Helicobacter pylori Possesses Two CheY Response Regulators and a Histidine Kinase Sensor, CheA, Which Are Essential for Chemotaxis and Colonization of the Gastric Mucosa

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Infection of the mucous layer of the human stomach by Helicobacter pylori requires the bacterium to be motile and presumably chemotactic. Previous studies have shown that fully functional flagella are essential for motility and colonization, but the role of chemotaxis remains unclear. The two-component regulatory system CheA/CheY has been shown to play a major role in chemotaxis in other enteric bacteria. Scrutiny of the 26695 genome sequence suggests that H. pylori has two CheY response regulators: one a separate protein (CheY1) and the other (CheY2) fused to the histidine kinase sensor CheA. Defined deletion mutations were introduced into cheY1, cheY2, and cheA in H. pylori strains N6 and SS1. Video tracking revealed that the wild-type H. pylori strain moves in short runs with frequent direction changes, in contrast to movement of cheY2, cheA2Y, and cheAY2 cheY1 mutants, whose motion was more linear. The cheY1 mutant demonstrated a different motility phenotype of rapid tumbling. All mutants had impaired swimming and greatly reduced chemotactic responses to hog gastric mucin. Neither cheY1 nor cheA2Y mutants were able to colonize mice, but they generated a significant antibody response, suggesting that despite impaired chemotaxis, these mutants were able to survive in the stomach long enough to induce an immune response before being removed by gastric flow. Additionally, we demonstrated that cheY1 failed to colonize gnotobiotic piglets. This study demonstrates the importance of the roles of cheY1, cheY2, and cheA in motility and virulence of H. pylori.

Helicobacter pylori is a human-specific gastric pathogen that colonizes the stomachs of at least half the world’s population (5). H. pylori survives largely within the gastric mucous layer without attaching to host cells (5). Most infected individuals are asymptomatic; however, for a significant number, infection with H. pylori is associated with the development of duodenal and gastric ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (21). Motility is a vital adaptation for many bacterial pathogens capable of colonizing mucosal surfaces. H. pylori has been shown to be extremely motile in viscous environments, such as that encountered in the gastric lumen (15). The bacterium’s sheathed flagella are composed of two proteins, FlaA and FlaB, connected to the basal body by the flagellar hook protein, which is a polymer of FlgE (34). Expression of both FlaA and FlaB is necessary for full motility and colonization of gnotobiotic piglets (9).

Chemotaxis, the purposeful movement of bacteria to and from chemical stimulants, has been studied most extensively in Escherichia coli and Salmonella enterica serovar Typhimurium, for which a model has been proposed for this important adaptation (33). Sensing of external stimulant and repellent ligands is achieved via methyl-accepting chemoreceptor proteins (MCPs), which transverse the inner membrane, possessing both a periplasmic ligand binding domain and a cytoplasmic signaling domain (24). Communication between the MCPs and the flagellar motor switch involves four proteins: CheA, CheY, CheZ, and CheW (10). CheA and CheY constitute a two-component regulatory system, although they deviate from the archetype in several ways, most notably in that CheY neither contains a DNA binding domain nor acts as a transcriptional activator (33). The effect of binding to a ligand causes a conformational change in an MCP which is recognized by an associated CheA-CheW complex, which binds to the MCP’s cytoplasmic signaling domain via CheW (10). CheA has autokinase activity that is inhibited by attractant-bound receptors and is stimulated by repellent-bound or attractant-free receptors. Stimulation of CheA initiates phosphorylation of the response regulator CheY. The phosphorylated CheY (CheY-P) interacts directly with FliM in the flagellar motor switch complex to cause clockwise rotation (30). This response is terminated by the action of CheZ, which accelerates the decay of the unstable CheY-P (10). In E. coli, the flagellar rotary motor turns clockwise upon interaction with CheY-P, resulting in a tumbling motion; otherwise, it turns counterclockwise, resulting in smooth swimming of the bacterial cell (10).

In H. pylori, a CheY orthologue has been identified as part of a stress-responsive operon, but chemotaxis studies were not reported (4). The annotated genome sequence of H. pylori 26695 contains nine putative chemotaxis orthologues: a bifunctional CheAY protein (HP0392); CheW (HP0391); three CheV proteins, proteins previously identified in Bacillus subtilis which contain an amino-terminal CheW homologous do-
main linked to a response regulator domain of the CheY family (13) (HP0393, HP0019, and HP0616); the previously identified CheY (HP1067); (4) and three classical MCPs (HP0099, HP0082, and HP1035) (35). No CheZ orthologue was identified. Additionally, a gene (HP0599) encoding a truncated soluble MCP orthologue (with amino acid sequence similarity to the classical MCPs restricted to the highly conserved domain) has been identified and its structure has been analyzed (2). In this study, the previously identified CheY (4) is referred to as CheY1, while the CheY protein fused to CheA is termed CheY2, and the gene that encodes the bifunctional CheA2 protein is referred to as cheA2.

Mizote et al. have demonstrated a chemotactic response to urea and bicarbonate by *H. pylori* CPY3401 (26). This response is increased in a high-viscosity environment, a condition that mimics the ecological niche of *H. pylori* (28). It was proposed that intracellular urea hydrolyzed by cytoplasmic urease may supply the proton motive force required to drive the bacterial flagellar motor and that *H. pylori* chemotaxis towards urea may serve to provide urea for hydrolysis by surface urease for gastric acid neutralization (28). Mucin, the principal component of mucus which is secreted from epithelial cells of intestinal, gastric, and gall bladder tissues, has been proposed as a che-motrattractant for *H. pylori* (37). Despite these studies, very little is understood about the mechanism of the chemotactic response in *H. pylori* or the role of chemotaxis genes in motility and virulence. In this study, we describe the construction and characterization of four chemotaxis mutants, cheY1, cheY2, cheA2, and cheAY2 cheY1 in independent *H. pylori* strains (N6 and SS1), showing the importance of these genes in the motility and virulence of *H. pylori*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *H. pylori* strains were minimal passaged, aliquoted, and stored at −80°C in brain heart infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) containing 15% (vol/vol) glycerol and 10% fetal calf serum (FCS) (Sigma, Poole, United Kingdom). Strains were grown in BHI broth supplemented with 10% FCS or on Helicobacter selective agar (DENT), consisting of Blood Agar Base No. 2 (Oxoid) supplemented with 7% (vol/vol) lysed defibrinated horse blood (TCS Microbiology, Botolph Claydon, United Kingdom) and DENT selective supplement (Oxoid) in a microaerophilic atmosphere at 37°C. *E. coli* strains were routinely grown in Luria-Bertani (LB) broth or on LB agar. The antibiotics used for selection purposes were ampicillin (100 μg/ml), kanamycin (20 μg/ml for *H. pylori* and 50 μg/ml for *E. coli*), and chloramphenicol (6 μg/ml).

**DNA manipulations.** Unless otherwise stated, plasmid and chromosomal DNA extractions, restriction enzyme digestions, and DNA ligations were performed by standard procedures (29) using enzymes supplied by Promega (Southampton, United Kingdom). Transformations into *E. coli* XL2-Blue MRF+ strain (Stratagene Europe, Amsterdam, The Netherlands) were performed using the manufacturer’s protocol. All chemicals were purchased from Sigma. The oligonucleotide primers used for PCRs were purchased from Genosys Biotechnologies (Europe) Ltd. (Cambridge, United Kingdom) and are summarized in Table 2. Sequencing of cloned DNA was performed by the dideoxynucleotide chain termination method with a PRISM sequencing kit (Applied Biosystems, War-ington, United Kingdom).

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<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
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<td></td>
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</tr>
<tr>
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<td>Typed laboratory strain</td>
<td>NCTC 11637</td>
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<tr>
<td>N6 cheY1</td>
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<tr>
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<tr>
<td>pSF6-TIK</td>
<td>pSF6-TI plus Kmr</td>
<td>This study</td>
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</tbody>
</table>

*Ap', ampicillin resistant; Kmr, kanamycin resistant; Cm', chloramphenicol resistant.*

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**TABLE 1. Bacterial strains and plasmids used in this study**

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cloned into pUC19, sequenced, and used to probe a λZAP library NCTC 11638 to identify the entire cheY1 gene sequence. Specific primers SPCY1 and SPCY2 were used to amplify a PCR product containing the entire cheY1 gene, which was cloned into pUC19. The H. pylori cheA gene was identified by partial sequencing of plasmid pLILCA, which contained a putative cheA gene on a 6-kb fragment from H. pylori 11637. Specific primers SPCAI and SPCA2 were used to amplify a fragment of cheA, which was cloned into pUC19. Following the release of the H. pylori 26095 genome sequence, specific primers SPF1 and SPF2 were designed to amplify the cheY2 gene from H. pylori 11637 chromosomal DNA, which was cloned into pUC19.

Construction of defined H. pylori cheY1, cheY2, cheY3, and cheY2 cheY1 mutants. Defined deletions and unique BglII sites were introduced into the cloned cheY1, cheA, and cheY2 genes by inverse PCR mutagenesis (IPCRM) using the primer pairs shown in Table 2, as described previously (7, 40). A 1.4-kb BglII restriction fragment of plasmid pJM30, containing a gene encoding resistance to chloramphenicol, was cloned into the unique BglII sites.

The constructs were introduced into H. pylori N6 or SS1 wild-type strain either by natural transformation (14) or by electroporation (31). For the construction of a defined cheA mutant, a 0.8-kb BglII restriction fragment of plasmid pCAT, containing a gene encoding resistance to chloramphenicol, was cloned into the unique BglII site in pCAIP2, which contains the mutated cheA gene on a 6-kb fragment from H. pylori N6 or SS1 wild-type strains. H. pylori cheY2 cheY1 lcl was cloned into pUC19, sequenced, and used to probe a ZAP library NCTC 11638 with a 30-s cooling period on ice between 10 mm of liquid content discarded. The number of bacteria remaining in each capillary tube was then determined by performing viable counts. All assays were performed in triplicate on at least three separate occasions. The results were expressed as the chemotaxis ratio $R_{chem}$ [(CFU/ml in taxin capillary]/(CFU/ml in control capillary)] to normalize experimental data (27).

Colonization of H. pylori gnotobiotic piglet model. Gnotobiotic piglet experiments were carried out essentially as described by Krakowska et al. (20). Large white hybrid piglets were delivered by cesarian section performed in a sterilized isolator unit. The piglets were maintained in sterile isolator units and were fed a sterile elemental diet. Soluble HGM (Sigma) was prepared as 1, 0.5, and 0.1% solutions in chemotaxis buffer. The tips of 50-μl-volume capillary tubes (Sigma) were then filled with HGM or chemotaxis buffer (control), sealed at one end, and inserted vertically into 0.5-ml tubes containing 300 μl of resuspended motile cells. These were incubated horizontally under microaerophilic conditions for 45 min at 37°C. After incubation, the tubes were disassembled, and the lower 10 mm of liquid content discarded. The number of bacteria remaining in each capillary tube was then determined by performing viable counts. All assays were performed in triplicate on at least three separate occasions. The results were expressed as the chemotaxis ratio $R_{chem}$ [(CFU/ml in taxin capillary]/(CFU/ml in control capillary)] to normalize experimental data (27).

Colonization of H. pylori mouse model. Female outbred mice (HSD/ICR strain; Harlan Ltd., Bicester, United Kingdom) with a body weight of approximately 20 g (4 to 6 weeks old) were challenged orally on successive days with SS1, SS1 cheY1, or SS1 cheY2. Prior to challenge, all strains were pretreated with acidified 5 mM urea (pH 2) in order to boost urease activity and thus optimize colonization potential (25). Challenge inocula were 1-ml volumes of 24-h tryptose soy broth cultures containing between $10^7$ and $10^8$ CFU. At 2 and 8 weeks, mice from each group were culled by CO₂ inhalation, and the stomachs were removed and opened along the greater curvature. After washing away the stomach contents, the entire mucosal surface was spread evenly over the surface of a Columbia chocolate agar plate containing selective antibiotics (amphotericin B [50 μg/ml], vancomycin [100 μg/ml], polymyxin B [3.3 μg/ml], bacitracin [200 μg/ml], and nalidixic acid [10.7 μg/ml]) for about 10 (25) before incubating microaerobically for 7 days at 37°C. The culture plates were then evaluated for H. pylori growth. Growth of even a single colony is sufficient to record an animal as being H. pylori positive (25). After 8 weeks, the mice were exsanguinated and the individual serum samples were stored at −20°C.

Whole-cell serum ELISA assay. H. pylori SS1 cells were harvested from DENT agar plates, washed twice with phosphate-buffered saline (PBS), and lysed with three 30-s bursts of ultrasound (Ultrasonic Processor; Jencons Scientific Ltd., Leighton Buzzard, United Kingdom) with a 30-μm cooling period on ice between
each burst. The insoluble material was removed (10,000 × g for 20 min), and the soluble material was used to coat wells of an enzyme immunoassay-radioimmuno-
noprecipitation assay plate (Corning Costar, High Wycombe, United King-
dom). After 18 h at 4°C (1 standard deviation in 0.1 M NaHCO₃, pH 9.5). The antibody levels within individual serum samples were determined by end point titration, as described previously (8). Essentially, antigen-coated wells were incubated with serum samples serially diluted twofold in PBS, and bound antibody was visual-
ized by using a polyvalent anti-mouse immunoglobulin horseradish peroxidase
conjugate (Sigma) and o-phenylenediamine as a substrate. Enzyme-linked im-
munosorbent assay (ELISA) titers were determined as the reciprocal of the
highest serum dilution that yielded an OD₄₉₀ value of 0.5 U above the back-
ground. Titer values of 10-6 or higher were considered positive. Student’s t tests were used to compare the data groups. Prob-
abilities of P < 0.05 were taken as significant. Statistical analysis was carried out with the InStat statistical package (Sigma).

RESULTS

Cloning of chemotaxis genes. PCR with degenerate primers was used to amplify a cheY1 gene fragment from H. pylori 11637 chromosomal DNA (39). The PCR product was cloned into pUC19 and sequenced. To isolate the entire cheY1 gene, a λZAP library NCTC 11638 was screened by radioactive colony
blot hybridization using the isolated gene fragment as a probe. A positive clone was identified and partially sequenced. Specific primers were designed to amplify a 941-bp fragment containing the entire cheY1 plus some flanking DNA, which was cloned into pUC19 and termed pCY110. Searches using
BLASTX software (3) revealed that the cloned fragment had a significant identity to CheY from several bacteria. Library screening and subsequent sequencing revealed that the com-
plete CheY1 codes for a protein of 124 amino acids with the highest homology (82% identity) to Campylobacter jejuni CheY (41). The four residues found in all CheY proteins to date (Asp12, Asp13, Asp56, and Lys109) are all conserved in H. pylori CheY1 (30). Asp56 is the site of phosphorylation by CheA. Amino acids 90 to 112 are also highly conserved, an
area of predicted interaction between CheY-P and FliM in E. coli (38). The EMBL accession number for the nucleotide sequence of H. pylori cheY1 is X81897.

Partial sequencing of pILLCA and subsequent searches re-
vealed that there was extensive homology to E. coli CheA. Specific primers were designed to amplify a 707-bp fragment from the putative cheA gene, which was cloned into pUC19 and termed pCA110. The highest level of homology (50% identity) was with Thermotoga maritima CheA. Comparison of the H. pylori 11637 cheA gene fragment with H. pylori 26695 cheA (HP0392) showed that the CheA proteins from the two strains were almost identical in the region 309 to 585 (35). Analysis of the whole CheA amino acid sequence from 26695 revealed a high level of
homology to CheA histidine protein kinases from a range of
enteric bacteria. The highest level of homology (47% identity) was with Pseudomonas putida CheA. The CheA active domains are conserved, including the area surrounding His48, the site of autophosphorylation, and the four blocks of residues in-
volved in kinase function found at the carboxyl end, which suggests that H. pylori CheA is functional as a transmitter. The main area of divergence between H. pylori CheA and E. coli CheA was between amino acids 109 and 250 (E. coli numbering). This region contains the P2 domain (124 to 257), which has CheY binding capability (33).

The cheY2 portion of cheAY2 was cloned with sequence informa-
tion from the annotated H. pylori 26695 genome (35). The four conserved CheY residues are also conserved in H. pylori CheY2 (30). BLASTX searches revealed that the highest homology (40% identity) was to the N terminus of the bifunc-
tional CheAY proteins from Streptomyces coelicolor and P. aeruginosa.

Construction of defined H. pylori cheY1, cheA2, cheY2, and
cheAY2 cheY1 mutants. Defined deletions were introduced into the H. pylori cheY1, cheA, and cheY2 cloned gene fragments by IPCRM, followed by the insertion of a kanamycin or chloram-
phenicol resistance cassette (Fig. 1) (7, 40). Mutation of the
cheA section of the cheAY2 gene results in a cheAY2 pheno-
type, as the cheY2 section is downstream of the cheA section. H. pylori cheY1, cheAY2, cheY2, and cheAY2 cheY1 mutants were constructed by allelic replacement, as described previ-
ously (12, 17). PCR using specific primer pairs and Southern
hybridization analysis confirmed that double recombination events had occurred (data not shown).

Motility assays. Analysis of the swarm plates showed that all the H. pylori N6 and SS1 chemotaxis mutants had reduced swarming ability compared to the abilities of the respective wild-type strains. The wild-type strain formed concentric rings that increased with the period of incubation. In contrast, the mutants formed irregular growth patterns of high density lim-
ited to the area of inoculation (data not shown).

Computerized tracking showed that the H. pylori N6 wild-
type strain moved with a speed of up to 20 μm/s, consistent with results reported by Karim et al. on several clinical isolates (18). In comparison, three of the deletion mutants (N6 cheY2, N6 cheA2Y, and N6 cheAY2 cheY1) had significantly higher
CLVs and SLVs than those observed for the wild-type strain. However, N6 cheY1 had lower CLVs and SLVs than those observed for N6 (Fig. 2a and b). The TL%es indicate that the
mutant strains N6 cheY2, N6 cheAY2, and N6 cheAY2 cheY1 are significantly straigher swimming than the wild type, N6 (Fig. 2c). The linearity of these mutant strains suggests that
both CheY2 and CheA contribute to tumbling motion. In con-
trast, the linearity of the N6 cheY1 mutant was less than that
observed for the wild-type strain. Analysis of the trail draw
diagrams showed that the wild-type H. pylori N6 strain moved in a random darting fashion, with frequent changes in direction and short straight runs. The mutants N6 cheY2, N6 cheAY2, and N6 cheAY2 cheY1 all moved in long straight runs or very wide circles with no sharp turns or changes in direction. In contrast, N6 cheY1 tumbled excessively, rarely moving out of the field of vision (Fig. 3a to c).

Capillary tube assays. Adler’s capillary assay (1), a standard for quantitative assessment of chemotactic proficiency in en-
teric bacteria, was carried out to study in greater detail the
phenotype of the chemotaxis deletion mutants. It has been shown that this method is applicable to H. pylori (26, 37). H. pylori N6 showed significant chemotaxis to 0.1, 0.5, and 1%
(wt/vol) HGM (Table 3). By contrast, the N6 cheY1 mutant failed to show significant taxis to 0.1% HGM, but at concentrations of 0.5 and 1% HGM, chemotactic responses representing, respectively, 90 and 86% reductions in chemotaxis were observed (Table 3). The N6 cheAY2 mutant showed a significant chemotactic response only to 1% HGM (Table 3), representing an 82% reduction in chemotaxis. No response was observed with either N6 cheY2 or N6 cheAY2 cheY1, except at the highest concentration of HGM (Table 3). However, the ratios observed were too close to the minimum $R_{che}$ value of 2 to be considered significant (Table 3).

Colonization of $H$. pylori in mice and anti-$H$. pylori serum responses. Three groups of 20 mice were infected with 1-ml volumes of overnight cultures of SS1 ($1.05 \times 10^8$, $6.4 \times 10^7$), SS1 cheY1 ($8.9 \times 10^7$, $7.0 \times 10^7$), or SS1 cheAY2 ($1.1 \times 10^8$, $7.9 \times 10^7$) (the number of viable bacteria administered to each mouse on successive days is in parentheses). All mice inoculated with SS1 were colonized at 2 and 8 weeks postinfection. Using the scoring system for colonization described previously, the 2-week time point showed 58% colonization, and the 8-week time point showed 94% colonization (25). This is in-
TABLE 3. Response of H. pylori N6 and chemotaxis mutant strains to HGM analyzed by capillary assays

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chemotactic responses to:</th>
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<tbody>
<tr>
<td></td>
<td>0.1% HGM</td>
</tr>
<tr>
<td>N6</td>
<td>0.84 ± 9.33</td>
</tr>
<tr>
<td>N6 cheY1</td>
<td>NR</td>
</tr>
<tr>
<td>N6 cheY2</td>
<td>0.20 ± 0.65</td>
</tr>
<tr>
<td>Ne cheAY2</td>
<td>NR</td>
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<tr>
<td>Ne cheAY2 cheY1</td>
<td>NR</td>
</tr>
</tbody>
</table>

Each data set represents a minimum of three experiments performed in triplicate. Results are expressed as the ratio of number of bacteria in attractant capillaries to that in the control capillary (Rvm). NR, no response.

dicative of growth in vivo. However, mice inoculated with SS1 cheY1 or SS1 cheAY2 showed no colonization at either 2 or 8 weeks postinfection.

Serum harvested from individual mice 8 weeks after oral inoculation with SS1, SS1 cheY1, and SS1 cheAY2 was analyzed for the presence of anti-H. pylori antibodies by ELISA. All strains tested generated an antibody response that was significantly (P < 0.05) higher than the titer seen in the control mice (Fig. 4). Six out of the 10 mice inoculated with SS1 cheY1 generated a significant anti-H. pylori serum response (Fig. 4). This response was not significantly different (P > 0.05) than that observed for the mice challenged with the SS1 wild-type strain, of which 9 out of the 10 mice challenged generated significant anti-H. pylori serum responses. Four of the mice challenged with SS1 cheAY2 positively seroconverted. This response was significantly lower (P < 0.05) than that observed for the SS1 and SS1 cheY1 strains.

**DISCUSSION**

H. pylori cells reside mainly in the mucous layer of the stomach or in the intestine in association with areas of gastric metaplasia. The ability to direct bacterial movement against the gastric flow towards the epithelial cell surface via chemotaxis is likely to be important in the colonization process. To determine the roles of the chemotaxis orthologues identified in the H. pylori 26695 genome sequence in motility and pathogenesis, defined cheY1, cheY2, and cheAY2 mutants were constructed in two independent strains (N6 and SS1) by using IPCRM and allelic replacement (12, 17). An H. pylori N6 cheAY2 cheY1 double mutant was also constructed.

Computerized tracking showed that H. pylori N6 moved with a speed of up to 20 μm/s, consistent with that reported by Karim et al. on several clinical isolates (18). The swimming pattern consisted of random darting movements, with frequent changes in direction and short runs. N6 cheY2, N6 cheAY2, and N6 cheAY2 cheY1 all moved in long straight runs or wide circles with no sharp turns or changes in direction. This suggests that, as in E. coli, CheY2 phosphorylated by CheA interacts with the flagellar motor switch, resulting in tumbling of H. pylori cells. This proposed system is further supported by the presence of a soluble MCP-like orthologue (HP0599) in H. pylori, which would allow the formation in the cytoplasm of a complex between CheW-CheAY2 and the truncated soluble MCP orthologue, thus allowing communication with the polar-located flagella. N6 cheY1 exhibited a tumbling phenotype closer to that of the wild type with respect to the frequency of directional changes. Swarming was not observed for the N6 cheY1 strain; therefore, the tumbling phenotype is unlikely to be due to suppression mutations in cheY1.

The recent sequencing of the C. jejuni 11168 genome has identified a similar configuration with a separate CheY and bifunctional CheAY orthologues (The Sanger Centre Campylobacter jejuni genome project [http://www.sanger.ac.uk/Project/C_jejuni/]). The H. pylori and C. jejuni CheY1 and CheY2 proteins show high sequence similarities (82 and 65%, respectively), suggesting that both proteins were derived from a common ancestral protein. The conservation of two divergent proteins suggests that these proteins have evolved vital functions. It is possible that both proteins are phosphorylated by CheA, which would explain the divergence in the P2 region between H. pylori and E. coli CheA proteins. CheY1-P and CheY2-P could then interact with a different site on the flagellar motor switch complex. Alternatively, CheY1 may act as a phosphate sink, accelerating the dephosphorylation of CheY2-P, thereby helping to terminate the clockwise tumbling response. This is consistent with the absence of an H. pylori CheZ orthologue, as CheZ accelerates the dephosphorylation of CheY-P in other bacteria (10), and also with the tumbling phenotype of N6 cheY1, which is similar to the phenotype observed for an E. coli cheZ mutant (16).

The small but significant chemotactic response observed for N6 cheAY2 reveals the importance of a functional CheY1 for the full chemotactic response of H. pylori N6 to HGM. This response in the absence of a functional CheA suggests that the CheY domains of the CheV orthologues may be phosphorylated by an alternative pathway in response to high levels of mucin. Alternatively, the CheY domains may be directly phosphorylated by small molecules linked to metabolism. In other bacterial systems, there is evidence that chemotaxis to dominant chemoattractants requires the transport into the cell and partial metabolism of these chemoattractants. In H. pylori, mucin may need to be transported into the cell in order to be recognized by the soluble MCP-like orthologue HP0599. This is consistent with the study of Nakamura et al. (28), which demonstrated that cytoplasmic urease activity was more important than external urease activity in chemotaxis. These results demonstrate that mucin is a chemoattractant for H. pylori N6 and that the chemotaxis components CheY1 and CheAY2 are involved in motility towards the mucus in the stomach.
N6 and SS1 cheY1 mutants were unable to colonize either gnotobiotic piglets or mice, respectively. Mutation of cheAY2 in H. pylori SS1 also prevented colonization of mice. Similar observations have been reported for a C. jejuni cheY (cheY1) mutant, which had a reduced ability to colonize mice and to cause disease in ferrets (41). The chemotaxis mutants of H. pylori N6 are motile, but their swimming behavior is altered; N6 cheY1 exhibits increased tumbling, whereas N6 cheY2 swims in straight lines. In addition, their chemotactic response to mucin was significantly reduced compared with that of the N6 wild-type strain.

Mutations in the chemotaxis system would appear to affect the ability of H. pylori to move in a controlled fashion towards the gastric mucous layer in the stomach. However, serology responses observed 8 weeks after infection with SS1 cheY1 were not significantly different from the responses of mice to the wild-type strain. Significant responses were also observed in mice immunized with SS1 cheY2. These findings suggest that chemotaxis is unnecessary for viability in vivo. It may be that significant numbers of chemotaxis-impaired bacteria remain in the mouse stomach for several days, but because they cannot maintain their position or penetrate the gastric mucosa, they are eventually washed out of the stomach with the gastric flow. Studies on C. jejuni have led to the proposal that active motility combined with chemotaxis should be regarded as a potential alternative to specific attachment (22). H. pylori adherence to epithelial cells is thought to produce attachment/effacement similar to that seen in the enteropathogenic E. coli EPEC strains (32). However, it has been proposed that only a small proportion of H. pylori cells, between 1 and 5%, attach to the epithelial surface (19). Full motility is essential for H. pylori cells to colonize the gastric mucosa (6, 9). A fully functional chemotaxis system must be required for colonization by maintaining H. pylori in the mucous layer close to the epithelial cell surface, thus reducing removal of bacteria from the stomach by gastric flow.

In this study we have demonstrated that CheY1 and CheA2 are necessary for flagellum-regulated movement and chemotaxis towards mucin. Additionally, the importance of chemotaxis in the pathogenicity of H. pylori has been demonstrated in two animal models. Chemotaxis in H. pylori appears to be distinct from the Synechocystis serovar Typhimurium and E. coli paradigm. The results of this study provide the framework for the full elucidation of the complex chemotaxis system of H. pylori.

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REFERENCES

23. Lee, A., J. L. O’Rourke, M. C. DeUngria, B. Robertson, G. Daskalopoulos, and M. F. Dixon. 1997. A standardized mouse model of Helicobacter pylori infection: cloning the Synechocystis serovar Typhimurium and E. coli paradigm. The results of this study provide the framework for the full elucidation of the complex chemotaxis system of H. pylori.

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