one to define different behaviours. Using this approach, along with others, we were able to determine that each of the CheV proteins plays a unique role in affecting flagellar rotation.

METHODS

Bacterial strains. The motile human isolate *H. pylori* SS1 was used for all experiments. *E. coli* strain DH10B was used for cloning, and derivatives of *E. coli* strain RP437 were used for motility studies. All strains are listed in Table 1.

Growth media and chemicals. For solid-medium culture, *H. pylori* was grown on Columbia blood agar (Becton Dickinson) plates with 5% defibrinated horse blood (Hemostat Laboratories), 5 µg trimethoprim ml⁻¹, 8 µg amphotericin B ml⁻¹, 10 µg vancomycin ml⁻¹, 50 µg cycloheximide ml⁻¹, 5 µg cefsulodin ml⁻¹, 2.5 U polymyxin B ml⁻¹ and 0.2% (w/v) β -cyclodextrin (Sigma) (CHBA) at 37 °C under conditions of 5–10% O₂, 10% CO₂ and 80–85% N₂. All antibiotics were from Sigma or ISC Bioexpress. For liquid culture, *H. pylori* strains were grown in Brucella broth (Becton Dickinson) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) (BB10) on a shaking incubator at 37 °C under conditions of 5–10% O₂, 10% CO₂ and 80–85% N₂. For selection of mutants, chloramphenicol was used at 5–10 µg ml⁻¹ (*H. pylori*) or 20 µg ml⁻¹ (*E. coli*).

For long-term storage, a thick 3–5-day growth of *H. pylori* from a CHBA plate was scraped into BB10/1% (w/v) β -cyclodextrin/25% glycerol/5% DMSO. The cells were dispersed by pipetting and vortexing, then frozen at –70 °C.

Table 1. Strains and plasmids used in this study

Strain/plasmid	KO no.	Genotype/description	Reference/source
<i>H. pylori</i>			
SS1		Wild-type	Lee <i>et al.</i> (1997)/J. O'Rourke
SS1 <i>cheV1</i>	KO864	SS1 $\Delta cheV1::cat_{mut}$	This study
SS1 <i>cheV2</i>	KO865	SS1 $\Delta cheV2::cat_{mut}$	This study
SS1 <i>cheV3</i>	KO856	SS1 $\Delta cheV3::cat_{mut}$	This study
SS1 <i>cheY</i>	KO631	SS1 $\Delta cheY::cat_{mut}$ 102	Terry <i>et al.</i> (2005)
SS1 <i>cheW</i>	KO852	SS1 $\Delta cheW::aphA3$	Terry <i>et al.</i> (2005)
<i>E. coli</i>			
DH10B		Wild-type	Gibco-BRL
RP437		Wild-type	D. E. Koshland, Jr
RP4315		<i>cheY201</i>	Clegg & Koshland (1984)/D. E. Koshland, Jr
RP3228		<i>cheW113</i>	Parkinson (1978)/D. E. Koshland, Jr
Plasmids			
pBluescript (pBS)			Stratagene
pCat-mut		pBS with <i>Campylobacter coli</i> <i>cat</i> gene lacking terminator	Terry <i>et al.</i> (2005)
pBS-CheV1		pBS:: <i>cheV1</i> _{SS1} plus 1863 bp flanking seq.	This study
pBS-CheV2		pBS:: <i>cheV2</i> _{SS1} plus 1897 bp flanking seq.	This study
pBS-CheV3		pBS:: <i>cheV3</i> _{SS1} plus 1846 bp flanking seq.	This study
pV1- <i>cat</i> _{mut}		pBS- $\Delta cheV1::cat_{mut}$	This study
pV2- <i>cat</i> _{mut}		pBS- $\Delta cheV2::cat_{mut}$	This study
pV3- <i>cat</i> _{mut}		pBS- $\Delta cheV3::cat_{mut}$	This study

Plasmid preparation was done using kits from Qiagen. For preparation of genomic DNA, DNeasy kits (Qiagen) or Wizard Genomic kits (Promega) were used. All restriction and DNA-modification enzymes were from New England Biolabs or Gibco. Amplification of DNA was carried out using *Pfu* or *Pfu*-Turbo polymerases (Stratagene) or Taq polymerase (a generous gift of D. Kellogg). All DNA sequencing was performed by the U. C. Berkeley sequencing facility.

Cloning *cheV* genes. To isolate the *cheV* genes for later use in allelic replacement vectors, each *cheV* gene and approximately 300 bp of flanking sequences was amplified using PCR and cloned into pBluescript (pBS). There are three *cheV* genes in each *H. pylori* genome; they were originally numbered in the J99 genome as *cheV1* (*hp0019/jhp0017*), *cheV2* (*hp0616/jhp0559*) and *cheV3* (*hp0393/jhp0988*) (Alm *et al.*, 1999), while the 26695 genome does not assign them numbers (Tomb *et al.*, 1997). Primers were designed to clone each *cheV* using the 26695 genome, and are listed in Table 2, as e.g. *cheV1up1* and *cheV1down1*. Each PCR product was gel purified and treated with T4 kinase (New England Biolabs). These products were then cloned into *EcoRV*-cut pBluescript, creating the vectors pBS-CheV1, pBS-CheV2 and pBS-CheV3. All insertions were verified by restriction analysis and sequencing.

Creation of $\Delta cheV::cat_{mut}$ mutants. pBS-CheV1, pBS-CheV2 or pBS-CheV3 were used as templates in inverse PCR, in which primers were chosen such that most of the *cheV* gene would be deleted from the product (Table 2; primers with iPCR in the name). Specifically, 879 bp were deleted from *cheV1*, leaving regions coding for five amino acids at the 5' end, and 30 amino acids at the 3' end; 660 bp were deleted from *cheV2*, leaving regions coding for 10 amino acids at the 5' end and 71 amino acids at the 3' end; 601 bp were deleted from *cheV3*, leaving regions coding for 25 amino acids at the 5' end and 56 amino acids at the 3' end. Each of these PCR products was gel purified and ligated with a chloramphenicol acetyltransferase gene lacking a terminator that had been excised from the vector pCat-mut

(Terry *et al.*, 2005) using *HincII*. These ligations created the vectors pV1-*cat*_{mut}, pV2-*cat*_{mut} and pV3-*cat*_{mut}. These plasmids were then transformed into *H. pylori* strain SS1 using natural transformation, and the veracity of each mutant determined by PCR of each locus (data not shown).

RT-PCR analysis of the expression of genes downstream of each *cheV*. To verify that our *cat*_{mut} insertions had not affected downstream transcription, we carried out reverse transcription (RT)-

Table 2. Primers used in this study

Name	Sequence (5'–3')
<i>cheV1up1</i>	ATTGGGGTTTGTGGAGGG
<i>cheV1down1</i>	ATTCGCTGGGGTTACTTCGC
<i>cheV2up1</i>	AGAAATGATCACTTCGCCCC
<i>cheV2down1</i>	TTTCAATCGGTGGGGTAGCG
<i>cheV3up1</i>	GATGGCTTGAAGTTGTTGACCGC
<i>cheV3down1</i>	TTGAGCGCCAAAACAGCCCC
<i>cheV1iPCRup1</i>	CGTAAGCTGATCAATGCCCGC
<i>cheV1iPCRdn1</i>	GGCCAGGAGTTTGAAGGCCG
<i>cheV2iPCRup1</i>	CGTTCGTTGTTAAGTGCAACG
<i>cheV2iPCRdn1</i>	GCTTTTCCAACAAGTTGGCGTGG
<i>cheV3iPCRup1</i>	GGGCATGGGGATGATTTCCTCT
<i>cheV3iPCRdn1</i>	GGCTATCATTTCTTATTCAAGCTC
HP0020_FORWARD	GGCTAAGCTCGCTTTTGAAG
HP0020_REVERSE	CTTAGTGATATGATGCCCCC
HP0617_FORWARD	GGAGTGAATTTGTGCTAGTG
HP0617_REVERSE	CATCTCTAAAGCAACGAGCG
HP0392_FORWARD	GATTACGCTAACATGAGCGC
HP0392_REVERSE	CTTACCCACTGGTTGCATCC

PCR on each downstream gene. A primer set comprising forward and reverse primers that would create 400–500 bp products was designed for the HP0020 (downstream of *cheV1*), HP0617 (downstream of *cheV2*) and HP0392 (downstream of *cheV3*) genes using the *H. pylori* 26695 sequence (Table 2). RNA was extracted from plate-grown *H. pylori* SS1 or each of its *cheV* mutants using Trizol extraction (Invitrogen) followed by RNeasy Mini kit (Qiagen) and stored at -20°C . The RNA was checked on an agarose gel, and 1 μg treated with 1–2 U DNase (Invitrogen), following the manufacturer's instructions. For the RT reaction, we used 66 ng DNase-treated RNA and Superscript RT-II Platinum Taq (Invitrogen), following the manufacturer's instructions. Control reactions were done with Taq alone, with its corresponding buffer. Cycling conditions were: (1) initial elongation step at 50°C for 30 min; (2) 94°C for 2 min; (3) 34 total cycles of 94°C for 1 min, 50 – 56°C for 1 min (depending on the primer set), 72°C for 1 min; (4) 72°C for 3 min.

Fixed-time diffusion motility data collection. *H. pylori* patches on CHBA were used to inoculate BB10. Cultures were typically grown with shaking for 12–16 h in 10% CO_2 /5% O_2 /85% N_2 to an OD_{600} of 0.05–0.3, and diluted in fresh BB10 to ~ 30 cells visible in the field. Wet mounts (50–70 μl) from these cultures were placed on sitting-drop vapour diffusion slides (Fisher). A minimum of three separate cultures, inoculated from different patches on different days, were used for all *H. pylori* motility analyses.

For *E. coli*, relevant strains were inoculated as single colonies into tryptone broth, and grown to exponential phase at 30°C .

Video was taken using a Nikon Eclipse E600 microscope with a Hamamatsu 1394 ORCA-285S camera using a $20\times$ phase-contrast objective. One minute of video was captured with an approximate frame rate of 13 frames s^{-1} , with 10–40 videos taken for each strain or mutant. This video was then analysed using the SimplePCI MTA software package (Compix) to track the bacteria and generate x,y positions of each cell over time.

Fixed-time diffusion data analysis. Before analysis could begin, the data were first filtered to capture only moving bacteria. Non-motile bacteria were removed first by eliminating any bacteria that moved less than 20 pixels s^{-1} . We empirically found the cutoff to work well for differentiating between motile and non-motile *H. pylori*, as well as working for *E. coli*. Fixed-time diffusion requires that all datasets be of the same length of time. We found 4 s to be a good tradeoff between acquiring enough tracks to analyse, and obtaining data in a linear range. All tracks shorter than 4 s were removed, while all tracks longer than 4 s were reduced to 4 s in length, by ignoring any part of the data after 4 s had passed. The usual time step for the data was 0.06–0.07 s, and a 4 s track had 52 points. The above-described filtering of data output from SimplePCI was done by first exporting the x,y positions of each tracked particle into Microsoft Excel formatted files. Data were then imported into R using the gdata package (the R Project for Statistical Computing, <http://www.r-project.org/>) (R Development Core Team, 2005). A custom R script was used for filtering the data. Flat text files were exported from R containing the filtered data.

Next, the filtered position data ($x(t)$, $y(t)$) collected for every bacterium were shifted from the initial position ($x(0)$, $y(0)$) to the origin (0, 0) by subtracting ($x(0)$, $y(0)$) from every ($x(t)$, $y(t)$). A transformation to polar coordinates was then done ($x(t)$, $y(t)$) $\rightarrow$ ($r(t)$, $\theta(t)$). We next calculated the mean square of the radius $\langle R^2(t) \rangle$ for each point in time. A least-square linear regression was then performed on the set of points ($\log(t)$, $\log(\langle R^2(t) \rangle)$). The slope of that curve gave the value of the diffusion exponent α . Confidence bands were produced by taking the standard error at the predicted value of $\log\langle R^2(t) \rangle$. This analysis was done in Matlab.

Soft-agar motility assay. Soft-agar plates were made with Brucella broth, 2.5% FBS and 0.35% agar. Plates were incubated at room temperature for 3 days before use. Strains were inoculated from CHBA plates into the soft-agar plates using a pipette tip, and migration was measured by measuring the colony diameter each day.

Direct observation of behaviour in soft agar. Low-percentage agar was prepared by autoclaving Brucella broth and 0.42% agar, and then allowing this solution to cool to 50°C . For preparing the samples, 900 μl was removed into a microcentrifuge tube and mixed with 100 μl FBS for a final agar concentration of $\sim 0.38\%$. This mixture was allowed to cool for 1 min at room temperature, at which point 20 μl of an overnight *H. pylori* culture was added, prepared as described for fixed-time diffusion motility data collection. Tubes were inverted several times to mix the bacteria with the agar solution and 100 μl of this mixture was then immediately placed into the well of a sitting-drop vapour diffusion slide (Fisher), and a coverslip quickly added. As heat tends to paralyse *H. pylori* movement (unpublished observations), under these conditions restoration of movement coincided with solidification of the agar. All observations were made within 15 min of adding bacteria to the agar. Microscopy and video data were collected as described above.

Mouse infection. For all infections, 6–8-week-old female FVB/N mice (Charles River) were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care-accredited facility in microisolator cages with free access to standard food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee, and carried out as described before (Ottemann & Lowenthal, 2002). For infection, bacteria were grown in BB10 microaerobically with shaking for 16 h, and 1×10^7 – 5×10^7 bacteria in 1 ml BB10 were introduced into each mouse by oral gavage. For competition experiments, a *cheV* deletion mutant and the wild-type SS1 strain were mixed together such that there were equal numbers (1×10^7 – 5×10^7 of each strain in 1 ml) based on the OD_{600} . All infection amounts were verified by plating the inoculum. For all mouse experiments, the wild-type SS1 was lab passaged the same number of times as the mutant. After 2 weeks, the mice were euthanized by inhalation of CO_2 , and the bacteria contained in one half stomach were plated onto CHBA with an additional 10 μg nalidixic acid ml^{-1} and 200 μg bacitracin ml^{-1} . For competitions, samples of the stomach homogenate were plated onto CHBA and onto CHBA plus chloramphenicol. The number of wild-type bacteria was determined using the following equation: (c.f.u. on CHBA) – (c.f.u. on CHBA-Cm). Statistical analysis was performed using a Student's *t*-test in Microsoft Excel for single-strain infections, or the Wilcoxon, matched-pair signed rank test for competition infections (http://www.fon.hum.uva.nl/Service/Statistics/Signed_Rank_Test.html).

Protein sequence analysis. Alignments were generated with CLUSTAL W (Thompson *et al.*, 1994), and Boxshade (<http://www.ch.embnet.org>) was used to visualize them in a readable format.

RESULTS

The CheV proteins of *H. pylori* contain all the conserved response-regulator and CheW elements

H. pylori possesses three CheV proteins, each composed of fused CheW and response-regulator/CheY domains, referred to as W- and RR-domains, respectively. Previous analysis of *H. pylori* behaviour found a motility role for CheV1 (Pittman *et al.*, 2001), but could detect no

phenotype for loss of *cheV2* or *cheV3*. CheV1 is relatively similar to CheV2 (40 % identical, 66 % similar), while CheV3 is less similar (only 24 % identical and 48–49 % similar to either CheV1 or CheV2). All of the CheVs retain the phosphorylation active-site residues, as shown by Pittman *et al.* (2001), but the conservation of the W-domains had not been examined. As shown in Fig. 1, the *H. pylori* CheVs retain conserved regions known to be important for CheW functions. For example, all of the *H. pylori* W-domains conserve an arginine residue that modulates CheA kinase activity (Boukhvalova *et al.*, 2002; Liu & Parkinson, 1991). There is also significant charge and residue conservation within CheW regions responsible for chemoreceptor binding. CheVs also contain

several in-frame insertions in both the W- and RR-domains (Fig. 1 and data not shown), although the function of these insertions is unknown. These findings thus suggest that the *H. pylori* CheVs likely retain CheW-like ability to interact with both chemoreceptors and CheA, and also that they are capable of being phosphorylated.

Deletion of any *cheV* alters *H. pylori* behaviour in soft agar

Our alignments suggested that all of the *H. pylori* CheVs should be able to interact with the chemotaxis machinery. We thus sought to thoroughly analyse the motility phenotypes of *H. pylori* lacking each *cheV*. To that end,

Fig. 1. Alignments of selected CheW-like domains. Labels have the following meanings: Ec is *Escherichia coli* K-12 MG1655, St is *Salmonella enterica* serovar Typhimurium LT2, Hp is *Helicobacter pylori* 26695, Hh is *Helicobacter hepaticus* ATCC 51449, Cj is *Campylobacter jejuni*, Tm is *Thermotoga maritima* MSB8, Ws is *Wolinella succinogenes* DSMZ 1740, Bs is *Bacillus subtilis*, Bg is *Borrelia garinii* PBI. The symbol # denotes areas where insertions tend to occur. X denotes regions involved in chemoreceptor binding. R is the conserved arginine thought to modulate CheA kinase activity. A are additional conserved residues governing CheA–CheW interaction. Black denotes identical residues and grey denotes similar residues.

we created *cheV* mutants in the common *H. pylori* strain SS1 by replacing each of the *cheV* genes individually with a copy of the chloramphenicol acetyltransferase (*cat*) gene lacking its intrinsic transcriptional terminator. This *cat* cassette has been shown to completely lack terminating function (Castillo *et al.*, 2008) and to be non-polar in other mutations (Terry *et al.*, 2005). We verified, using RT-PCR, that each downstream gene was expressed as expected (Fig. 2). *cheV1* is predicted to be the first gene in a two-gene operon, *cheV2* is predicted to be the first gene in a five-gene operon, and *cheV3* is predicted to be the 11th gene in a 13-gene operon. It is of note that *cheV3* is followed by the *cheA* and *cheW* genes.

We next analysed the behaviour of these mutants, first under non-steady-state conditions in soft-agar assays. For this assay, a small amount of bacteria is inoculated into a rich medium with a low percentage of agar. The gel of the agar creates a three-dimensional structure analogous to a maze, in which bacteria with no ability to change direction cannot navigate. *H. pylori* are believed to migrate out from the starting point in response to changes in nutrient or waste-product status as they carry out metabolism and growth. *H. pylori* mutants lacking flagellar motility or chemotaxis do not spread significantly through the agar (Eaton *et al.*, 1992; Foyne *et al.*, 2000; Ottemann &

Lowenthal, 2002; Pittman *et al.*, 2001; Terry *et al.*, 2006). The relative rate of migration, in millimetres per day, reflects the ability of the bacteria to exhibit chemotaxis, swim and grow. The wild-type was found to migrate at a rate of 4.0 mm per day (Table 3). In agreement with Pittman *et al.* (2001), we found that deletion of *cheV1* resulted in a severe decrease in soft-agar migration (average of 1.5 mm per day) (Table 3) (Pittman *et al.*, 2001). We observed that both the *cheV2* (3.5 mm per day) and *cheV3* (4.3 mm per day) mutants had subtle but significant alterations in their soft-agar migration, with *cheV2* showing a slight decrease in soft-agar motility and *cheV3* a slight increase (Table 3). None of these mutants displayed growth defects (data not shown). These results thus suggest that all of the CheVs might play roles in chemotaxis.

***cheV* mutants have chemotaxis defects when analysed in a fixed-time diffusion model**

To learn more about the role of each CheV in *H. pylori* chemotaxis, we developed a mathematical model to describe swimming behaviour based on a fixed-time diffusion measurement. Einstein (1905) first described a mathematical model of small particle diffusion, and we utilized this concept, as well as current theories (Metzler & Klafter, 2000) to construct our model.

Bacterial motion is characterized by two behaviours: changes in direction and relatively straight swimming. For our analysis, we modelled the motion of swimming bacteria to that of a particle diffusing in medium. From the theory of Brownian motion, we know that after a long enough period of time, the mean square $\langle R^2(t) \rangle$ of the bacterial position is proportional to time, t , according to the equation $\langle R^2(t) \rangle = Dt^\alpha$ (D , diffusion constant). We assume that when measurements take place in a sufficiently small amount of time, T , the mean square positions of the bacteria will be proportional to t^α , with α ranging between 1 and 2. The value of α measures how close the bacterial behaviour is to pure diffusion ($\alpha=1$). If a bacterium swims straight for a relatively large fraction of the time up to T , the value of α will be close to 2. If up to the time T the bacterium swims with frequent direction changes, the value of α will be near 1. Note that the exponent α depends on the value of T . If T is too large, any bacteria that change direction will have an α near 1. Conversely, if T is too small the value of α will be near 2. We fixed T empirically by observing the trajectories of different bacterial mutants. We chose T of 4 s because this length of time allowed us both to film sufficient numbers of bacteria (i.e. the bacteria remained within the field and plane of focus), and to generate α values that differed between wild-type and known chemotaxis mutants. Of particular benefit is that the model does not require accurate measurements of tumbling phenomena.

Our basic approach was to capture swimming *H. pylori* using video microscopy and analyse the bacterial beha-

Fig. 2. RT-PCR analysis shows that the genes downstream of each *cheV* are expressed in *cheV* mutants. RNA (66 ng per lane) was analysed using RT-PCR and primers designed for the gene downstream of each *cheV*. Control lanes lack reverse transcriptase but retain Taq. The mutant analysed is indicated above each gel section; arrowheads indicate the RNA-derived PCR product. Each section shows results of RT-PCR of RNA isolated from SS1 wild-type *H. pylori* (A); RT-PCR of RNA isolated from SS1 *cheV* mutant *H. pylori* (B); control reaction (Taq only) of RNA isolated from SS1 wild-type *H. pylori* (C); and control reaction (Taq only) of RNA isolated from SS1 *cheV* mutant *H. pylori* (D). M, size markers. The HP0020 product (downstream of *cheV1*) is predicted to be 487 bp; the HP0617 product (downstream of *cheV2*) is predicted to be 486 bp; the HP0392 product (downstream of *cheV3*) is predicted to be 450 bp.

Table 3. Properties of the *H. pylori cheV* mutants

Soft-agar migration indicates the colonial expansion rates of *H. pylori* SS1 and mutants on Brucella broth/2.5% FBS soft agar. The *cheW* and *cheY* rates have been reported previously (McGee *et al.*, 2005; Pittman *et al.*, 2001; Terry *et al.*, 2006). The number in parentheses indicates the number of independent colony measurements. Each mutant was compared to a wild-type on the same plate; the migration of *cheW/cheY* mutants is due to growth only. ND, Not determined. The migration rates of all three *cheV* mutants were significantly different from that of wild-type using Student's *t*-test. α is the diffusion exponent in liquid medium. Mouse colonization is the number of *H. pylori* in the stomach after 2 weeks of infection, with the 95% confidence interval in parentheses.

Strain	Soft-agar migration (mm per day)	α	$10^{-6} \times$ Mouse colonization
<i>H. pylori</i> SS1 (wild-type)	3.97 ± 0.01 ($n=127$)	1.6122 ± 0.0012	3.32 (1.53)
SS1 <i>cheW</i>	0.5*	1.8651 ± 0.0004	0.83*
SS1 <i>cheY</i>	0.5*	1.9640 ± 0.0003	1.7*
SS1 <i>cheV1</i>	1.48 ± 0 (97)	1.8986 ± 0.0011	0.84 (0.33)
SS1 <i>cheV2</i>	3.45 ± 0.01 (65)	1.7851 ± 0.0010	2.52 (0.36)
SS1 <i>cheV3</i>	4.31 ± 0.01 (127)	1.1221 ± 0.0019	2.29 (0.74)
<i>E. coli</i> RP437	ND	1.6512 ± 0.0021	–
<i>E. coli</i> RP3228 (<i>cheW</i>)	ND	1.9811 ± 0.0006	–
<i>E. coli</i> RP4315 (<i>cheY</i>)	ND	1.8483 ± 0.0007	–

*Data taken from Terry *et al.* (2005).

viour. Under our conditions, the bacteria and media components freely diffuse, such that the bacteria respond to a relatively constant set of conditions. For these measurements, we took videos of exponential-phase bacteria in their growth medium (BB10) and then used tracking software to generate position (x,y) and time coordinates for each object that was consistent in size with a bacterium. A bacterial 'track' was defined as a set of positional (x,y) and time points that the software assigned to the same bacterium, because a given object was close to the same position in consecutive video frames. The tracking data were filtered to remove non-motile bacteria, and tracks were then trimmed to be 4 s in length. The positional data were then transformed to move the beginning of each track to the origin (0,0), with all changes in position relative to the origin preserved. The average change in distance over time was used to calculate the diffusion exponent, α , for each bacterial strain, as described in Methods and above.

We first tested our quantitative diffusion description with wild-type *E. coli* and found it had a diffusion exponent α of 1.6512 (Table 3). This value is intermediate between 1 and 2, suggesting that *E. coli* behaviour is set between smooth swimming and changing direction, as expected. We next analysed *E. coli* with mutations in the chemotaxis genes *cheW* or *cheY*. Mutants lacking these proteins constantly swim and almost never change direction (Blair, 1995). Using our fixed-time diffusion method, we found that these mutants were more smooth swimming than wild-type, with α values closer to 2, as expected (Table 3). Thus, our method works and appears sensitive to directional changes of the analysed bacteria.

We next used this methodology to analyse the behaviour of *H. pylori*. Wild-type bacteria again demonstrated an

intermediate diffusion exponent ($\alpha=1.6122$), while the *cheY* mutant ($\alpha=1.9640$) and the *cheW* mutant ($\alpha=1.8651$) were more smooth swimming (Table 3, Fig. 3). These measurements show that cells bearing the *cheY* mutant allele were significantly more smooth swimming than those with the *cheW* mutation. Furthermore, these data support the notion that CheY-P is involved in creating direction changes in *H. pylori*, along the lines of the *E. coli* model, because mutants unable to make CheY, and hence CheY-P, are smooth swimming. We then extended this analysis to our *cheV* mutants. Surprisingly, we found that one of them, the *cheV3* mutant, was heavily biased towards changing direction ($\alpha=1.1221$), while the *cheV1* mutant ($\alpha=1.8986$) and the *cheV2* mutant ($\alpha=1.7851$) were both smooth swimming (Table 3, Fig. 3). This analysis suggests that all three CheVs participate in controlling flagellar rotation, but that they play different roles. Loss of either of CheV1 or CheV2 is similar to loss of CheW or CheY, and results in cells that are less able to change direction than is the wild-type. Loss of CheV3, on the other hand, causes cells to change direction much more frequently than wild-type, akin to mutants that have elevated CheY-P.

We were somewhat puzzled by the apparently different phenotypes of the *cheV* mutants in the soft-agar assay versus the fixed-time diffusion swimming assay. For example, the *cheV2* mutant rarely changes direction in the diffusion assay, but traverses the soft-agar plates relatively well. Other straight-swimming mutants such as *cheW*, *cheA* or *cheY* cannot migrate through these soft-agar plates (Beier *et al.*, 1997; Foyne *et al.*, 2000; Pittman *et al.*, 2001), suggesting that the *cheV* mutants might display condition-dependent phenotypes in the soft-agar assay. To assess the actual swimming behaviour of the mutants in the

Fig. 3. Motility patterns of *H. pylori* SS1 and its chemotaxis mutants. The left panels show the un-rotated 4 s swimming tracks of the strain indicated on the left; each is set to the same scale. The middle panels show the same tracks rotated to originate from the origin; each is set to the same scale. The right panels show graphs of $\langle R^2(t) \rangle$ vs time (s) for these tracks. The diffusion exponent, α , is derived from the slope of the line and is given on the left. Light grey lines on each side of the plotted lines denote error. The number of tracks analysed for each strain is as follows: WT, >50; *cheY*, 13; *cheW*, >30; *cheV1*, 21; *cheV2*, 18; *cheV3*, 18.

soft-agar assay, we created miniature versions of this assay in hanging-drop microscope slides. We were able to observe *H. pylori* in these mini soft-agar assays, and saw that the wild-type and each of the *cheV* mutants was able to reverse directions. An example with the *H. pylori cheV1* mutant is shown in the supplementary movie available with the online version of this paper. As *H. pylori* cells are elongated and not spherical, it was easy to observe direction changes, although differences between the individual mutant strains were too subtle to quantify in this particular assay. The *cheY* mutant was never observed to move in the agar, consistent with it being immobilized at the end of channels (data not shown). However, smooth swimming motility was noted in the agar close to glass before solidification was complete, indicating that the mutant was capable of swimming in this assay.

CheV proteins play roles in mouse infection

As it was shown previously that chemotaxis is important for *H. pylori* infection of mice, we analysed how the *cheV* mutants would fare in the stomach. For these experiments we infected mice with $\sim 5 \times 10^7$ cells of each *cheV* mutant. After 2 weeks of infection, we analysed the number of *H. pylori* in the stomach, and compared this amount to mice infected with wild-type alone. Infection with wild-type SS1 bacteria resulted in colonization at an average of 3.32×10^6 c.f.u. per g stomach (Table 3). Both the *cheV2* and *cheV3* mutants were able to infect to levels that were not different from wild-type, while the *cheV1* mutant had a somewhat decreased ability to colonize mouse stomachs (Table 3). As stated above, these mutants did not display *in vitro* growth rate differences.

A more sensitive assay of colonization ability was employed by infecting the mice with mixtures of wild-type and one of the *cheV* mutants. Again, we allowed the infection to proceed for 2 weeks and determined the number of mutant and wild-type bacteria remaining in the stomach. Using this assay, we found that the *cheV1* mutant had the most severe defect, and was outcompeted >1000 fold by the wild-type (Fig. 4). The *cheV2* mutant was slightly and significantly outcompeted (1.8-fold). Strains lacking *cheV3* were also significantly outcompeted (8.5-fold) by wild-type. Thus, all three of the *cheV* genes appear to play roles in mouse-stomach colonization.

DISCUSSION

CheV proteins are widespread throughout bacteria but remain relatively understudied. *H. pylori* is unusual in that it has three CheVs with only one set of the core chemotaxis proteins, and no CheR or CheB. We have shown here that the *H. pylori* CheVs all affect flagellar rotation, and play distinct roles in the process.

Wild-type *H. pylori* exhibits many different swimming behaviours which could affect chemotaxis. These beha-

Fig. 4. Competitive index (CI) of *H. pylori* SS1 *cheV* mutants coinfecting with wild-type (WT). CI = mutant/WT_{output} divided by mutant/WT_{input}. A CI of 1 indicates a mutant with the same infection ability as WT. For *cheV1*, $n=10$ and the P -value is <0.01 . For *cheV2*, $n=6$ and the P -value is <0.05 . For *cheV3*, $n=10$ and the P -value is <0.01 .

viours include reversals, stops with and without reorientations, and changes in velocity. As it was unclear which of the many observed *H. pylori* behaviours was important for direction changes, and the importance of any particular behaviour could vary between wild-type and a mutant, we sought a method in which neither the method of direction change nor the velocity of bacteria would bias the results. Fixed-time diffusion was chosen as it fits these requirements, is intuitive, and is sensitive to small changes in behaviour. Using the equation $\langle R^2(t) \rangle = Dt^\alpha$, where the coefficient α describes the behaviour as it relates to Brownian (α close to 1, or many changes in direction) or ballistic (α close to 2, or no changes in direction), we were able to describe the behaviour of motile bacteria without the need to assume the extent to which various behaviours contributed to direction change. Use of this method facilitated our analysis of the roles for the CheV proteins.

Using the fixed-time diffusion approach to study *H. pylori* behaviour in liquid media, in which bacteria and media components diffuse freely, we found that each of the three CheVs plays a role in affecting flagellar rotation. Mutants lacking CheV1 are extremely smooth swimming, with only *cheY* mutants having a more smooth bias. Mutants lacking CheV2 are very smooth swimming as well. The soft-agar phenotypes mirror these phenotypes, albeit imperfectly. The *cheV1* mutants have a significant soft-agar defect, while mutants lacking *cheV2* have only a marginal defect. The finding that the *cheV2* mutant soft-agar phenotype is not as severe as might be expected from the diffusion exponent suggests that there could be a compensatory occurrence in the soft-agar assay. Similarly, a *B. subtilis cheV* mutant shows altered steady-state bias and time to adapt but retains the ability to adapt (Garrity & Ordal,

1995; Rosario *et al.*, 1994). The two chemotaxis assays used here have quite different conditions. For the diffusion analysis, the assay is relatively short (in the order of minutes), does not require the bacteria to generate a gradient and occurs in a liquid milieu. The soft-agar assay, in contrast, takes days, requires bacterial metabolism and growth, and occurs in a gel-like medium that has barriers to bacterial movement. Bacteria do not form chemical gradients in liquid culture, while the relatively slow forward progress in agar may allow them to generate a gradient through metabolism of attractants or release of repellants. We found that the two smooth-biased *cheV* deletion mutants were able to reverse direction in soft agar even when they had been embedded in it for a short period of time. One explanation is thus that the smooth-biased *cheV1* and *cheV2* mutants are capable of changing direction when a large enough chemical gradient is applied.

Mutants lacking *cheV3* display a strikingly different phenotype from those of the other *cheV* mutants. We found that these mutants switched direction frequently and migrated in soft agar at the same rate as wild-type. *E. coli* mutants with similarly elevated directional changes, such as those lacking *cheZ* or *cheB*, do better in the soft-agar assay than their smooth-locked counterparts, although not better than wild-type (Wolfe & Berg, 1989). Strictly smooth-swimming cells would be expected to get stuck in the soft-agar channels, while tumble mutants might be able to make their way through the matrix to some degree. It is possible that the swimming bias of this mutant is slightly modulated by conditions in the soft-agar assay, as hypothesized for the *cheV2* mutant above. Alternatively, because *H. pylori* migrates about 25 times slower in the soft-agar assay than does *E. coli*, perhaps tumble-bias mutants fare better in this bacterium.

We further utilized mouse-infection phenotypes to determine how the *cheV* mutants compare with known chemotaxis mutants. The mouse-infection phenotypes mirrored the soft-agar phenotypes more closely than they mirrored the liquid-swimming phenotypes. We hypothesize that the *cheV* mutants are able to adjust their swim/tumble biases under some conditions, such as in soft agar and the mouse stomach. The *cheV1* mutant was the most impaired *cheV* mutant in soft agar and similarly was the most impaired in the mouse stomach. This phenotype is similar to other swim-bias mutants such as those lacking *cheA*, *cheW* or *cheY* (Terry *et al.*, 2005). The *cheV2* mutant was only moderately impaired in the soft agar, and had only a slight defect in the mouse model. This phenotype suggests that these mutants are able to change directions under conditions encountered in both settings. Mutants lacking *cheV3* are moderately outcompeted in the mouse model. These mutants have an extreme bias towards frequent direction changes and migrate somewhat more quickly than wild-type in soft agar. The behaviour in mice is consistent with only moderately impaired chemotaxis in this setting, although it has not been determined how *H. pylori* mutants with a non-swim bias fare in this setting.

Vibrio cholerae tumble-bias mutants perform much better than swim-bias mutants during infection (Butler & Camilli, 2004).

In conclusion, we have shown that each *H. pylori* CheV affects swimming behaviour, as postulated by others (Jimenez-Pearson *et al.*, 2005; Pittman *et al.*, 2001; Szurmant & Ordal, 2004). We favour a model in which each CheV performs chemoreceptor–CheA kinase coupling that is regulatable by the phosphorylation state of the CheY domain of the CheV, as in *B. subtilis* (Rao *et al.*, 2008). *H. pylori* CheVs, however, can exist in either CheA-activating or CheA-deactivating states, and phosphorylation switches them toward the opposite form. Indeed, two of the *H. pylori* CheVs seem to activate CheA, while the third seems to deactivate CheA. We have noticed that the CheV proteins fall into families with insertions conserved in size, position and sequence within the *Helicobacteraceae* (Fig. 1). These sequences may have some relevance to function, as they are found close to the chemoreceptor binding pocket (data not shown).

It seems most likely that all the CheVs plus CheW exist as couplers in a presumed chemoreceptor supercomplex. Based on mutant phenotypes, CheW plays a pivotal role (Pittman *et al.*, 2001; Terry *et al.*, 2005). The phosphorylation state of each CheV may be a way for *H. pylori* to adapt, as shown with the *B. subtilis* CheV (Karatan *et al.*, 2001; Rao *et al.*, 2008). This role in *H. pylori* seems especially likely given that there seem to be no other proteins involved in adaptation in this microbe.

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