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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* 700-*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* 700-*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 mucusa (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.

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MATERIALS AND METHODS

H. pylori strains

Bacterial strains used in this study were *H. pylori* strains ATCC 700392 and ATCC 43504. In addition, in this study *H. pylori* 700*tlpA::cat* was developed. Frozen stocks and replated cultures of the *H. pylori* strains were used. As recommended by ATCC, the strains were cultivated on TSA agar plates [trypticase soy agar plates (Becton Dickinson Biosciences) supplemented with 5% sheep blood (Public Health Institute of Chile), culture supplement Vitox (Oxoid) and antibiotic culture supplement Dent (Oxoid)] for 24 h at 37 ºC in 5.5% CO_2 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 x 10^8 cells per ml (OD_{560} = 0.4). The chemotaxis assay was done as previously described by Cerda et al. (2003). Briefly, 100 μl of bacterial suspension were placed into a 200-μl disposable pipette tip. On the other hand, a 100 μl volume of a solution containing 10 mM of the compound to be tested for chemotactic response (buffer alone served as control) was aspirated through a 25 G stainless-steel needle (0.254 mm ID x 20 mm long) into a 1-ml tuberculin syringe. 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 incubated at 30 ºC for 45 min. Finally, the needle-syringe system was separated from the bacterial suspension, cleaned externally and 10-fold serially diluted in chemotaxis buffer. Dilutions were plated onto 4% (w/v) trypticase soy agar plates supplemented with 5% horse serum (HyClone), culture supplement Vitox (Oxoid) and antibiotic culture supplement Dent with 5.5% CO_2 and 85% humidity. Those culture conditions enhanced visualization of colonies. After 24 h incubation at 37 ºC the number of colony-forming units (CFUs) per plate was counted. Each assay was performed in duplicate. Results were expressed as the mean of at least five independent assays. To ascertain whether a test compound was or was not an attractant, a relative chemotaxis response (RCR) was calculated as the ratio between the number of bacteria entering the needle-syringe system in a dilution dependent manner and the number of bacteria in the control condition. A relative chemotaxis response of 2 or greater was considered significant (Adler, 1973; Cerda et al., 2003; Mazumder et al., 1999; Moulton and Montie, 1979). Differences between groups were analyzed statistically by using the Student's t-test.

Motility assay

Bacterial cells grown in 5.5% CO_2 and 85% humidity at 37 ºC for 5 days on TSA agar plates were scrapped and suspended in phosphate saline buffer pH 7.2 (PBS). The suspended cells were stab inoculated with toothpicks into plates containing 0.3% agar (Difco), trypticase soy broth (Becton Dickinson Biosciences), 5% horse serum (HyClone), culture supplement Vitox and antibiotic culture supplement Dent. Cells were cultured at 37 ºC for 48 h in 5.5% CO_2 and 85% humidity. Motility was scored by measuring the diameter of the growth zone after 48 h (Cerda et al., 2003).

DNA manipulations and genetic techniques

Chromosomal DNA from *H. pylori* was isolated as previously described (Owen and Bickley, 1997). To produce a *tlpA* knockout *H. pylori* mutant, a PCR *tlpA* amplicon from *H. pylori* strain 700392 (Cerda et al., 2003) was firstly cloned into pBR322. Then, the chloramphenicol acetyl transferase gene (*cat*) from *C. coli* (Wang and Taylor, 1990) was inserted at a *Sac*I restriction site of *tlpA* to create the plasmid pBR322-*tlpA::cat*. Log phase recipient cells were prepared from overnight TSA agar plates. To do so, bacteria were scraped from the agar surface, washed twice in 1 ml of 10% cold glycerol and recovered after spun 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 μg of pBR322-*tlpA::cat* plasmid DNA and the suspension was spotted onto bacterial TSA agar plates followed by incubation for 12-16 h in 5.5% CO_2 and 85% humidity to enhance transformation. Bacteria were scrapped from the agar surface and suspended in a minimal volume 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 the *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).

TABLE 1
Primers used in the study

Primer	Sequence	Reference
TlpA-F	5′ CGATTGGACGTCTTTTTAATCC 3′	Cerda et al, 2003
TlpA-R	5′ CCCGCAAAAGCTTCTTTAGC 3	Cerda et al, 2003
TlpB-F	5´ CCGCATATGATGTTTTCTTCAATGTTTGC 3´	This study
TlpB-R	5´ CCGGGATCCATTAAAACACGCCGTGATCAC 3´	This study
TlpC-F	5´ ATG AAA TC TACA AGA ATT GG 3`	This study
TlpC-R	5´ TTC TTT TAA GGT AAT AGA GG 3´	This study
16S-F	5´GCTAAGAGATCAGCCTAT 3´	This study
16S-R	5´CCTACCTCTCCCACACTCTA 3´	This study

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-*IS605* 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).

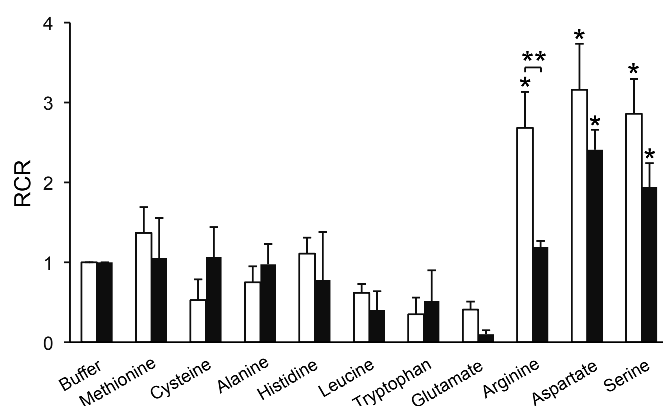

Figure 1. *H. pylori* 700392 is attracted by aspartate, serine and arginine. Relative chemotactic response (RCR) of *H. pylori* 43504 (filled bars) and 700392 (empty bars) to 10 mM amino acids. A buffer solution served as a negative control and 10 mM aspartate and 10 mM serine as positive controls, as 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 (*$p < 0.05$, ** $p < 0.01$).

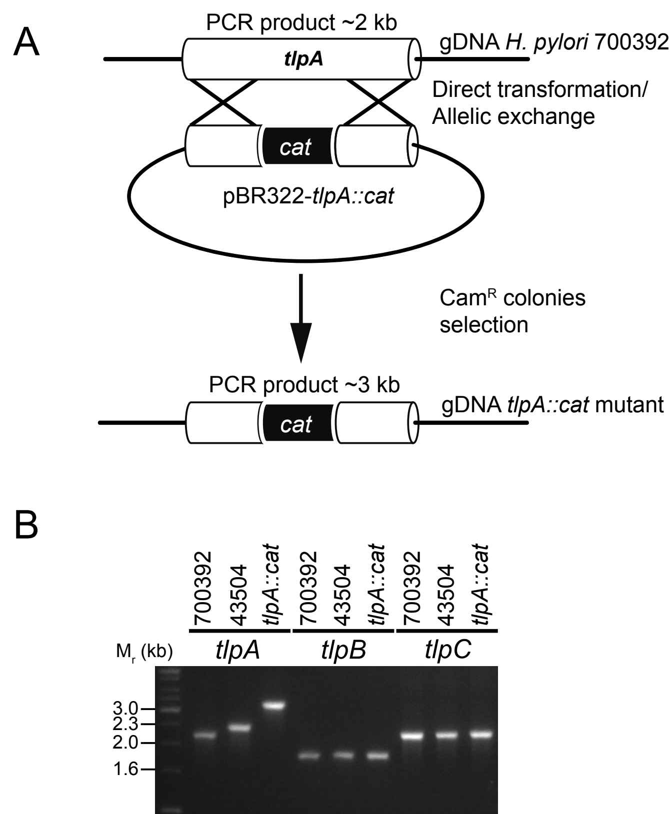

Figure 2. Construction of the *tlpA::cat* mutant in *H. pylori* 700392. **A.** Schematic outline of *H. pylori* 700*tlpA::cat* mutant construction through the allelic replacement of *tlpA* gene in *H. pylori* 700392. Predicted PCR amplicons with TlpA-F/TlpA-R primers from genomic DNA (gDNA) are shown. **B.** *tlpA*, *tlpB* and *tlpC* PCR amplification from *H. pylori* 700392, 43504 and 700*tlpA::cat* gDNA demonstrates the replacement of *tlpA* gene in *H. pylori* 700*tlpA::cat* mutant. No differences between *tlpB* and *tlpC* amplicon sizes were observed.

Synthesis of *tlpA* mRNA in the *H. pylori* 700-*tlpA::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-*tlpA::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-*tlpA::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-*tlpA::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-*tlpA::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
Chemotactic response of *H. pylori* to arginine and bicarbonate

Condition	N° of CFUs*/syringe (mean ± SD) at 45 min	
	H. pylori 700392	*H. pylori* 700*tlpA::cat*
Buffer	637 ± 25	343 ± 17
Bicarbonate	1.400 ± 38	345 ± 10
Arginine	1.705 ± 43	296 ± 20

(*) CFUs: colony-forming units.

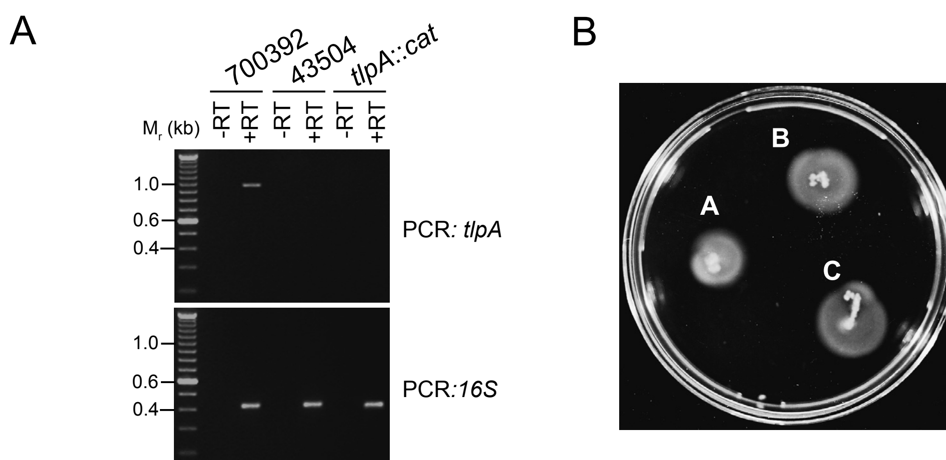

Figure 3. *tlpA* loss-of-function does not alter motile phenotype in *H. pylori 700tlpA::cat* mutant. **A.** *tlpA* RT-PCR from 700392, 43504 and 700*tlpA::cat* total mRNA. Note the lack of *tlpA* expression in both *H. pylori* 43504 and *H. pylori* 700*tlpA::cat* mutant due to mini-*IS605* and *cat* cassette insertions, respectively. No reverse transcriptase in the reaction mix (-RT) with *H. pylori* 700392 mRNAs was used as negative control. **B.** Motility assays in soft agar. Cell suspensions of *H. pylori* 700392 (A), *H. pylori* 43504 (B) and *H. pylori* 700*tlpA::cat* (C) were stabbed on 0.3% agar TSA plates and incubated as described under Materials and Methods. Both mutant strains spread in clear concentric rings because of their motility (representative experiment).

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/Blast.cgi). 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.

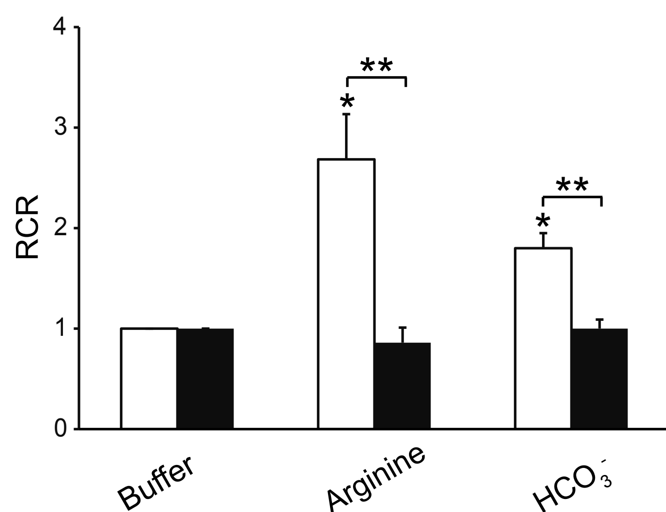

Figure 4. *tlpA* null mutant shows loss of arginine and sodium bicarbonate chemotactic response. Relative chemotactic responses (RCR) of *H. pylori* 700392 (empty bars) and 700*tlpA::cat* (filled bars) are shown. Chemotactic properties of the *tlpA* null strain differed significantly (*p<0.05, **p<0.01)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

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