Analysis of the microbial ecology between *Helicobacter pylori* and the gastric microbiota of Mongolian gerbils

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Animal models are essential for in vivo analysis of *Helicobacter*‐related diseases. Mongolian gerbils are used frequently to study *Helicobacter pylori*‐induced gastritis and its consequences. The presence of some gastric microbiota with a suppressive effect on *H. pylori* suggests inhibitory gastric bacteria against *H. pylori* infection. The aim of the present study was to analyse the microbial ecology between *H. pylori* and the gastric microbiota of Mongolian gerbils. Gastric mucosa samples of *H. pylori*‐negative and -positive gerbils were orally inoculated to five (Group 1) and six (Group 2) gerbils, respectively, and the gerbils were challenged with *H. pylori* infection. The colonization rate (40 %) of *H. pylori* in Group 1 gerbils was lower than the rate (67 %) in Group 2 gerbils. Culture filtrate of the gastric mucosa samples of Group 1 gerbils inhibited the *in vitro* growth of *H. pylori*. Three lactobacilli species, *Lactobacillus reuteri, Lactobacillus johnsonii* and *Lactobacillus murinus*, were isolated by anaerobic culture from the gerbils in Groups 1 and 2, and identified by genomic sequencing. It was demonstrated that the three different strains of lactobacilli exhibited an inhibitory effect on the *in vitro* growth of *H. pylori*. The results suggested that lactobacilli are the dominant gastric microbiota of Mongolian gerbils and the three lactobacilli isolated from the gastric mucosa samples with an inhibitory effect on *H. pylori* might have an anti-infective effect against *H. pylori*.

**INTRODUCTION**

*Helicobacter pylori* is one of several bacterial microbiota capable of colonizing the human stomach (Bik et al., 2006). Whilst most individuals remain asymptomatic (Aviles-Jimenez et al., 2004), ~15 % of *H. pylori* infections result in peptic ulcers and 0.5–2 % of infected individuals develop gastric adenocarcinoma (Atherton, 2006). It has been estimated that *H. pylori* colonization increases the risk of gastric cancer ~10-fold (Suzuki et al., 2007).

There have been many challenges to establishing experimental infection with *H. pylori* in animals. Several experimental animal models, such as gnotobiotic piglets (Eaton et al., 1992), C57/BL6 mice (Kim et al., 2008) and Mongolian gerbils, are helpful in understanding the pathogenesis of human *H. pylori* infection. The Mongolian gerbil model, in which *H. pylori* is able to colonize long-term, is particularly easy to handle. It is useful as a small-animal model for the severe inflammation and obvious ulceration caused by *H. pylori* (Matsumoto et al., 1997; Hirayama et al., 2002). *H. pylori* is inhibited by a number of commensal bacterial species as well as opportunistic human pathogens (Krausse et al., 2005). Probiotics including live bacterial cells can also improve the intestinal microflora and modulate immune functions in beneficial ways (Gill & Guarner, 2004; Borchers et al., 2009). Probiotics have been shown to function as antimicrobial effectors (Cross, 2002). Oral administration of certain lactic acid bacteria can prevent pathogenic infection by microbes such as *Listeria monocytogenes* (Popova et al., 1993), *Escherichia coli* (Ishida-Fujii et al., 2007), *Klebsiella pneumoniae* (Gonchar et al., 2009) and *Salmonella* serotype Enteritidis (Jain et al., 2009) through the regulation of inflammatory cytokines. Although the immunomodulatory effects of fermented products have been reported elsewhere (Michetti et al., 1999; Halper et al., 2003; Kim et al., 2008; Kato-Mori et al., 2010), the mechanism by which cell-free products, i.e. fermentation metabolites, stimulate the immune system remains poorly understood. In addition, these probiotic bacteria can be used to supplement eradication therapy for patients with *H. pylori* infection, either to increase the eradication rate or to prevent the occurrence of side-effects of antimicrobial drugs (International Agency for Research on Cancer, 1994; Ferrero & Fox, 2001). Based on the results of those studies, it is possible that gastric bacteria might affect the colonization of *H. pylori* in the gastric mucosa.

In our previous study (Zaman et al., 2010), *Lactobacillus* spp. were isolated from the gastric mucosa of a gerbil...
uninfected with H. pylori. The uninfecivity of the gerbil was detected on the basis of the least frequency of detection of H. pylori ureA in the faecal sample. The presence of Lactobacillus spp. in the gastric mucosa of that uninfected gerbil represents the possibility of an inhibitory effect by this species of gastric bacteria on colonization by H. pylori. It was suspected that some gastric bacteria may inhibit persistent infection of H. pylori, and thus the gastric bacterial microflora isolated from the gastric mucosa were analysed and compared.

In the present study, the gastric microflora of Mongolian gerbils was analysed by 16S rRNA gene sequencing after inoculating the gerbils with the gastric mucosa samples of H. pylori-positive and -negative gerbils, dividing them into two separate groups. All of the gerbils were challenged with H. pylori. It was also noted that further study to examine the direct effect of the isolated Lactobacillus strains separately or combined is necessary.

**METHODS**

**Animals.** We purchased 5-week-old female Mongolian gerbils (MGS/ Sea; specific-pathogen-free; body weight 25–35 g) from Kyudou, and bred them under specific-pathogen-free conditions in plastic cages and under standard laboratory conditions (room temperature 23 ± 2 °C; relative humidity 40–60%; 12 h light/dark cycle) in the animal facility of Kyorin University. Standard rodent food pellets (CE-2; Clea Japan) and sterilized tap water were provided ad libitum in micro-isolator units as described previously (Krause et al., 2005). The experiments were approved by the Experimental Animal Ethics Committee of Kyorin University School of Medicine on 1 April 2008 (approval no. 75).

**Bacterial strain and culture.** H. pylori strain TK1402 was isolated from gastric biopsy specimens of patients with gastric and duodenal ulcers (Osaki et al., 1998, 2006). This strain exhibits infectivity in germ-free mice (Osaki et al., 1998), C57/BL6 mice (Yamaguchi et al., 2003) and Mongolian gerbils (Nakagawa et al., 2005). The TK1402 strain was cultured for 2 days in Brucella Broth containing 1.5% agar (Difco) and 7% horse serum (SBHS-agar; Sigma) under microaerobic conditions at 37 °C using Anaero Pack (A28; Mitsubishi Gas Chemical) containing 85% N2, 10% CO2 and 5% O2.

**Animal experiments.** The stocked gastric mucosa samples of the H. pylori-negative and -positive gerbils of our previous experiment were inoculated into another five and six Mongolian gerbils (5 weeks old) in Groups 1 and 2, respectively. One week after this inoculation, all of the gerbils were inoculated with H. pylori TK1402 (1 × 10^9 c.f.u.). H. pylori TK1402 was harvested in Hanks' balanced salts solution (HBSS; Sigma) after incubation and 1 ml aliquots from the prepared bacterial suspension containing 1 × 10^7 c.f.u. were used. All gerbils were sacrificed 4 weeks after the inoculation of H. pylori and gastric mucosa samples were collected. The mucus layer of the stomach was scraped off with a spatula, collected into 500 µl HBSS and homogenized for determination of the number of micro-organisms in the mucus layer (mucosa). H. pylori-selective medium (Nissui Pharmaceutical) was inoculated with 50 µl of the gastric sample and incubated at 37 °C for 5 days for the identification of H. pylori. Purple colonies were counted and the number of viable H. pylori cells was expressed as c.f.u. g⁻¹ of the gastric mucosa. Brucella Agar medium supplemented with 7% horse serum was inoculated with a single colony for identification of the bacteria. The isolated strain was shown to be positive for urease, catalase and oxidase with a Gram-negative helical form, and was thus identified as H. pylori. All the gastric mucosa samples were used for culture and also stocked at ~80 °C for future experiments, such as identification of gastric flora and PCR examination.

**Isolation of H. pylori from the gastric mucosa.** H. pylori was isolated from the gastric mucosa samples of the two separate groups of Mongolian gerbils (Groups 1 and 2) after killing the gerbils at 4 weeks post-inoculation with H. pylori TK1402. Group 1 gerbils were inoculated with gastric mucosa samples of H. pylori-positive gerbils and Group 2 gerbils were inoculated with gastric mucosa samples of H. pylori-negative gerbils (Zaman et al., 2010).

**DNA extraction from gastric mucosa samples.** For DNA extraction, 200 µl gastric mucus sample suspension was added to a solution containing 250 µl extraction buffer (200 mM Tris/HCl, 80 mM EDTA; pH 9.0) and 50 µl of 10% SDS. Then, 300 mg of glass beads (diameter 0.1 mm) and 500 µl buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously at 4200 r.p.m. for 30 s using a Mini Bead Beaker (Wakennaku). After centrifugation at 14,000 g for 5 min, 400 µl supernatant was collected, phenol/chloroform extractions were performed and 250 µl supernatant was subjected to 2-propanol precipitation. Finally, the DNA was suspended in 1 ml Tris/EDTA buffer.

Total DNA (1 µl) was amplified using PCR primers for the 16S rRNA of H. pylori, HP-16-F (5'-CGCTAAGAGATCCGCTTC-3') and HP-16-R (5'-CGGTTGCTTCTGTCATGTCC-3') for the detection of H. pylori (Osaki et al., 2006), and g-Lact-F (5'-ACCA-GTCCATGCGCATAC-3') and g-Lact-R (5'-TCCACCCCTTGTTGCTGA-3') primers for the detection of lactobacilli (Rinttilä et al., 2004).

**Quantitative real-time PCR.** A real-time PCR assay was performed using the method reported by Rinttilä et al. (2004), with some modifications. Quantitative analysis was performed using SYBR Green methods. Each reaction mixture (10 µl) was composed of 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 200 µM, a 1:75 000 dilution of SYBR Green I, 11 ng Taq Start antibody (Clontech) µl⁻¹, 0.005 U Taq DNA polymerase (Takara) µl⁻¹, each of the specific primers at a concentration of 0.25 µM and 1 µl of × 1 or × 10 diluted template DNA. The amplification program consisted of one cycle at 94 °C for 5 min, followed by 45 cycles at 94 °C for 30 s, 60 °C for 20 s and 72 °C for 35 s, and finally one cycle at 94 °C for 30 s. We used an absolute quantification in which DNA target genes were compared with data from a standard curve, which was generated by amplifying serial dilutions of a known number of H. pylori TK1402 or Lactobacillus gasseri YIT 0192. For each primer set, PCR was performed in parallel reactions using different amounts of H. pylori TK1402 chromosomal DNA. Quantification data were analysed using 7600 quantification software (Applied Biosystems). In this analysis, the background fluorescence was removed by manually setting a noise band. The long-linear portion of the standard amplification curve was identified, and the crossing point was the intersection of the best-fit line through the long-linear region and the noise band. The standard curve was a plot of the crossing points versus the log bacterial number (c.f.u. ml⁻¹). The quantification software determined the unknown concentration by interpolating the noise band intercept of an unknown sample against the standard curve of known concentrations. The quantitative data were calculated from the standard curve of the PCR. For this approach, the identity and specificity of the PCR product were confirmed by dissociation curve analysis, which is part of the 7600 quantification program. To confirm the specificity of the PCR product, a melting curve analysis was performed after amplification to distinguish the targeted PCR product from the
non-targeted PCR product. The melting curves were obtained by slow heating at temperatures from 60 to 95 °C at a rate of 0.2 °C s⁻¹, with continuous fluorescence collection. The presence of lactobacilli among both Groups 1 and 2 was confirmed by performing real-time PCR. The number of lactobacilli quantified by real-time PCR was measured per gram of the gastric mucosa.

**Isolation of gastric bacteria and their identification using API 20E and the 16S rRNA gene sequencing system.** Gastric bacteria were isolated from the gastric mucosa samples of both Group 1 and Group 2 gerbils. Identification of the isolated gastric bacteria was performed using API 20E and the 16S rRNA gene sequencing method. The 16S rRNA gene sequencing method is more reliable than the API 20A system for the identification of anaerobic bacteria. To confirm the identities of bacterial species, extracted DNA samples after PCR amplification were used for the sequencing of 16S rRNA genes. DNA was extracted from the biopsy samples.

Facultative anaerobic bacteria were isolated from the gastric mucosa samples of the 11 gerbils (1-1-1-5 and 2-1-2-6) by aerobic culture using DHL plates. To isolate the dominant bacterial species from each gerbil, the gastric specimen and 10-fold dilutions of the suspension were inoculated on Gifu anaerobic medium (GAM) agar (Nissui Pharmaceutical), phenyl ethyl alcohol (PEA)-blood agar (Nissui Pharmaceutical) supplemented with 5 % horse blood and DHL agar (Nissui Pharmaceutical), GAM agar and PEA-blood agar were incubated under anaerobic conditions at 37 °C in an anaerobic incubator (Hirasawa) for 48 h. DHL and PEA-blood agar were aerobically incubated at 37 °C for 24 h. After incubation, it was noticed that the growth of bacteria under the anaerobic conditions was more prominent than under the aerobic conditions. Each different type of colony was inoculated for single-colony isolation, and incubated under aerobic and anaerobic conditions at 37 °C. The facultative anaerobic isolates were identified using the API 20E system (bioMerieux), whereas the obligate anaerobes were identified using the genomic sequencing method. These obligate anaerobes isolated from the Mongolian gerbil gastric mucosa samples were cultured in Man–Rogosa–Sharpe (MRS) broth or agar (Difco), and incubated under anaerobic conditions in an atmosphere of 80 % N₂, 10 % H₂ and 10 % CO₂ at 37 °C. Genomic DNA from the cultured bacteria of the gastric mucosa samples was extracted using a MagExtractor (Toyobo).

PCR was performed in thin-walled 0.5 ml Gene Amp reaction tubes (PerkinElmer). Aliquots of 1 µl extracted genomic DNA were mixed with each primer solution (5 pmol) and 0.5 U Taq polymerase in a total volume of 20 µl. Reactions were performed for 30 cycles of 94 °C for 5 min, 94 °C for 1 min, 61 °C for 20 s and 72 °C for 1 min with a thermal cycle (Gene Amp PCR system 9600-R; PerkinElmer). We used two universal primers (27F, 5'-AGAGTTTGTATCMTGGCTCAG-3'; 1492R, 5'-TACGTYACCTGTAGACTCAT-3') (DeLong, 1992) and two primers (518F, 5'-CAACAGCGCCGGTATACTACG-3'; 808R, 5'-TACGGGATCATCTAAC-3') (Stackebrandt & Goodfellow, 1991) for PCR. After the PCR products were obtained, the mixture was purified using a PCR clean-up system (Promega) for the sequencing study. Sequencing reactions were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler using ABI BigDye Terminator v3.1 Cycle Sequencing kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using universal primers (518F and 808R). The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The sequencing of each PCR was analysed by Sequence Scanner (ABI). A BLAST search was performed using Lasergene 7 (DNASTAR).

**Preparation of culture filtrates of lactobacilli.** Lactobacilli cultures were maintained as frozen stocks held at −80 °C in MRS broth (Difco) plus 20 % glycercine (Fisher Scientific). Lactobacilli cultures were propagated and transferred once before use. MRS agar was prepared by the addition of 1.5 % (w/v) granulated agar (BBL, Microbiology Systems) to the broth medium. Lactobacilli were inoculated on MRS agar and incubated under anaerobic conditions at 37 °C in an anaerobic incubator (Hirasawa) for 48 h. Liquid culture of lactobacilli was performed using MRS broth (Oxoid) at 37 °C in 5 % CO₂ for another 2 days. After centrifugation of the liquid culture broth of the lactobacilli for 48 h, culture filtrates were collected and preserved at −20 °C for performing the growth inhibition assay of *H. pylori*.

As *H. pylori* was not detected in gerbil 1-2 and gerbil 1-5, and their gastric mucosa samples inhibited the growth of *H. pylori*, two lactobacilli strains (*Lactobacillus reuteri* and *Lactobacillus johnsonii*) were chosen from lactobacilli isolated from gerbil 1-2 and three different lactobacilli strains (*L. reuteri*, *Lactobacillus murinus* and *L. johnsonii*) were chosen from gerbil 1-5 as representative strains with inhibitory activity against *H. pylori*.

**Growth inhibition of *H. pylori* TK1402 using brain heart infusion (BHI) broth culture filtrates of gastric mucosa samples of Group 1 gerbils.** The growth-inhibitory effect on *H. pylori* TK1402 was analysed using BHI broth culture filtrates of the gastric mucosa samples of the gerbils in Group 1. *H. pylori* TK1402 was cultured overnight and 32-fold dilution of BHI broth culture filtrates of gastric mucosa samples of Group 1 gerbils was prepared with 7 % horse serum. An aliquot of 50 µl of suspension of the pre cultured *H. pylori* was inoculated in 100 µl of diluted samples into 96-well plates after setting the final OD₅₉₀ as 0.05. Shaking culture using the culture filtrates of the *H. pylori*-negative gerbils was done for 48 h. After the incubation, OD₅₉₀ of each sample was measured by a microplate reader (Mithras LB940; Berthold Technologies). Growth of *H. pylori* in BHI broth supplemented with 7 % horse serum was used as control.

**Growth inhibition of *H. pylori* TK1402 using MRS broth culture filtrates of lactobacilli strains.** *H. pylori* TK1402 was propagated and transferred once before use. *Brucella* Agar medium supplemented with 7 % horse serum was used for the culture of *H. pylori* TK1402 at 37 °C. The lactobacilli strains were isolated from gastric mucosa samples of gerbil 1-5 of Group 1 and gerbil 2-2 of Group 2. Various dilutions of the MRS broth culture filtrates of five lactobacilli were prepared with 7 % horse serum. An aliquot of 50 µl of suspension of the pre cultured *H. pylori* was inoculated in 100 µl of various diluted samples into 96-well plates after setting the final OD₅₉₀ as 0.05. Shaking culture using the culture filtrates of five lactobacilli was performed for 48 h. After incubation, OD₅₉₀ of each sample was measured by a microplate reader.

**Statistical analysis.** The data were analysed using StatView software. Group differences were tested with Student’s t-test or the χ² test. P<0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Quantification of *H. pylori* and lactobacilli by real-time PCR and isolation of gastric bacteria**

In the gastric mucosa samples of the Group 1 gerbils, no *H. pylori* was observed in three gerbils (1-2, 1-4 and 1-5), although two gerbils (1-1 and 1-3) showed the presence of
The presence of *H. pylori* was observed in four (2-2, 2-3, 2-4 and 2-6) of six gerbils in Group 2 inoculated with the gastric mucosa samples of *H. pylori*-positive gerbils, but another two gerbils (2-1 and 2-5) showed no colonization by *H. pylori*. There was no significant difference in the number of *H. pylori* as determined by real-time PCR between Groups 1 and 2. The colonization rate of *H. pylori* in Group 1 gerbils (2/5, 40 %) was lower than that in Group 2 gerbils (4/6, 67 %).

For the inoculation of gerbils, *H. pylori* TK1402 was selected as described previously (Nakagawa et al., 2005). In this study, we used Mongolian gerbils to analyse the microflora in the stomach of the gerbil infected with *H. pylori* previously (Zaman et al., 2010). Marchetti et al. (1995) reported the successful colonization by clinical isolates of *H. pylori* strains, but not NCTC 11637 (an established laboratory strain), in conventional and specific-pathogen-free mice. Oral administration of *H. pylori* TK1402 induced colonization and gastric inflammation of the stomach of Mongolian gerbils (Nakagawa et al., 2005). The difficulty of colonization in experimental animals such as piglets and mice by *H. pylori*, except when maintained in germ-free and decontaminated conditions, has been reported previously (Krakowka et al., 1987; Ohnishi, 1996).

There was no significant difference in the number of lactobacilli between Group 1 (1-1, 1-2, 1-3 and 1-5) and Group 2 (2-1–2-6) gerbils (Table 1). This result shows *Lactobacillus* spp. to be the dominant bacteria in the stomach of Mongolian gerbils as the presence of lactobacilli was observed in both groups of gerbils. The difficulty of colonization by *H. pylori* in conventional mice may be explained by the large number of indigenous lactobacilli in their stomachs (Kabir et al., 1997). However, a very small number of lactobacilli inhabiting the stomachs of humans may permit *H. pylori* to colonize this organ easily.

*Lactobacilli* are components of the normal intestinal flora of healthy humans that exert antagonistic activities against pathogens. The reason for the presence of lactobacilli in both of the two groups of gerbils is not clear yet. The composition of the gastric microflora, including lactobacilli, may have an influence on the colonization of *H. pylori*.

In particular, it is known that the primary microorganisms associated with the stomach belong to the genus *Lactobacillus*. *Lactobacillus* shows a particular capacity to survive and develop in an acidic environment, and can live as an indigenous bacterium in the gastric mucosa, which can effectively inhibit the colonization of *H. pylori* (Peek, 2008; Chen et al., 2012). In other words, it is rational to prevent and control *H. pylori* infection by regulating the balance of the flora in the stomach. Thus, *Lactobacillus* can be a choice to replace antibiotics or as an adjuvant to antibiotics in treating *H. pylori* infection.

### Table 1. Detection of *H. pylori* in Group 1 and Group 2 gerbils

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th></th>
<th>Group 2</th>
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<tbody>
<tr>
<td></td>
<td>1-1</td>
<td>1-2</td>
<td>1-3</td>
<td>1-4</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>52.8</td>
<td>59.3</td>
<td>53.0</td>
<td>58.5</td>
</tr>
<tr>
<td>Stomach weight (g)</td>
<td>1.9</td>
<td>2.0</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Gastric pH</td>
<td>2.5</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>No. (log_{10}) <em>H. pylori</em> (real-time PCR)</td>
<td>5.31</td>
<td>ND</td>
<td>4.31</td>
<td>ND</td>
</tr>
<tr>
<td>No. (log_{10}) of <em>Lactobacillus</em> spp. g⁻¹ mucus (real-time PCR)</td>
<td>10.58</td>
<td>12.18</td>
<td>10.76</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detected.

**Stomach weights and gastric pH of Mongolian gerbils after inoculation with the gastric mucosa samples**

Although there was some variation of the pH, there was no significant difference in the pH between the two groups (Fig. 1a). The stomach weight of Group 1 gerbils was significantly lower than that of Group 2 gerbils (Fig. 1b). Although the reason for the difference is unclear, it is possible that colonization of *H. pylori* in the gastric mucosa induced various inflammatory changes, including cell...
Table 2. Identification of facultative anaerobes isolated by aerobic culture from the gastric mucosa samples of Mongolian gerbils

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
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<tbody>
<tr>
<td>1-1</td>
<td>E. coli Kluyvera spp.</td>
</tr>
<tr>
<td>1-2</td>
<td>E. coli Kluyvera spp.</td>
</tr>
<tr>
<td>1-3</td>
<td>E. coli Kluyvera spp.</td>
</tr>
<tr>
<td>1-4</td>
<td>E. coli Kluyvera spp.</td>
</tr>
<tr>
<td>1-5</td>
<td>E. coli Kluyvera spp.</td>
</tr>
</tbody>
</table>

Identification of different gastric bacteria after isolation from the gastric mucosa samples

Isolated facultative anaerobic bacteria were identified by API 20E (Table 2). E. coli were isolated from 10 out of 11 gerbils (except gerbil 2-5) and Kluyvera spp. were isolated from nine out of 11 gerbils (except gerbils 1-3 and 2-1).

Using anaerobic cultivation of the gastric mucosa of the 11 gerbils, bacterial colonies were formed from the gastric mucosa samples of all of the gerbils (Table 3).

Three species of lactobacilli were isolated from the gastric mucosa samples, and they were identified by genome sequencing as L. murinus, L. reuteri and L. johnsonii. Table 3 shows three different strains of lactobacilli (L. murinus, L. reuteri and L. johnsonii) in Group 1 gerbils and two strains of lactobacilli (L. reuteri and L. johnsonii) in Group 2 gerbils. Some of the bacterial strains could not be to be determined (shown as undetermined in Table 3). Although L. reuteri and L. johnsonii strains were isolated in all Group 1 and Group 2 gerbils, L. murinus was present only in gerbil 1-5. According to the genome sequencing, L. reuteri was grouped into two subtypes: LR1 and LR2 (Fig. 2). There were several differences in base sequences in the 16S rRNA gene between LR1 and LR2 subtypes of L. reuteri. In contrast, there was no difference in the 16S rRNA gene sequence of L. johnsonii strains. Sequencing of 16S rRNA genes that were PCR-amplified from DNA extracted from the biopsy samples was performed to confirm the identities of different bacterial species.

The bacterial species isolated differed between the previous study (Actinomyces spp. or Bifidobacterium spp.) and the present study. The reason for the difference is not clear, but the following two possibilities are suggested. (1) The identification method was different; we used the API system previously, but real-time PCR in this study. It is well known that the gene sequencing method is more reliable than the API system for the identification of bacteria. We have already obtained a result where the three strains of L. reuteri, L. johnsonii and L. murinus identified by genome sequencing were identified as Actinomyces spp. or Bifidobacterium spp. in the API system (data not shown). (2) Inoculation with the gastric mucosa samples may eliminate gastric bacteria other than lactobacilli in our present study.

BHI broth culture filtrates of gastric mucosa samples of Group 1 gerbils exhibit growth inhibition of H. pylori

On the basis of the results of the real-time PCR (Table 1), gerbils 1-2, 1-4 and 1-5 were determined as H. pylori-

Table 3. Identification of different lactobacilli isolated by anaerobic culture from the gastric mucosa samples of Mongolian gerbils

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Gerbil</th>
<th>Isolated lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>L. reuteri*,‡</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>1-2</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>1-3</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>1-4</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>1-5</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>2-1</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>2-2</td>
<td>–</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>2-3</td>
<td>–</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>2-4</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>2-5</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
<tr>
<td>2-6</td>
<td>L. reuteri</td>
<td>L. johnsonii</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Gerbil</th>
<th>Isolated lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>L. reuteri*,‡</td>
<td>Undetermined</td>
</tr>
<tr>
<td>2-2</td>
<td>–</td>
<td>L. reuteri2†,‡</td>
</tr>
<tr>
<td>2-3</td>
<td>–</td>
<td>L. reuteri2</td>
</tr>
<tr>
<td>2-4</td>
<td>L. reuteri</td>
<td>L. reuteri2</td>
</tr>
<tr>
<td>2-5</td>
<td>L. reuteri</td>
<td>L. reuteri2</td>
</tr>
<tr>
<td>2-6</td>
<td>L. reuteri</td>
<td>L. reuteri2</td>
</tr>
</tbody>
</table>

*Subtype 1 according to genomic sequence.
‡Subtype 2 according to genomic sequence.
†Difference in genomic sequence was detected between L. reuteri1 and L. reuteri2.
negative among the five gerbils in Group 1. As the colonization rate of \(H.\) \(pylori\) in gerbils 1-1 and 1-3 pretreated with \(H.\) \(pylori\)-negative gastric mucosa samples was relatively low, the direct effect of the gastric mucosa samples of the five gerbils of Group 1 on the growth of \(H.\) \(pylori\) was examined. It is possible that the gastric mucosa samples may have an inhibitory effect on the growth of \(H.\) \(pylori\). It was shown that the culture filtrates (1:32 dilution) of two gerbils (1-2 and 1-5) inhibited significantly the growth of \(H.\) \(pylori\) TK1402 (Fig. 3). In particular, it was indicated that the gastric mucosa sample of gerbil 1-5 exhibited the strongest inhibitory effect on the growth of \(H.\) \(pylori\); the gastric mucosa sample of gerbil 1-2 also exhibited a strong inhibitory effect.

**Inhibition of growth of \(H.\) \(pylori\) TK1402 by culture filtrates of five lactobacilli strains**

The inhibitory effects of the five lactobacilli strains were clarified through the use of the growth inhibition assay using \(H.\) \(pylori\) TK1402 with MRS broth culture filtrates of these lactobacilli with 7% horse serum (Fig. 4). The growth of \(H.\) \(pylori\) co-cultured with the culture filtrates of five

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**Fig. 2.** Sequencing analysis of \(L.\) \(reuteri\)1 (LR1) and \(L.\) \(reuteri\)2 (LR2) isolated from different Mongolian gerbils. LR1-4, LR1 isolated from gerbil 1-4; LR1-5, LR1 isolated from gerbil 1-5; LR2-7, LR2 isolated from gerbil 2-2; LR2-8, LR2 isolated from gerbil 2-3.

**Fig. 3.** Growth inhibition of \(H.\) \(pylori\) by culture filtrates (BHI broth) of Group 1 Mongolian gerbils.
lactobacilli was inhibited compared with the control (MRS broth only) on the basis of the inhibition of bacterial growth (OD₅₉₅). The substances of the culture filtrates of lactobacilli may have a suppressive effect on the growth of *H. pylori*. The inhospitable acidic milieu in the stomach provides an effective barrier, killing many of the microbes that enter the gastrointestinal tract. It was shown in various studies that *Lactobacillus* spp. were the dominant bacteria in the stomach of *H. pylori*-infected, as well as control, gerbils (Osaki et al., 2012; Sun et al., 2003). *L. gasseri* and *L. reuteri*, which are present in the stomach of most Mongolian gerbils, were also shown to inhibit the growth of some *H. pylori* strains. *L. gasseri* OLL 2716 promoted the elimination of *H. pylori* in humans (Johnson-Henry et al., 2004) and exerted a protective effect against the generation of lesions in a rat gastric ulcer model. In addition, these probiotic bacteria can be used to supplement eradication therapy for patients with *H. pylori* infection, either to increase the eradication rate or to prevent the occurrence of the side-effects of the antimicrobial drugs (Mégraud, 2004), and are utilized in yogurts that are specifically labelled as health foods (Wang et al., 2004).

Eradication therapy of *H. pylori* infection by triple association of two antibiotics and a proton pump inhibitor has been reported. However, the treatment may fail in 10–35% of cases due to *H. pylori* resistance, antibiotic side-effects and other reasons (Tursi et al., 2004). The search for new or additional therapeutic agents is necessary to overcome treatment failure. Probiotic bacteria are defined as commensals that, when administered to humans, have an inherent benefit over and above nutrition (Guarner & Schaafsma, 1998). Some probiotics, including lactobacilli, have been shown previously to decrease inflammatory markers in *H. pylori* infection models both *in vitro* and *in vivo* (Johnson-Henry et al., 2004; Tamura et al., 2006).

We studied the microbial ecology in the stomach of Mongolian gerbils with *H. pylori* infection. Observations in

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**Fig. 4.** Growth inhibition assay of *H. pylori* TK1402 by culture filtrates (MRS broth) of five lactobacilli at various dilutions: (a) *L. murinus* isolated from gerbil 1-5, (b) *L. reuteri* isolated from gerbil 1-5, (c) *L. johnsonii* isolated from gerbil 1-5, (d) *L. reuteri* isolated from gerbil 1-2, (e) *L. johnsonii* isolated from gerbil 1-2 and (f) control (MRS broth).
vitro indicated that spent supernatant of *L. acidophilus* La1 and *L. reuteri* contained a bactericidal activity effective on *H. pylori* (Michetti et al., 1999; Ojetti et al., 2012). Non-pathogenic lactobacilli have been used for decades because of their health benefits and their ability to increase resistance to infectious disease (Gill et al., 2000; Chen et al., 2012). Eradication of *H. pylori* was recently shown to decrease the incidence of gastric cancer (Tatematsu et al., 2007; Fukase et al., 2008). These studies suggest that different lactobacilli might be effective in producing a suppressive activity against *H. pylori*. Further studies found that lactobacilli can also have a beneficial effect in conjunction with standard antibiotic based triple therapies against *H. pylori*. Future research is required to clarify the suppressive effect of these microflora against *H. pylori* colonization.

**CONCLUSIONS**

On the basis of the result of the real-time PCR measurements of the number of lactobacilli per gram of gastric mucosa samples, the presence of lactobacilli was observed in almost all of the gerbils, except in one gerbil, although there was no significant difference in the number of lactobacilli between the two groups (Groups 1 and 2) used in this study. Some strains of lactobacilli can colonize the gastric mucosa and exhibit anti- *H. pylori* colonization. Due to differences in species and specificity, these strains can lead to different anti- *H. pylori* activities. Although different strains of lactobacilli, such as *L. murinus*, *L. reuteri* and *L. johnsonii*, were isolated from the Mongolian gerbil gastric mucosa samples, the three strains did not display the same anti- *H. pylori* activity.

The results of the present study suggest that lactobacilli are the dominant gastric microflora of Mongolian gerbils and three different lactobacilli exerted strong growth-inhibitory effects against *H. pylori* in the *in vitro* study. *Lactobacillus* itself is considered to be a harmless organism in the gastric mucosa when it colonizes the stomach. This work provides bacterial targets for further studies on the direct mutual interaction between *H. pylori* and the Mongolian gerbil’s gastric microbiota. Future studies examining the direct effect of *L. murinus*, *L. johnsonii* and *L. reuteri* on the colonization of *H. pylori* in the stomach of Mongolian gerbils are necessary. It would be of great interest to further explore the role of such probiotic strains in the complex regulation of anti- *H. pylori* activities and screen for more efficient potential clinical agents.

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