Note

Reduction of Overall *Helicobacter pylori* Colonization Levels in the Stomach of Mongolian Gerbil by *Lactobacillus johnsonii* La1 (LC1) and Its *in Vitro* Activities against *H. pylori* Motility and Adherence

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Received December 1, 2011; Accepted January 15, 2012; Online Publication, April 7, 2012
[doi:10.1271/bbb.110921]

The effects of *Lactobacillus johnsonii* La1 (LC1) on *Helicobacter pylori* colonization in the stomach were investigated. *H. pylori* colonization and gastritis in LC1-inoculated Mongolian gerbils were significantly less intense than those in the control animals. LC1 culture supernatant (>10-kDa fraction) inhibited *H. pylori* motility and induced bacterial aggregation in human gastric epithelial cells, suggesting the potential of clinical use of LC1 product.

**Key words:** *Lactobacillus johnsonii* La1 (LC1); *Helicobacter pylori*; Mongolian gerbil; motility; adherence

*Helicobacter pylori* is a spiral-shaped, Gram-negative microaerophilic bacterium that colonizes the gastric mucosa of more than 50% of individuals worldwide. It is closely associated with gastrointestinal diseases, including gastritis and peptic ulcers, and is a bacterial risk factor for gastric cancer.1,2 Currently, increased eradication failure due to resistance to current chemotherapy and the side effects of chemotherapy (e.g., diarrhea) has become problematic.3 *H. pylori* motility, conferred by polar flagella, is necessary for colonization and pathogenesis in gastric mucosa.3,4 Motile *H. pylori* colonizes more intensely and survives longer in the gastric mucosa than non-motile mutant. Therefore, inhibitors of *H. pylori* motility and adherence have potential for clinical use in managing *H. pylori* infection.

*Lactobacillus johnsonii* La1 (LC1) is a well-described probiotic lactic acid-producing bacterium (LAB).5,6 Several LC1 products are known to have bactericidal and immunomodulatory effects.7–9 Clinical studies have described the inhibitory effect of LC1 administration to *H. pylori*-positive individuals on *H. pylori* colonization, but this has been evaluated mainly by urease activity using the 13C-breath test and bacteriological and pathological examination using limited biopsy samples.10 At present, the effect of LC1 on initial colonization by *H. pylori* is unclear. In this study, we evaluated the protective effect of LC1 colonization on overall colonization by *H. pylori* using an LC1-inoculated Mongolian gerbil model. We also investigated the *in vitro* effects of LC1 culture supernatants on *H. pylori* motility and adherence.

The Mongolian gerbil is the only animal model that develops gastritis, followed by peptic ulcer and eventually gastric cancer, when infected with *H. pylori*.10 Outbred, specific pathogen-free, male Mongolian gerbils (4 weeks old; mean body weight, 36 ± 4 g) were purchased from Kyudo (Kumamoto, Japan). The study protocol was approved by the Ethics Committee for Animal Experiments of Niigata University. Nine animals were assigned to each group. The animals were inoculated orally with LC1 (2.0 × 10⁹ CFU each time) or sterile water 3 times at 2-d intervals. Three days after the final LC1 inoculation, the animals were infected orally with *H. pylori* ATCC43504 (6 × 10⁷ CFU each time)10 twice at a 2-d interval. One, 2, 3, and 4 weeks after *H. pylori* inoculation, the animals were inoculated orally with LC1 (2.0 × 10⁹ CFU each time) or sterile water. One week after the final LC1 inoculation, colonization by *H. pylori* of the stomach and gastritis levels were evaluated, essentially as described previously.10 Fecal and gastric lactobacilli were isolated, and LC1 in the isolated lactobacilli was detected by specific PCR, essentially as described previously.3

The LC1 culture supernatant was fractionated. LC1 was grown in de Man-Rogosa-Sharpe (MRS) broth (Difco, Sparks, MD) for 13 h at 37°C in an anaerobic atmosphere. After removal of bacterial cells, part of culture supernatant was concentrated (up to 10 times) using polyethylene glycol 6000 (Wako Pure Chemical Industries, Osaka, Japan) and dialyzed with Dulbecco’s Phosphate-Buffered Saline (PBS [—], pH 7.3–7.7). This was designated CS (4.85 mg of protein/mL). Fresh MRS broth was concentrated by the same method. The culture supernatant was brought to 50% saturation by the slow addition of solid ammonium sulfate, keeping it on ice with stirring. The precipitate was collected by centrifugation at 15,000 g at 4°C for 20 min, resolved, and dialyzed with PBS (—). The supernatant of 50% ammonium sulfate precipitation was concentrated up to 25 times and dialyzed with PBS (—) (designated AS50; 5.02 mg of protein/mL). Using an Amicon Ultra-15 Centrifugal Filter with a 10-kDa cutoff filter device

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Abbreviations: LAB, lactic acid-producing bacterium; LC1, *Lactobacillus johnsonii* La1; MRS broth, de Man-Rogosa-Sharpe broth
Table 1. Effects of LC1 Administration on H. pylori Colonization in Mongolian Gerbils

<table>
<thead>
<tr>
<th>Probiotic treatment</th>
<th>Infection</th>
<th>n</th>
<th>Gross appearance</th>
<th>Histological findings</th>
<th>H. pylori (CFU/Stomach)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
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<td>2+</td>
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<td>1c</td>
<td>5</td>
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<tr>
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<td>6d</td>
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<td>9</td>
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</tr>
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*aGastritis scores, as described previously,10 with modifications. Gross appearance: none, no detectable lesions; 1+; edematous lesions of mucosa only in the antrum; 2+, lesions in the antrum as well as part of the corpus; 3+, lesions in the antrum and most of the corpus. Histological findings: none, normal, or nearly normal; 1+, infiltration of inflammatory cells (mainly neutrophils) and mononuclear cells (mainly lymphocytes) only in the antrum; 2+, the +1 level plus regenerative hyperplasia of glandular structure in the antrum as well as part of the corpus; 3+, the +1 level plus remarkable dilatation of mucosal tissue (due to regenerative hyperplasia of glandular structure) in the antrum and most of the corpus.

*bMean ± SD. *p < 0.05 (Fisher’s exact test). **p < 0.05 (Fisher’s exact test). †p < 0.05 (Student’s t test). ††Animals were not inoculated with H. pylori.

Next we examined the in vitro effect of the LC1 products on H. pylori motility. Incubation with fresh medium did not alter H. pylori motility (Fig. 1A, control). Mean swimming speed after 90 min of incubation was about 140 μm/s. H. pylori motility was markedly reduced by the concentrated culture supernatant (CS), in a dose-dependent manner (Fig. 1A). Incubation with CS at 50 times dilution for 90 min reduced motile bacteria by 7.3% (Fig. 1A, p < 0.05). Mean swimming speed after 90 min of incubation was also reduced (about 30 μm/s). Most of the inhibitory activity was concentrated in the >10-kDa fraction of 50% ammonium sulfate precipitation. After 90 min of incubation with the >10-kDa fraction at 50 times dilution, bacterial motility was completely inhibited (Fig. 1A). In contrast, after 90 min of incubation with the <10-kDa fraction or the supernatant (AS50-S) of 50% ammonium sulfate precipitation at 50 times dilution, more than 80% of H. pylori cells remained motile (mean swimming speed, about 110 μm/s and 100 μm/s respectively). For all fractions, the viability of H. pylori was not significantly reduced after incubation (Fig. 1B).

After incubation with H. pylori alone and in the presence of control medium, H. pylori diffusely adhered (Millipore, Tokyo), molecules larger than 10 kDa were concentrated (designated the >10-kDa fraction; 3.32 mg of protein/mL). The flow-through fraction was designated the <10-kDa fraction (0.263 mg of protein/mL).

To examine the effects of LC1 on H. pylori motility, H. pylori was incubated with LC1 culture supernatant fractions in brain-heart infusion (BHI) broth (Difco) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) at 37 °C for 10 or 90 min. The residual motility of H. pylori (10⁶ to 10⁷ CFU/mL) was examined under an inverted, phase-contrast microscope with a microwarm plate (Kitazato, Tokyo), as described previously.11 The swimming speed of each bacterial cell in a specimen was measured using a motion analysis-system with the program C-Imaging C-MEN (Complix, Cranberry, PA). Mean speed above 4.0 μm/s (a speed 10 times higher than that of the Brownian motion of fixed H. pylori) was judged to mean positive motility. The swimming speed given in the text represents the mean speed of the motile bacteria. After incubation, the viability of H. pylori was also examined by plating the bacterial dilutions onto blood agar plates (trypticase soy agar supplemented with 5% sheep blood, Becton Dickinson, Tokyo) and counting colonies.

The adherence of H. pylori to human gastric epithelial MKN74 cells (obtained from RIKEN, Tsukuba, Japan) in the presence of LC1 culture supernatant fractions was also examined by scanning electron microscopy, essentially as described previously.12

Statistical comparison was done by Fisher’s exact test for the gastritis score and Student’s t test for other analyses. p < 0.05 was considered significant.

The average viable count of H. pylori in the stomachs of the LC1-inoculated animals was 2.1 × 10⁶ CFU/g stomach, significantly lower than that of the LC1-untreated animals (1.2 × 10⁷ CFU/g stomach, p < 0.05, Table 1). Gastritis was also evaluated by gross appearance and histological findings. Corresponding to the results for viable counts, the gastritis scores of the LC1-inoculated animals were also lower than those of the LC1-untreated animals (Table 1). LC1 itself did not induce inflammation of the gastric tissue (Table 1). From the LC1-inoculated animals, a total of 1.7 × 10¹² CFU/g (SD, 3.1 × 10¹²) of lactobacilli were isolated from the fecal microflora and, of those, 6.0% (SD, 3.8) were positive for LC1 PCR. In contrast, LC1 was below detectable levels (<0.5%) in the total 6.7 × 10⁷ CFU (SD, 5.6 × 10⁷) of the gastric lactobacilli.
to human gastric epithelial MKN74 cells (Fig. 2A and B). When *H. pylori* was incubated in the presence of the >10-kDa fraction (at 50 times dilution), aggregative adherence was seen, with no detectable morphological damage to *H. pylori* cells (Fig. 2C, arrows). In contrast, incubation in the presence of the <10-kDa fraction and the supernatant of 50% ammonium sulfate precipitation did not induce any detectable change in *H. pylori* adherence (data not shown).

Clinical studies have reported beneficial effects of oral administration of LC1 on *H. pylori*-infected individuals, although this was evaluated only by 13C-breath test and bacteriological and pathological examination using limited biopsy samples.13,14) In the present study, we found direct evidence that LC1 reduced total viable *H. pylori* cells in the Mongolian gerbil stomach. Although we administrated LC1 Mongolian gerbils before and after inoculation with *H. pylori*, LC1 administration after *H. pylori* inoculation might also reduce colonization levels of *H. pylori*. This possibility is under investigation.

Our animal study strongly suggests that LC1, which mainly colonizes the intestinal mucosa, inhibits initial colonization by *H. pylori*, as well as growth, in gastric mucosa, although the exact mechanisms of the action of intestinal LC1 on *H. pylori* remain unclear. LC1 products (e.g., bacteriocin, teichoic acid, and GroEL) are known to have bactericidal and immunomodulatory effects.2–9) For example, lactacin F is a 2.5-kDa bacteriocin that has a bactericidal effect on certain Gram-positive bacteria, including *L. delbrueckii* and *Enterococcus faecalis*.7) Lactacin F is known to be concentrated in the precipitate under 40% ammonium sulfate saturation and to form an aggregate with a molecular weight of 180 kDa.7) Thus, it is possible that lactacin F was present in the >10-kDa fraction of 50% ammonium sulfate precipitation in this study, but no effect of it on *H. pylori* has been reported. In this study, the >10-kDa product of LC1 had an inhibitory effect on *H. pylori* motility and adherence without any reduction in *H. pylori* viability. To our knowledge, this is the first evidence of inhibitory activity by the LC1 product on *H. pylori* motility and adherence.

Modulation of host innate and humoral immunity by probiotic LABs (including *L. johnsonii*) has also been suggested.15) It is possible, then, that such immunomodulation induced by LC1 colonizing in the intestinal mucosa also contributes to the reduction in *H. pylori* colonization and the improvement of gastritis. This possibility is also under investigation.

In conclusion, intestinal LC1 reduced subsequent infection by *H. pylori* in a Mongolian gerbil model. Although the in vivo role remains unclear, we confirmed inhibition of the >10-kDa fraction of the LC1 culture supernatant on *H. pylori* motility and adherence. Purification of this product is under investigation. In our preliminary study, this activity was stable after exposure to lower pH levels. Although further analysis under conditions representing gastric environments is required, this LC1 product might have great potential for clinical use.

**Acknowledgments**

We thank Dr. Olga Razvina, Ms. Yukiko Maruyama, and Mr. Keisuke Suzuki for helpful discussion during manuscript preparation. This study was supported in part by a grant from Nestlé Japan, Ltd.

**References**