

Two Predicted Chemoreceptors of *Helicobacter pylori* Promote Stomach Infection

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***Helicobacter pylori* must be motile or display chemotaxis to be able to fully infect mammals, but it is not known how this chemotaxis is directed. We disrupted two genes encoding predicted chemoreceptors, *tlpA* and *tlpC*. *H. pylori* mutants lacking either of these genes are fully motile and chemotactic in vitro and are as able as the wild type to infect mice when they are the sole infecting strains. In contrast, when mice are coinfecting with the *H. pylori* SS1 *tlpA* or *tlpC* mutant and the wild type, we find more wild type than mutant after 2 weeks of colonization. Neither strain has an in vitro growth defect. These results suggest that the *tlpA*- and *tlpC*-encoded proteins assist colonization of the stomach environment.**

Many bacterial pathogens have the ability to move via flagella, but little is known about how microbes use this ability inside animal hosts. A bacterium presumably benefits if it regulates its flagellar activity so as to direct its motility, a process called chemotaxis. One pathogen that requires flagella and chemotaxis is the ulcer-causing bacterium *Helicobacter pylori* (11, 14).

Infection by *H. pylori* causes chronic gastritis and gastric and duodenal ulcers and can lead to the development of adenocarcinoma of the distal stomach and gastric mucosa-associated lymphoid tissue lymphoma (8, 24, 30). This bacterium infects approximately 50% of the world's human population (5), but only a subset of those infected develop disease.

Previous studies have identified a number of *H. pylori* factors that contribute to animal infection, including flagellum-directed motility (8, 10, 28). *H. pylori* carries five sheathed flagella located at one pole. Mutational loss of any of the flagellar filament components or of the ability to move renders *H. pylori* motility deficient and less able to infect a variety of animal models (11, 15, 18, 25). Many flagellated bacteria perform chemotactic motility by coupling the recognition of environmental conditions, such as amino acids or pH, with regulation of swimming behavior, a process that has been studied extensively in *Escherichia coli* (reviewed in references 2 and 13). In *E. coli*, environmental cues are sensed first by transmembrane chemoreceptor proteins that regulate a kinase cascade composed of the CheA kinase, the CheW coupling protein, and the flagellar-motor regulator CheY. The output of these events alters the direction of flagellar rotation to allow the bacteria to respond to environmental conditions.

H. pylori contains orthologues of several *E. coli* chemotaxis proteins, including CheW, CheY, and a CheA-CheY hybrid (1, 29), suggesting that this portion of the chemotactic signaling pathway is similar in these two bacteria. In support of this,

elimination of the *cheY*, *cheA*, or *cheW* gene in *H. pylori* yields bacteria that have a reduced chemotactic response in vitro (14, 27). Furthermore, the *cheA* or *cheY* mutants are unable to infect mouse stomachs (14). Although chemotaxis is needed for mouse stomach colonization by *H. pylori*, neither its in vivo targets nor the chemoreceptors that direct it are known. Scrutiny of the *H. pylori* genomes identified four genes predicted to code for putative chemoreceptors (1, 29). These chemoreceptors were identified on the basis of the presence of a conserved domain in the chemoreceptor that interacts with CheW (1, 29). To begin to understand how *H. pylori* chemotaxis is controlled in vivo, we constructed mutations that eliminated two of these potential chemoreceptors, TlpA and TlpC, and analyzed their behavior.

Construction of *tlpA* and *tlpC* mutants in *H. pylori* strains SS1 and G27. For this study, the human-isolated type I *H. pylori* strains G27, SPM342f, and SS1 were used (Table 1). *H. pylori* was grown as previously described on Columbia blood agar plates supplemented with *H. pylori*-selective antibiotics and 5% defibrinated horse blood (CHBA) under microaerobic conditions (25). Liquid culture was done in brucella broth containing 10% fetal bovine serum (BB10) in anaerobic jars with Campy Paks (Oxoid) and shaking at 37°C. Chloramphenicol was used at concentrations of 5 to 10 µg/ml (*H. pylori*) or 20 µg/ml (*E. coli*), while kanamycin was used at 15 µg/ml (*H. pylori*) or 30 µg/ml (*E. coli*). Long-term storage of *H. pylori* was as previously described (25). DNA was amplified by using *Pfu*-Turbo polymerases (Stratagene) or *Taq* polymerase (generous gift of D. Kellogg). All DNA sequencing was performed by the sequencing facility at the University of California at Berkeley.

tlpA, *tlpC*, and *cheY* were cloned from *H. pylori* genomic DNA using PCR. Primers to amplify each gene were designed on the basis of the sequence of *H. pylori* strain 26695 (Table 2) (29). *tlpA* (HP0099 from the *H. pylori* 26695 genome [29]) was amplified using primers *tlpA*1 and *tlpA*3, *H. pylori* strain SPM342f genomic DNA, and *Pfu* polymerase. The resultant 2.3-kb PCR product was ligated with pCR2.1-topo (Invitrogen) to create pKO102. An *Eco*RI-containing *tlpA* fragment was subcloned into pBluescript KS to create pKO112. *tlpC* (HP0082)

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Antibiotic resistance	Source or reference
<i>E. coli</i> DH10B	Cloning strain		Lab stock
<i>H. pylori</i>			
G27	Wild type, type I		N. Salama (6)
SS1	Wild type, type I		J. O'Rourke (19)
SPM342F	Wild type, type I		N. Salama (21)
TAG101	G27 $\Delta tlpA::cat-1$	Cm ^r	This study
TAG102	G27 $\Delta tlpA::cat-2$	Cm ^r	This study
TAS101	SS1 $\Delta tlpA::cat-1$	Cm ^r	This study
TCG568	G27 $\Delta tlpC::cat-1$	Cm ^r	This study
TCS565	SS1 $\Delta tlpC::cat-1$	Cm ^r	This study
CYG487	G27 $\Delta cheY::aphA3-1$	Km ^r	This study
CYS481	SS1 $\Delta cheY::aphA3-1$	Km ^r	This study
Plasmids			
pBluescript	Cloning vector	Ap ^r	Stratagene
pBS-cat	pBluescript::cat (from <i>Campylobacter coli</i>)	Ap ^r Cm ^r	N. Salama
pBS-kan	pBluescript::aphA3 (from <i>C. coli</i>)	Ap ^r Km ^r	N. Salama
pKO112	pBluescript with <i>tlpA</i> (SPM342F)	Ap ^r	This study
pTA101	pKO112 $\Delta tlpA::cat-1$	Ap ^r Cm ^r	This study
pTA102	pKO112 $\Delta tlpA::cat-2$	Ap ^r Cm ^r	This study
pTC100	pBluescript with <i>tlpC</i> (SS1)	Ap ^r	This study
pTC200	pTC100 $\Delta tlpC::cat-1$	Ap ^r Cm ^r	This study
pKO126	pBluescript with <i>cheY</i> (SS1)	Ap ^r	This study
pKO126K	pKO126 <i>cheY::aphA3-1</i>	Ap ^r Km ^r	This study

was amplified from strain SS1 using *Pfu*-Turbo polymerase and oligonucleotides *tlpC4* and *tlpC5* to obtain a 2.8-kb PCR product. This PCR product was ligated into *EcoRV*-cut pBluescript KS to create pTC100. *cheY* (HP1067) was cloned using SS1 genomic DNA and primers *cheY6* and *cheY7*. This reaction yielded a 2.6-kb fragment that was cloned to create pKO126, using an approach similar to that used for *tlpC*. We used restriction enzyme analysis, DNA sequencing, and database comparison to confirm the construction of each plasmid.

Next, a portion of each gene was deleted, using inverse PCR. For *tlpA* in pKO112, we used primers *tlpA4* and *tlpA5* to delete 1,374 bp (encoding amino acids 29 to 487). For *tlpC*, we used primers pTC2-1 and pTC2-2 and pTC100 DNA to remove 1,005 bp (encoding amino acids 175 to 510). After the deletion mutants were created, the *cat* gene was excised from pBS-cat by digestion with either *HincII* alone or *KpnI* and *SacI* with additional blunting. This *cat* fragment was ligated with the *tlpA* inverse PCR product to create two plasmids, pTA101 and pTA102, that contained both orientations of *cat* within the deleted *tlpA* gene (Table 1). Similarly, the *tlpC* inverse PCR

product was ligated with the *cat* gene to create pTC200. A *cheY* insertion mutant was created by digesting pKO126 with *Bpu1102I*, which cuts at the DNA sequence corresponding to amino acid 46. The *aphA3* gene was prepared by restriction digestion of pBS-kan with *XhoI* and *EagI*, and blunt ends were created with T4 polymerase. These two DNA pieces were ligated together to create pKO126K.

pTA101, pTA102, pTC200, and pKO126K were used individually to transform *H. pylori* G27 and SS1 to chloramphenicol or kanamycin resistance using natural transformation as previously described (9, 28). PCR and Southern blotting verified the correct nature of all these mutations and the insertion of only one *cat* or *aphA3* gene (data not shown).

Analysis of in vitro and mouse-infecting characteristics of *H. pylori* mutants lacking TlpA or TlpC. We used phase-contrast microscopy of wet mounts (grown in CHBA for 6 to 9 h) of G27 and SS1 $\Delta tlpA::cat$ or $\Delta tlpC::cat$ mutants suspended in BB10 to analyze motility and found no observable defect of these mutants compared to their wild-type parents. Swim rates in soft agar were comparable between the wild-type and *tlpA* or *tlpC* mutant *H. pylori*, suggesting that the mutants retained wild-type chemotaxis (Table 3). In contrast, a mutant lacking chemotaxis entirely by virtue of loss of *cheY* was motile but did not migrate in soft agar, as shown previously (3, 14).

To determine whether TlpA or TlpC is required to colonize animal stomachs, we infected 4- to 6-week-old male FVB/N mice with SS1 $\Delta tlpA::cat$ or $\Delta tlpC::cat$ mutants as described previously (25) and compared levels of colonization to that of the wild-type strain. For these experiments, we used two separate isolates of the *H. pylori* SS1 *tlpA* mutants and one SS1 *tlpC* mutant. After 2 weeks of infection, the mice were sacrificed, and the numbers of *H. pylori* in their stomachs were determined by plating the homogenized stomach onto CHBA plates supplemented with nalidixic acid and bacitracin as de-

TABLE 2. PCR primers used in this study

Primer	Sequence
Colicat1.....	5'-GTATAGTCTGCTGTAACCTCAGTCC-3'
tlpA1.....	5'-TGAAAGATCTGCCTTTTGGGGCGTTG-3'
tlpA3.....	5'-CCGCAAGCTTGAACCTGCTTTTATTTCACATC-3'
tlpA4.....	5'-GCTGTTTAAGGACACCCC-3'
tlpA5.....	5'-TTAGCGAACGATAGCGCG-3'
tlpC-4.....	5'-ACCCCAACTAAGTCCCTTAAG-3'
tlpC-5.....	5'-CAGAGCTTGAATCAATGTTGGG-3'
pTC2-1.....	5'-CAAGAAAGGAGTCTGAAAAC-3'
pTC2-2.....	5'-TTGATTCTTACCCATTAGGGG-3'
cheY6.....	5'-ATCGCACAAAGATAGAAACGG-3'
cheY7.....	5'-GCTGTTCTAAACCTCAAATCCATT-3'

TABLE 3. In vitro motility of *H. pylori* *tlpA*, *tlpC*, and *cheY* mutants^a

Strain	Swim rate (mm/day)
SS1	4.5 ± 0.3
SS1 $\Delta tlpA::cat-2$	4.1 ± 0.8
SS1 $\Delta tlpC::cat-1$	4.1 ± 0.1
SS1 <i>cheY::aphA3-1</i>	0.4 ± 0.1
G27	5.2 ± 0.3
G27 $\Delta tlpA::cat-1$	5.1 ± 0.9
G27 $\Delta tlpA::cat-2$	5.0 ± 0.7
G27 $\Delta tlpC::cat-1$	4.7 ± 0.4
G27 <i>cheY::aphA3-1</i>	0.75 ± 0.15

^a Colonial expansion (swim) rates were determined by measuring the colony diameter each day for 7 days of cells grown in brucella broth, 2.5 to 5% fetal bovine serum, and 0.35% agar.

scribed previously (25). Infection with wild-type SS1 resulted in mice carrying an average of 5.85×10^6 CFU/g of stomach (Fig. 1). Infection with either the *tlpA* or *tlpC* mutant resulted in similar levels of *H. pylori* in mouse stomachs (Fig. 1). Statistical analysis using the paired *t* test (available at <http://www.graphpad.com/calculators/ttest1.cfm>) revealed no significant difference ($P > 0.05$) in the colonization abilities of the wild type and the $\Delta tlpA::cat$ or $\Delta tlpC::cat$ mutant.

***tlpA* and *tlpC* mutants are defective for mouse colonization when they are coinfecting with wild type.** We examined mice infected with a mixture of approximately equal amounts of

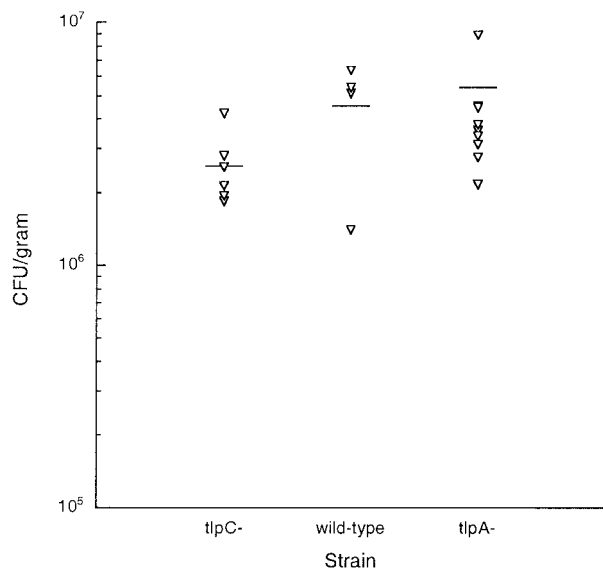


FIG. 1. The numbers of *H. pylori* recovered from mice infected with mutants lacking *tlpA* or *tlpC* do not differ from those from mice infected with the wild type. Each point represents the bacterial numbers per gram of stomach in mice infected for 2 weeks with SS1 $\Delta tlpC::cat-1$ mutant (left), wild-type SS1 (middle), or SS1 $\Delta tlpA::cat-1$ mutant (right). Mice infected by wild-type SS1 were given 1×10^8 CFU ($n = 4$), mice receiving SS1 $\Delta tlpA::cat$ ($n = 9$) were inoculated with 4×10^7 to 13×10^7 CFU, and mice receiving SS1 $\Delta tlpC::cat$ ($n = 6$) were given 1×10^7 to 6×10^7 CFU. Two different isolates of $\Delta tlpA::cat-1$ were used but their colonization levels did not differ, so their data are shown together in one column. The short horizontal lines represent the geometric mean CFUs per gram. The levels of colonization do not differ significantly from each other ($P > 0.05$).

TABLE 4. In vitro competition of wild-type SS1 with its *tlpA*, *tlpC*, or *cheY* mutants^a

Mutant	Mutant/wild-type strain ratio after growth for the following time:						
	0 h	12 h	21–24 h	31–32 h	36 h	40 h	48 h
<i>tlpA</i>	0.8	– ^b	1.05	1.0	–	–	1.1
<i>tlpC</i>	0.91	0.47	0.47	–	0.45	–	0.47
<i>cheY</i>	0.5	–	0.39	–	–	0.41	–

^a Bacteria were mixed together and grown in BB10 as described in the text. At the time points indicated, a sample was withdrawn and plated onto CHBA and CHBA supplemented with chloramphenicol or kanamycin. Data are representative of two separate experiments.

^b –, not determined.

wild-type SS1 and either SS1 $\Delta tlpA::cat$ or SS1 $\Delta tlpC::cat$ mutant, because previous studies had shown that some *H. pylori* mutants appear to have wild-type colonization ability when they are the sole infecting strain but have a colonization defect when coinfecting with the wild type (28). After 2 weeks, we collected the stomachs and determined the amounts of each of the two strains that remained. The wild-type strain was better able to colonize the mouse stomach than either the *tlpA* or *tlpC* mutant strain (Fig. 2). After dividing by the input ratio, there was 52-fold more wild type than the *tlpA* mutant and 25-fold more than the *tlpC* mutant. In contrast, when wild-type SS1 and SS1 $\Delta tlpA::cat$ or SS1 $\Delta tlpC::cat$ were grown together in vitro, the ratio of wild type to mutant did not change over 48 h (Table 4). Consistent with this, loss of *tlpA* or *tlpC* did not change the growth rate of either SS1 or G27 $\Delta tlpA::cat$ or $\Delta tlpC::cat$ compared to their wild-type parents when grown singly (data not shown). These results suggest that both TlpA and TlpC confer an advantage in vivo in a competitive setting but are not needed in vitro for growth, motility, or chemotaxis.

Because loss of either the *tlpA*- or *tlpC*-encoded chemoreceptors resulted in *H. pylori* that were not completely outcompeted by their wild-type parent, we competed a totally nonchemotactic mutant (*cheY* mutant) with the wild type for comparison. After coinfection of this mutant with wild-type SS1, we found that there were no *cheY* mutants after 13 days (Fig. 2). Similar to the results with the *tlpA* or *tlpC* mutants, when this *cheY* mutant was grown in vitro together with wild-type SS1, the ratio of the two strains did not appreciably change (Table 4), nor was the growth rate of either G27 or SS1 *cheY::aphA3* mutant different from those of their parents (data not shown). These findings are consistent with the notion that loss of *cheY* does not affect in vitro growth, but its function (chemotaxis) is necessary in vivo.

Conclusions. We have created and characterized *H. pylori* mutants that lack the *tlpA*- or *tlpC*-encoded gene products which are predicted to encode chemoreceptors. We found that bacteria lacking either of these proteins were less fit than their wild-type parents for mouse stomach colonization but have no in vitro growth or chemotaxis defects. Because chemotactic motility has been shown to be required for full stomach colonization (14), an interpretation of these findings is that *tlpA* and *tlpC* encode chemoreceptors that direct the chemotactic response of the bacterium upon sensing mouse stomach conditions.

Chemoreceptor-encoding genes are usually identified on the basis of a highly conserved domain (HCD) of the encoded

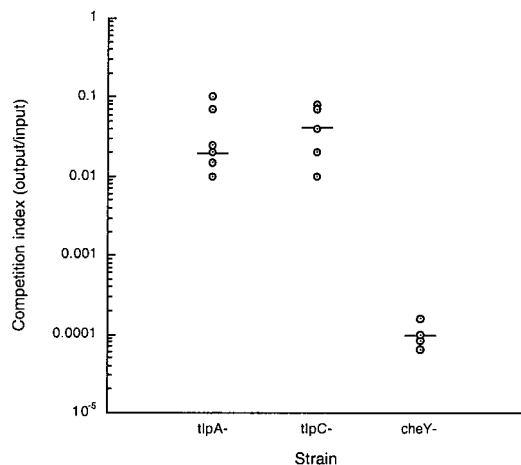


FIG. 2. Number of bacteria in the stomachs of mice infected with mixtures of wild-type and mutant *H. pylori* after 2 weeks. Each point represents one mouse. The competitive index (CI) is the mutant/wild-type ratio for output divided by the same ratio for input. The short horizontal lines represent the geometric means. The number of mice (n) used follows: for $\Delta tlpA::cat$ mutant (left), $n = 10$; for $\Delta tlpC::cat$ mutant (middle), $n = 8$; and for $cheY::aphA3$ mutant (right), $n = 4$. We were unable to detect any *cheY* mutants in the output and used 250 CFU/g (the estimated detection limit) for these calculations. Statistical analysis using the Wilcoxon matched-pair signed-rank test (available at http://www.fon.hum.uva.nl/Service/Statistics/Signed_Rank_Test.html) showed that all strains are significantly different from a strain with no competition defect (CI = 1) ($P < 0.01$). On the basis of observations with other mutants and wild-type strains, two strains with equal abilities yield a CI of 1 (data not shown).

protein sequence, a portion that interacts with CheW (1, 29). Although the HCD allows identification of putative chemoreceptors, it does not indicate to what those chemoreceptors respond, because sensing of environmental cues occurs outside the HCD, commonly in the extracellular or periplasmic domains. The periplasmic portions of both TlpA and TlpC are not homologous to any proteins with known functions but are homologous to each other (data not shown). This homology suggests they sense similar compounds or contain similar structures, although this remains to be determined. Furthermore, this similarity may suggest redundancy and may explain why loss of either one of these proteins has only a minor colonization defect: the remaining (TlpA or TlpC) protein can substitute to some degree.

Other groups have found that *H. pylori* moves in vitro towards several chemicals and conditions, including urea, several urea analogs including fluorofamide and hydroxyurea, sodium and potassium bicarbonate, sodium chloride, glutamate, and methionine (22). In addition, strains with functional urease respond positively to viscosity (23). Although several of these chemicals are found in vivo, their relevance for *H. pylori* chemotaxis in vivo has not been shown. In addition, it remains to be determined if TlpA or TlpC participate in sensing these chemicals. We have been unable to reproduce the capillary assay as reported by Mizote and colleagues (22) and so have not analyzed our mutants in this fashion.

Mutation of either *tlpC* or *tlpA* resulted in *H. pylori* that behaved as their wild-type parent with regard to chemotaxis in vitro. This is not surprising, given that loss of any one *E. coli*

chemoreceptor does not change its behavior in rich media such as used here. Furthermore, although both TlpA and TlpC contain a chemoreceptor signature, the HCD, it remains to be proven that these proteins function as chemoreceptors. While the vast majority of HCD-containing proteins are chemoreceptors, at least two appear to regulate gene expression (4, 7; J. Kirby and D. Zusman, unpublished data). It seems likely that the TlpA and TlpC proteins participate in chemotaxis, but this remains to be shown.

Most characterized HCD-containing proteins (referred to as chemoreceptors) participate in directed motility, and some of these proteins from pathogenic bacteria have been shown to participate in colonization or virulence. For example, the legume-nodulating bacterium *Rhizobium leguminosarum* contains several chemoreceptors, two of which (McpB and McpC) appear to be important for plant colonization (31). Mutants lacking either of these proteins had a 10-fold plant colonization defect only when coinoculated with the wild type. The causative agent of cholera, *Vibrio cholerae*, is predicted to contain 43 HCD-containing proteins (17), some of which have been implicated in virulence using a variety of approaches. Peterson and Mekalanos (26) identified *acfB*, a gene that encodes a chemoreceptor. This gene product is coordinately expressed with known *V. cholerae* virulence factors and is required for full colonization (12, 26). Using a different approach, Lee and coworkers looked for genes that affected cholera toxin expression in the mouse intestine and found that several of these genes encoded proteins involved in chemotaxis, including one gene that coded for a HCD-containing protein, McpX (20). Loss of this gene created bacteria that outcompeted their parents, a phenotype that is common to *V. cholerae* chemotaxis mutants (16, 20). Taken together, these studies are consistent with the notion that chemoreceptors are important for infection in *V. cholerae*, *R. leguminosarum*, and as we show here, for *H. pylori*.

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