tlpA gene expression is required for arginine and bicarbonate chemotaxis in Helicobacter pylori

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ABSTRACT

About half of the human population is infected with Helicobacter pylori, a bacterium causing gastritis, peptic ulcer and progression to gastric cancer. Chemotaxis and flagellar motility are required for colonization and persistence of H. pylori in the gastric mucus layer. It is not completely clear which chemical gradients are used by H. pylori to maintain its position. TlpA, a chemotaxis receptor for arginine/bicarbonate, has been identified. This study aimed to find out whether tlpA gene expression is required for the chemotactic response to arginine/bicarbonate. Wild-type motile H. pylori ATCC 700392 and H. pylori ATCC 43504, a strain having an interrupted tlpA gene, were used. Also, a tlpA-knockout mutant of H. pylori 700392 (H. pylori 700392-tlpA::cat) was produced by homologous recombination. Expression of tlpA was assessed by a Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assay. Chemotaxis was measured as a Relative Chemotaxis Response (RCR) by a modified capillary assay. H. pylori 700392 presented chemotaxis to arginine and sodium bicarbonate. H. pylori 700392-tlpA::cat showed neither tlpA gene expression nor chemotaxis towards arginine and bicarbonate. Besides confirming that TlpA is a chemotactic receptor for arginine/bicarbonate in H. pylori, this study showed that tlpA gene expression is required for arginine/bicarbonate chemotaxis.

Key words: tlpA, chemotaxis, Helicobacter pylori, arginine, bicarbonate.

INTRODUCTION

Helicobacter pylori, a motile Gram-negative human pathogen that causes gastritis and duodenal/gastric ulcers and represents a high risk of gastric cancer, inhabits the gastric mucus layer (McGowan et al., 1996). Most of these bacteria live deep in the layer of mucus gel and close to the surface of the epithelium. Mucus is continuously secreted by surface epithelial cells of the gastric glands and is degraded at the luminal surface of the mucus layer (Schreiber and Scheid, 1997). Because of a rapid mucus turnover, H. pylori cells need motility and spatial orientation to avoid being dragged into the lumen, where the acidic pH inhibits growth and paralyzes cell motility (Schreiber et al., 1999; Worku et al., 1999). Accordingly, orientation plays a central role both in acute colonization and chronic persistence of H. pylori.

Motile bacteria sense chemical gradients by means of chemoreceptor proteins that relay the information to the flagellar motor (Bren and Eisenbach, 2000). All gastric Helicobacter species are highly motile. In recent years, comparative genomics in various Helicobacter species and related bacteria has facilitated the analysis of genes. Experiments with H. pylori in different animal models have shown that flagellar motility is essential to colonize the gastric mucus (Ernst and Gold, 2000). H. pylori shows taxis response towards urea, amino acids and bicarbonate whereas it moves away from H+ (Cerda et al., 2003; Croxen et al., 2006; Mizote et al., 1997; Worku et al., 2004). In addition to motility, recent studies in in vivo systems have shown that H. pylori chemotaxis is required for colonization and inflammatory response induction in gastric mucosa (Andermann et al., 2002; Williams et al., 2007). However, it is still unclear which combination of chemical gradients H. pylori uses in vivo to maintain an optimal position in the gastric mucus layer (Schreiber et al., 2004). By using genomic analysis it has been shown that the chemotaxis system of H. pylori is genetically similar to the one in Salmonella. However, extensive functional analysis of potentially participating proteins is still necessary. Only four genes with homology to chemotaxis receptors have been identified in H. pylori: tlpA, tlpB, tlpC, tlpD (Tomb et al., 1997). Sensing specificities of these four annotated H. pylori chemosensors have not been comprehensively described. In vitro negative taxis to acidic pH was found to be dependent on the sensor protein TlpB (Croxen et al., 2006). On the other hand, Schweinitzer et al. (2008) reported that TlpD is a receptor for energy taxis. Positive taxis to arginine and bicarbonate have been observed in vitro (Cerda et al., 2003; Mizote et al., 1997; Worku et al., 2004) and reported to be dependent on TlpA function (HP0099, according to the annotated genome sequence of H. pylori strain 26695) (Cerda et al., 2003). The H. pylori sensor TlpA has been expressed heterologously in E. coli and found to provide tactic movement towards arginine, bicarbonate and urea (Cerda et al., 2003). Interestingly, the tlpA gene was found to be interrupted by a mini IS605 sequence in the H. pylori 43504 strain, which fails to recognize either arginine or sodium bicarbonate as chemoattractants (Cerda et al., 2003). However, strain-dependency has not been discarded yet. In this work, we present further evidence on the role of TlpA as a chemotactic receptor by showing that tlpA disruption in the H. pylori wild-type strain ATCC 700392 causes loss of in vitro chemotactic response to arginine and bicarbonate.
Bacterial cells grown in 5.5% CO₂ and 85% humidity at 37 ºC

Motility assay

Bacterial cells were scraped from the plates and suspended in 5.5% CO₂ and 85% humidity.

Chemotaxis assay

Bacterial cells were scraped from the plates and suspended in chemotaxis buffer (10 mM potassium phosphate, pH 7.0; 3.0% polyvinylpyrrolidone) at a concentration of 3.0 in chemotaxis buffer (10 mM potassium phosphate, pH 7.2) and incubated at 30 ºC for 45 min. Finally, the needle-syringe system was fitted to the pipette tip in such a way that most of the needle became immersed into the bacterial suspension. The system was positioned horizontally and diluted externally and 10-fold serially diluted in chemotaxis buffer. Dilutions were plated onto 4% (w/v) trypticase soy agar plates followed by incubation for 12-16 h in 5.5% CO₂ and 85% humidity to enhance transformation. Bacteria were scraped from the agar surface and suspended in 1 ml of 10% cold glycerol and recovered after spin down at 2935 xg for 6 min in an Eppendorf centrifuge 5415C. The bacterial sediment was resuspended in 0.5 ml of 10% glycerol, mixed with 3-8 μl of PBS to inoculate TSA agar plates containing 15 μg ml⁻¹ of chloramphenicol. Transformed colonies (H. pylori 700-tlpA::cat) were isolated from the plates after incubation for 4-5 days. Further details of the procedure for insertion mutation were obtained from Croxen et al. (2006) and Andermann et al. (2002). Correct allelic replacement was confirmed by PCR of genomic DNA isolated from resistant colonies, using TlpA-F and TlpA-R primers (Table 1). Treatments of DNA with restriction enzymes, T4 DNA ligase and T4 DNA polymerase were performed according to protocols recommended by the supplier (Promega).

mRNA extraction and RT-PCR analysis

Total mRNA from H. pylori 700395, H. pylori 43504 and the H. pylori 700-tlpA::cat mutant were isolated and purified using RNeasy Mini Kit (Qiagen). Total cDNA was synthesized using cDNA CoreKit (Bioline) following manufacturer’s instructions. PCRs were performed in a PTC-100 MJ Research thermal cycler using cTlpA-F and cTlpA-R primers and 16S-F and 16S-R as internal control (16S rDNA H. pylori-specific primers) (Table 1).

RESULTS AND DISCUSSION

Metabolic reconstitution experiments based on genomics data of H. pylori showed the essential character of at least eight amino acids (i.e. alanine, arginine, histidine, leucine, methionine, phenylalanine, valine and cysteine) in the absence of sulphate as sulfur source (Schilling et al., 2002). Against this background, we tested the chemotactic response of H. pylori 43504 and 700392 strains aiming to identify new TlpA ligands. In these experiments, seven of ten tested amino acids proved to be non attractants in both strains. In accordance with previous results (Cerda et al., 2003), both strains recognized L-serine and L-aspartate as attractants. However, L-arginine was attractant for H. pylori 700392 but non attractant for H. pylori 43504 (Fig. 1).
Previously, we had found that tlpA (ORF HP0099) codes for a receptor protein that recognizes arginine and sodium bicarbonate as attractants in H. pylori 700392. In addition, we found that the lack of chemotactic behavior of H. pylori 43504 strain towards arginine and bicarbonate was associated with a mini-16S605 insertion in the tlpA gene. This observation provided a knockout model for the TlpA function. In order to confirm that the loss-of-function of the tlpA gene in the H. pylori 43504 strain was not a strain-dependent phenomenon we assayed the effect of disrupting the tlpA gene in H. pylori 700392. This strain is chemotactic to arginine/bicarbonate. To this end, we inserted a cat cassette into the tlpA gene (Fig. 2A). Insertion into tlpA was confirmed by PCR amplification and observation of either the expected ~2 kb, 2.3 kb or 3 kb bands in H. pylori 700392, H. pylori 43504 and H. pylori 700-tlpA::cat mutant, respectively (Fig. 2B). No differences in amplicon size were observed in the MCPs genes tlpB (ORF HP0103) and tlpC (ORF HP0082) from H. pylori 700392, H. pylori 43504 and H. pylori 700-tlpA::cat strains, thus showing a single allelic replacement of the tlpA gene (Fig. 2B).

<table>
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<tr>
<th>Primer</th>
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<tr>
<td>TlpA-F</td>
<td>5’ CGATTGGAGCTCTTTTCAATCC 3’</td>
<td>Cerda et al, 2003</td>
</tr>
<tr>
<td>TlpA-R</td>
<td>5’ CCCGCAAAGGCTCTTTAAGC 3’</td>
<td>Cerda et al, 2003</td>
</tr>
<tr>
<td>TlpB-F</td>
<td>5’ CCGGATATGCTGTTTCTTCAATGGTAGTC 3’</td>
<td>This study</td>
</tr>
<tr>
<td>TlpB-R</td>
<td>5’ CCGGGATCCATTTAAAAACACGGCGGATCAGATGCT 3’</td>
<td>This study</td>
</tr>
<tr>
<td>TlpC-F</td>
<td>5’ ATG AAA TC TACA AGA ATT GG 3’</td>
<td>This study</td>
</tr>
<tr>
<td>TlpC-R</td>
<td>5’ TTC TTT TAA GGT AAT AGA GG 3’</td>
<td>This study</td>
</tr>
<tr>
<td>16S-F</td>
<td>5’ CTCAGAGATCACCTAT 3’</td>
<td>This study</td>
</tr>
<tr>
<td>16S-R</td>
<td>5’ CCTACCTCTCCACACTCTA 3’</td>
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### Table 1

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<td>5’ CCCGCAAAGGCTCTTTAAGC 3’</td>
<td>Cerda et al, 2003</td>
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<tr>
<td>TlpB-F</td>
<td>5’ CCGGATATGCTGTTTCTTCAATGGTAGTC 3’</td>
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<tr>
<td>TlpB-R</td>
<td>5’ CCGGGATCCATTTAAAAACACGGCGGATCAGATGCT 3’</td>
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<tr>
<td>TlpC-F</td>
<td>5’ ATG AAA TC TACA AGA ATT GG 3’</td>
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<tr>
<td>TlpC-R</td>
<td>5’ TTC TTT TAA GGT AAT AGA GG 3’</td>
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</tr>
<tr>
<td>16S-F</td>
<td>5’ CTCAGAGATCACCTAT 3’</td>
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<td>16S-R</td>
<td>5’ CCTACCTCTCCACACTCTA 3’</td>
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**Figure 1.** H. pylori 700392 is attracted by aspartate, serine and arginine. Relative chemotactic response (RCR) of H. pylori 43504 (filled bars) and 700392 (empty bars) to 10 mM amino acids. A buffer solution served as a negative control and 10 mM aspartate and 10 mM serine as positive controls, as described for strain 700392 (Cerda et al., 2003). Chemotactic responses were tested using a capillary assay, as described under Materials and Methods. Each bar represents average and corresponding standard deviation of at least 5 independent experiments (*p < 0.05, ** p < 0.01).
Synthesis of tlpA mRNA in the *H. pylori* 700-tpmA::cat mutant was evaluated by RT-PCR. From the analysis of total cDNA, no expression was detected in *H. pylori* 43504 and *H. pylori* 700-tpmA::cat mutant, thus showing that the mini-IS605 and the cat insertions cause loss of tlpA expression on both *H. pylori* strains (Fig. 3A). Next, the motile behavior was tested as to whether tlpA loss-of-function caused a negative motile phenotype in the bacterium. Soft agar assays showed that the *H. pylori* 700-tpmA::cat mutant and the *H. pylori* 43504 and 700392 strains present a similar motility behavior. The diameter of growth halo for the three *H. pylori* strains ranged between 18 and 24 ± 2 mm after 48 h (Fig. 3B), thus demonstrating that the tlpA insertion mutation in *H. pylori* 700-tpmA::cat does not alter the swimming behavior of the bacteria. Accordingly, we assayed the chemotactic response towards sodium bicarbonate and L-arginine using the *H. pylori* 700-tpmA::cat mutant. This strain was found to exhibit a similar chemotactic phenotype as that of *H. pylori* 43504, that is, no chemotactic response either to sodium bicarbonate or arginine (Fig. 4, Table 2). These results confirm our previous conclusion that tlpA codes for a chemotactic receptor that in *H. pylori* recognizes arginine and bicarbonate as attractants.

Motility and chemotaxis have been considered two important processes in colonization, persistence and inflammatory response (Andermann et al., 2002; Williams et al., 2007; Pittman et al., 2001; Ottemann and Lowenthal, 2002; McGee et al., 2005; Terry et al., 2005; Wunder et al., 2006; Castillo et al., 2008; Lowenthal et al., 2009). Tlps chemotactic receptors constitute a well known group of proteins playing an adaptive role in *H. pylori*. Various authors have described the roles of TlpA, and TlpB in *H. pylori* colonization and persistence (Croxen et al., 2006; Andermann et al., 2002).

*H. pylori* niche is the stomach mucus layer in which a pH gradient is established between lumen (pH 3.0) and epithelium (pH 7.0). Local pH variations may represent a limit condition for *H. pylori* chemotaxis in its niche, thus restricting the local stomach colonization (Schreiber et al., 2004). *H. pylori* infection is predominant in antrum and corpus. Positive taxis towards arginine and bicarbonate could participate in territory preferences of *H. pylori* in stomach colonization. On the other hand, Croxen et al. (2006) demonstrated the role of TlpA in pH negative taxis and colonization. Urease is the major factor in acid resistance (Mendz and Hazell, 1996). This enzyme hydrolyzes urea to ammonia and carbon dioxide, thus favoring proton neutralization. In addition, bicarbonate secretion by gastric epithelia is related to local pH neutralization. Bicarbonate is secreted into the gastric mucosa by a chloride-bicarbonate exchanger that is localized in parietal cells whereas Na+ is secreted by a Na+-H+ exchanger that is localized in the mucous neck cells, chief cells and surface mucous cells (Stuart-Tilley et al., 1994). The chemotactic response to sodium bicarbonate may also contribute to the persistence of *H. pylori*. Since the bicarbonate anion is one of the reaction products of urease activity, this response might be important in the absence of urea. Arginine uptake may constitute an important survival mechanism of *H. pylori* in the stomach niche. In *H. pylori*, arginine is both an essential amino acid (Schilling et al., 2002).

**TABLE 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>N° of CFUs*/syringe (mean ± SD) at 45 min</th>
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<tbody>
<tr>
<td></td>
<td><em>H. pylori</em> 700392</td>
</tr>
<tr>
<td>Buffer</td>
<td>637 ± 25</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>1.400 ± 38</td>
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<tr>
<td>Arginine</td>
<td>1.705 ± 43</td>
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(*) CFUs: colony-forming units.
and a substrate for urea cycle, a metabolic pathway implicated in nitrogen metabolism in this organism (Mendz and Hazell, 1996). Therefore, positive taxis towards arginine could favor its uptake in the gastric environment, thus producing metabolic effects. By both avoiding low pH zones, as a primary mechanism, and approaching regions of the stomach with high levels of arginine, bicarbonate and other aminoacids, as a secondary one, bacteria could improve their colonization fitness. In this regard, crosstalk signaling between TlpA and TlpB pathways could play a major role in antrum colonization.

It is well known that MCPs may form different arrays and organize complex networks between different receptors, in which CheW, CheA, CheR and CheB proteins are involved, thus enhancing signal transduction. Even though in H. pylori CheB/CheR enzymes have not been yet identified, other adaptive proteins may play related roles in this organism. For instance, the CheV paralogs CheV1, CheV2 and CheV3, which have been proposed as MCPs interacting proteins, have been found to modulate CheA autophosphorylation (Lowenthal et al., 2009; Pittman et al., 2001). Future insights on TlpA/ TlpB and accessory protein arrangements will be necessary to clarify possible cooperative roles of these proteins in H. pylori colonization.

TlpA seems to be a ubiquitously distributed protein among the Helicobacter sp., including H. hepaticus, H. mustelae, H. felis and other sixteen H. pylori strains (http://blast.ncbi.nlm.nih.gov/Blastcg). In addition, Andermann et al. (2002) have shown that tlpA loss-of-function impairs colonization capability of H. pylori. This evidence suggests a strong role of TlpA in H. pylori survival, inflammatory evasion and in re-population after antibiotic treatment, marking it a possible target for inhibitor drug design against this receptor and/or protein partners involved in TlpA signal transduction. Future research in this field will open opportunities for new H. pylori eradication therapies.

ACKNOWLEDGMENTS

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REFERENCES


Figure 4. tlpA null mutant shows loss of arginine and sodium bicarbonate chemotactic response. Relative chemotactic responses (RCR) of H. pylori 700392 (empty bars) and 700HlpA::cat (filled bars) are shown. Chemotactic properties of the tlpA null strain differed significantly (*p<0.05, **p<0.01) from the isogenic parent strain. Averages and means from at least 5 independent experiments are shown.

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