A Study on the Regulation and Energy Requirements of *Helicobacter pylori* Motility and Its Inhibitors

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**Summary.** The motility conferred by the flagella is necessary for colonization of the gastric mucosa by *Helicobacter pylori*, a bacterial risk factor for gastric cancer. In this study, we investigated characteristics of *H. pylori* movement. The motility of *H. pylori* was observed at a swimming speed of around 70 mm/s or more in vitro. The motility was up-regulated by H+ at a slightly acidic pH (pH 6.0 > 5.0 > 4.0 or 7.4). The addition of a proton-ionophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), immediately inhibited the *H. pylori* motility. In contrast, amiloride, monensin, and orthovanadate had little or no effect on the motility. Some agents inhibited the motility without affecting bacterial growth. In human gastric biopsy specimens, two modes of *H. pylori* flagella morphology were observed: a spiral flagellar bundle consisting of multiple, polar flagella, and a non-spiral flagellar bundle or multiple, unentwined polar flagella. These results demonstrate not only a feature of *in vivo* *H. pylori* flagella morphology and motility, but also that the driving force of the *H. pylori* flagellar motion is, at least in part, the electrochemical proton gradient across the membrane, and a slightly acidic pH (~pH 6.0) provides the optimal condition for *H. pylori* motility. A potential benefit of anti-*H. pylori* motility agents for eradication therapy is discussed.

**Key words—** *Helicobacter pylori*, swimming motility, regulation, pH, inhibitors.

**INTRODUCTION**

*Helicobacter pylori* colonizes the gastric mucosa of most children by the time they are 10 years of age in most developing countries and that of approximately 50% of adults by 60 years of age in the developed countries10. *H. pylori* infection is thought to persist over several decades2, and is a cause of gastritis and peptic ulcers4,5. Moreover, chronic *H. pylori* infection is associated with an increased risk of gastric adenocarcinoma5.

*H. pylori* is a gram-negative, spiral-shaped bacterium with one to two turns along the axis, and has multiple (four to six) polar flagella with terminal bulbs3. Each flagellum consists of a flagellar filament (which is composed of the major flagellin, FlaA, and a minor flagellin, FlaB) and a flagellar sheath6. The motility conferred by the flagella is necessary for colonization of the gastric mucosa and the development of gastritis by the bacterium7. The motility of *H. pylori* is strictly regulated by temperatures, being maximum at 37°C but ceasing at environmental temperatures (<20°C)9. *H. pylori* is generally considered to move through the mucous layer with a pH gradient10 between 1.4 and neutral, and *H. pylori* migrates on the gastric mucosa during the treatment of patients11.

The effect of pH on *H. pylori* motility has yet to be studied in detail. Moreover, the mode of infection of *H. pylori* has yet to be examined from the viewpoint
of motility. This study was designed to investigate the nature of the H. pylori motility in vitro and in vivo. The action of an agent that inhibits H. pylori movement was also given attention.

MATERIALS AND METHODS

Bacterial strains

Four clinical strains (MCP5, MCP14, MCP30, and MCP34) of H. pylori were isolated from patients with chronic gastritis or peptic ulcers. The Vibrio cholerae O1 strain EO8 (El Tor biotype) was isolated from a cholera patient\(^\text{15}\).

Media and bacterial growth

Bacteria were grown on either tryptic soy agar (TSA) containing 5% sheep blood (Nippon Becton Dickinson, Tokyo) or on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich., USA) containing 5% sheep blood for 3 to 4 days at 37°C in a microaerophilic atmosphere (5% O\(_2\) and 10% CO\(_2\)). Bacteria were also grown in brain heart infusion (BHI) broth (Difco) containing 10% fetal bovine serum (FBS) for 1 to 2 days at 37°C in a microaerophilic atmosphere. The pH of the media was adjusted with HCI\(^\text{12}\).

Chemicals

Sodium orthovanadate was purchased from Wako Pure Chemical Industries (Osaka). Amiloride, monensin, and carboxylycyanide-m-chlorophenylhydrazone (CCCP) were obtained from Sigma Chemical (St. Louis, MO, USA). Troxipide, \((\pm)\) 3,4,5-trimethoxy-N-3-piperidylbenzamide (a gastric mucosal protective agent), was a gift from Kyorin Pharmaceutical Co., Tokyo.

Susceptibility testing

Susceptibility testing of bacterial strains was performed using the agar dilution method with Mueller-Hinton agar containing 5% sheep blood in accordance with NCCLS standard procedures\(^\text{19}\). The plates were incubated for 3 days at 35°C in a microaerophilic atmosphere.

Chemotaxis test

A capillary method\(^\text{14}\) was employed. H. pylori cells were suspended in BHI broth containing 10% FBS (pH 7.4) at 5×10\(^6\) CFU/ml. A 10 μl capillary (Drummond Scientific Co., Broomall, PA, USA) filled with the fresh medium adjusted to various pH values was then inserted into the broth and incubated for various periods at 37°C in a microaerophilic atmosphere. The bacteria in the capillary tube was fixed with 10% Formalin, and the total bacterial cell counts per ml were determined with a bacteria counter (Kayagaki Irika Kogyo, Tokyo).

Table 1. Swimming motility of H. pylori in a liquid medium at different pH\(^\text{a}\)

<table>
<thead>
<tr>
<th>pH</th>
<th>Motile cell population (%)</th>
<th>Mean swimming speed (μm/s) of motile cells(^\text{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>92</td>
<td>65±5(^\text{c})</td>
</tr>
<tr>
<td>6.0</td>
<td>100</td>
<td>95±8(^\text{c})</td>
</tr>
</tbody>
</table>

\(^\text{a}\)Swimming motility was examined in BHI broth containing 10% FBS at 37°C. \(^\text{b}\)The data are presented as mean±SD of experiments with four H. pylori strains. \(^\text{c}\)Significantly different (P<0.01).
Inhibition of *H. pylori* swimming motility by a proton-ionophore, CCCP. Bacterial strains: *Circle, H. pylori* strain MCP14; *Square, V. cholerae* 01 strain EO8. A. Bacterial swimming population in the presence of various concentrations of CCCP; B. Mean swimming speed of the motile bacteria in the presence of various concentrations of CCCP. CCCP at a concentration of 10 μM completely inhibited the motility of all four *H. pylori* strains used at both pH 6 and 7.4.

Table 2. Inhibition by various chemical agents of the swimming motility of *H. pylori* in a liquid medium

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Swimming speed (SS, μm/s) in the absence of chemical agents</th>
<th>Addtion:</th>
<th>( IC_{30} ) (μM)</th>
<th>( IC_{100} ) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em></td>
<td>68±14</td>
<td>0</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td><em>V. cholerae</em> O1</td>
<td>85±18</td>
<td>63±11</td>
<td>30</td>
<td>600</td>
</tr>
</tbody>
</table>

*aSwimming motility was examined in BHI broth containing 10% FBS at 37°C, *bBacterial strains used were *H. pylori* strain MCP14 and *V. cholerae* 01 strain EO8, \( IC_{30} \), concentration (μg/ml) necessary to inhibit 30% of the motility; \( IC_{100} \), concentration (μg/ml) necessary to inhibit 100% of the motility; *ND, not determined.*

Swimming motility analysis

Bacterial motility was examined under an inverted, phase-contrast microscope with a microwarm plate (Kitazato Co., Tokyo) that regulates temperatures of specimens. The motility speed (μm/s) was measured by using a motion analysis system with the program C-Imaging C-MEN (Complix Inc., PA, USA), essentially as described previously*. Bacterial swimming in a liquid layer of brain BHI broth containing 10% FBS (10⁶ to 10⁷ CFU/ml) between a glass slide and a glass cover were continuously recorded 15 times with
A B

Fig. 3. Inhibition of the swimming colony formation of H. pylori by a gastric mucosal protective agent, troxipide. Strain MCP14 was inoculated at the center of soft agar plates (0.3% BHI agar containing 10% FBS, pH 7.4) with and without troxipide, and the plates were incubated for 3 days at 37°C in a microaerophilic atmosphere. A. without troxipide (diameter of a colony, 2.3 cm); B. with troxipide at 10 μM (2.9 μg/ml) (diameter of a colony, 1.0 cm). Similar results shown in the figure were also obtained with three other H. pylori strains.

for 0.05 s each (a total of 0.75 s), and the swimming speed (μm/s) of each bacterial cell in a specimen was obtained. This was performed in at least 5 different fields of a specimen, the swimming speeds of ca. 300 bacterial cells were collected for each specimen, and the percentage of motile bacteria was determined. Brownian motion of bacteria was estimated to be 0.4±0.3 μm/s using heated or formalin-treated, non-motile bacteria (H. pylori and V. cholerae), and the mean speed of ≥4.0 μm/s (speed 10 times higher than that of Brownian motion) was judged to be motility-positive; bacterial motility was also judged with the naked eye under a phase-contrast microscope. The data were presented as the mean±standard deviations (SD) of at least three trials.

In another method, H. pylori was inoculated at the center of 0.3% BHI agar containing 10% FBS (with or without inhibitory substances) and incubated for 3 to 4 days at 37°C in a microaerophilic atmosphere. Formation of a swimming colony (at the center of the agar plate) was judged with the naked eye.

Scanning electron microscopy
Gastric biopsy specimens obtained from patients with gastritis or gastric ulcers were washed in saline, fixed, and analyzed by scanning electron microscopy, as previously described\(^1\).

Statistical analysis
Data were evaluated by Mann-Whitney's U test. The level of significance was \(P<0.05\).

RESULTS

Chemotaxis of H. pylori
H. pylori chemotaxis toward different pH values was examined in a liquid medium (BHI broth containing 10% FBS). In this experiment, H. pylori was first suspended in a pH 7.4 liquid medium, and then a capillary containing the same liquid medium adjusted to various pH values was inserted into the bacterial suspension and the accumulation of H. pylori cells in a capillary was determined. The level of H. pylori accumulation was the highest in the pH 6.0 capillary (Fig. 1). Accumulation of H. pylori cells in the pH 5.0 capillary was slightly lower, and that in the pH 4.0 and pH 7.4 capillaries was considerably lower.
Swimming patterns and up-regulation of swimming motility by an acidic pH

When *H. pylori* was examined for swimming motility under a phase-contrast microscope after being cultured in BHI broth containing 10% FBS, *H. pylori* rotated on its axis, and swam linearly or in a zigzag fashion with a circular motion. Such *H. pylori* motility was pH dependent, and *H. pylori* displayed a higher mean swimming speed at pH 6.0 than at pH 7.4 (Table 1). The swimming speed of *H. pylori* at pH 6.0 reached 95 μm/s (a mean swimming speed).

Effect of CCCP and other chemical agents on the *H. pylori* swimming motility

The addition of CCCP, a proton-ionophore, in *H. pylori* liquid cultures resulted in a rapid decrease of
Effect of a gastric mucosal protective agent on *H. pylori* motility

The inhibition of swimming motility was also examined using soft (0.3%) agar. In this experiment, *H. pylori* was inoculated at the center of the soft agar, and the formation of a swimming colony was judged. A gastric mucosal protective agent, troxipide, at a concentration of 2.9 μg/ml (1 × 10^{-4} M) inhibited the swimming motility of *H. pylori* in soft agar (pH 7.4), as shown in Fig. 3. This inhibitory effect was observed even at pH 6.0 (data not shown). The minimal inhibitory concentration (MIC) of troxipide against the *H. pylori* strains was ≥ 256 μg/ml, indicating that the drug had no effect on the *H. pylori* growth at concentrations of < 256 μg/ml.

Scanning electron microscopic analysis of *H. pylori* and its flagella in gastric biopsy specimens

Although *H. pylori* strain MCP5 grown on blood agar had five individual flagella at one end (Fig. 4A), some *H. pylori* cells found in biopsy specimens (from which strain MCP5 was isolated) possessed multiple-polar flagella that were united into a single spiral bundle (Fig. 4B). Such *H. pylori* cells with a spiral flagellar bundle were found in the crevice between two neighboring gastric epithelial cells.

*H. pylori* with a non-spiral flagellar bundle, or with multiple, unentwined polar flagella was also present on the mucosa (Fig. 4C). Those bacteria were, in many cases, tightly attached to the epithelial cell surface, surrounded by the microvilli (Fig. 4C). Occasionally, large groups of *H. pylori* cells lined up in a single direction were found on the mucosa (Fig. 4D).

**DISCUSSION**

The low permeability of the mucus layer and the secretion of HCO₃⁻ gives rise to a pH gradient that ranges from about pH 1.4 at the stomach lumen to about neutral pH at the surface epithelium. *H. pylori* has been considered to move through such a mucus layer. This study demonstrated that a slightly acidic pH (pH 6.0) is an attractant for *H. pylori* swimming in vitro. In addition, acidic pH environments were found to up-regulate *H. pylori* motility. *H. pylori* may move rapidly through the mucus layer at a pH 6.0 position (which is located slightly above the epithelium of the gastric mucosa) and spread its infection.

*H. pylori* grows in broth culture within a broad pH range (pH 5.0 to pH 7.0 or more) with optimal in vitro growth occurring at pH 5.5[12]. In addition, the surface...
CagA protein of H. pylori (which is strongly associated with duodenal ulceration and interleukin-8 production from infected epithelial cells) has been shown to be best expressed at pH 6.0\textsuperscript{48}. Moreover, the adherence of H. pylori to tissue culture cells represents a fourfold increase at pH 5.4 over that at pH 7.4\textsuperscript{37}. Environments at pH 6.0 appear to trigger H. pylori's full virulence. In addition, it has been reported that an exposure of H. pylori to acidic stress results in a transcriptional switch that results in the increased expression of class I genes, which affects activity of the flagellum, and that H. pylori is more motile at acidic pH than at pH 7.0\textsuperscript{49}.

This study also demonstrated two distinct modes of H. pylori flagellar morphology in human gastric biopsy specimens. One of these is a dynamic mode in which H. pylori cells are thought to move on the mucosa by rotating one thick spiraling flagellar bundle composed of multiple entwined polar flagella (Fig. 5A). In Escherichia coli and Salmonella Typhimurium (bacteria possessing peritrichous flagella), the rotation of a flagellar bundle propels the bacterial cell and enables swimming\textsuperscript{30}. H. pylori cells with a spiral flagellar bundle were found in the crevice of the epithelial cells (a space between two epithelial cells) in this study. H. pylori cells in the other mode (with a non-spiral flagellar bundle or with multiple, unentwined polar flagella, Fig. 5B) were, in many cases, tightly attached to (locked on) the epithelial cell surface, and surrounded by the microvilli. This pattern of adherence is similar to that of enteropathogenic E. coli, a causative agent of chronic diarrhea in infants\textsuperscript{50}, seen on the small intestinal epithelium\textsuperscript{31}.

In liquid media, H. pylori rotated on its axis, and swam either linearly, in a zigzag fashion, or in a circular motion. These characteristic movements (e.g., a strong drill-like movement with occasional drastic zigzag turns) are vital to substantial H. pylori colonization on the gastric mucosa, as H. pylori digs itself into areas such as the crevice of the epithelial cells. In addition, in this study, large groups of H. pylori were found swimming on the gastric mucosa in a single direction, in response to stimulation on the mucosa (proceeding toward or escaping from this stimulation). This phenomenon is either the beginning or the end of swimming because H. pylori cells during swimming (swimming) seem to be washed out under experimental conditions. For scanning electron microscopy, biopsy specimens have to be washed out to remove most mucus layers; otherwise, adherent H. pylori on the epithelial cells can not be seen. This is also the reason why H. pylori cells with a spiral flagellar bundle (most likely swimming H. pylori cells) were found only in the crevice of the epithelial cells.

The above observations found in this study may be consistent with the clinical observation that H. pylori migrates from the antrum to the fundus during treatment with omeprazole\textsuperscript{30}.

CCCP collapses the electrochemical proton gradient and inhibits the motility (H\textsuperscript{+}-driven motors) of Rhodobacter sphaeroides (which swims vigorously by the rotation of a single sub-polar flagellum) at a concentration of 40 \mu M\textsuperscript{22}. The motility of H. pylori was completely inhibited at much lower concentrations of CCCP (e.g., 10 \mu M). This, along with the fact that amiloride (the inhibitor of Na\textsuperscript{+}-driven flagellar motors\textsuperscript{32}), monensin (the inhibitor of sodium pumps), and sodium orthovanadate (the inhibitor of ATP synthesis) had very little or no effect on the motility of H. pylori, strongly suggests that the motility of H. pylori is, at least in part, due to its H\textsuperscript{+}-driven flagellar motors. This action of flagellar motion would be beneficial especially for bacteria living under acidic environments, that is, H. pylori colonizing gastric mucosa. These results also offer insights into the regulation and energy requirements of H. pylori motility. It has been reported that the chemotactic activity of H. pylori toward urea (chemotactic responses to urea) is inhibited by CCCP, although in those experiments, the effect of CCCP on the H. pylori motility itself was not examined\textsuperscript{33}.

In Japan, gastric mucosal protective agents have been used for the treatment of patients with gastritis. In this study, one of the gastric mucosal protective agents (troxipide) was found to inhibit H. pylori motility at concentrations that did not affect H. pylori growth. This agent inhibits interleukin-8-induced migration of human neutrophils in vitro\textsuperscript{40}. The mechanisms by which troxipide manifests inhibitions are not known; however, there may be a common mechanism(s) between the H. pylori motility inhibition and neutrophil migration inhibition. Since the motility of H. pylori is important for its successful colonization on the gastric mucosa\textsuperscript{21}, gastric mucosal protective agents that inhibit H. pylori motility may be useful from the viewpoint of developing a new approach to eradication therapy for H. pylori infections. The new therapy would include a combination therapy using an anti-H. pylori agent that kills H. pylori and an anti-motility agent that inhibits H. pylori migration, e.g., from the antrum to the fundus, within the stomach.

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REFERENCES