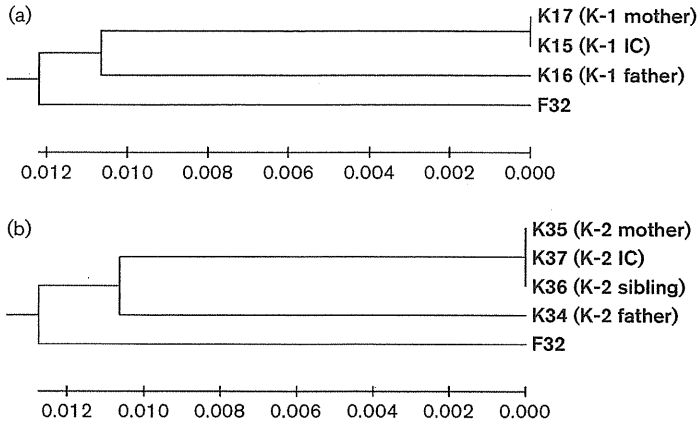


**Fig. 2.** Phylogenetic tree showing the correlation of isolates from family K-1 (a) and family K-2 (b) based on sequence arrangements of seven loci. Paternal isolates are K17 and K34 displayed as K-1 father and K-2 father, respectively. Maternal isolates are K17 and K35 displayed as K-1 mother and K-2 mother, respectively. The K-2 sibling isolate is K37 displayed as K-2 sibling. The index child (IC) isolates are K15 and K37 displayed as K-1 IC and K-2 IC. The genome sequence of Japanese *H. pylori* strain F32 was used for the comparison as a control and displayed as F32. The scale bar indicates distance between the strains tested (substitutions/site).

The MLST results were confirmed by RAPD fingerprinting (Fig. S1). There was no discrepancy between the results from MLST and RAPD-PCR.

## DISCUSSION

MLST is a standard method of molecular typing for pathogenic and non-pathogenic bacteria. We have conducted this study using MLST to clarify the mode of intra-familial transmission among several *H. pylori*-positive family members with an index child.

In this study, four cases (families K-1, K-2, K-3 and K-4) of mother-to-child transmission were demonstrated in five families. The alleles of all loci in *H. pylori* isolates matched almost those of all family members including the father and the sibling in family K-3 and K-4, therefore the possibility of paternal or sibling infection cannot be excluded in two of the cases. There have been several studies concerning the intra-familial transmission of *H. pylori* evaluated by molecular analysis (Georgopoulos *et al.*, 1996; Han *et al.*, 2000; Kivi *et al.*, 2003; Konno *et al.*, 2005, 2008; Nahar *et al.*, 2009; Nwokolo *et al.*, 1992). The major causative mode of infection was suggested to be mother-to-child transmission in these studies and our previous study (Osaki *et al.*, 2013). Father-to-child transmission was not detected clearly, as the father and the mother had similar genotypes of *H. pylori* in families K-3 and K-4. It has been previously reported that maternal, paternal and sibling infection are all strongly and significantly related to infection of the child with *H. pylori* in bivariate analyses (Weyermann *et al.*, 2009). Phylogenetic analysis revealed *H. pylori* isolated from the father of the K-3 (K26) and K-4 (K29) family were located upstream from the each node compared to *H. pylori* strains from other family members (family K-3, K27 and K28; family K-4, K30 and K33), implying that the father was infected with the original *H. pylori* strain.

In two families (K-3 and K-4), the original *H. pylori* strain was not only related to infection in the children but also inter-spousally. A previous study reported this type of transmission previously by using *H. pylori* 16S rRNA ribotyping, showing that eight of 18 couples were colonized with a single *H. pylori* strain (Georgopoulos *et al.*, 1996). An additional study reported six cases of inter-spousal infection using RAPD-fingerprinting analysis (Konno *et al.*, 2008). It is difficult to clarify the time when inter-spousal infection occurred, but it is unlikely that the couples were

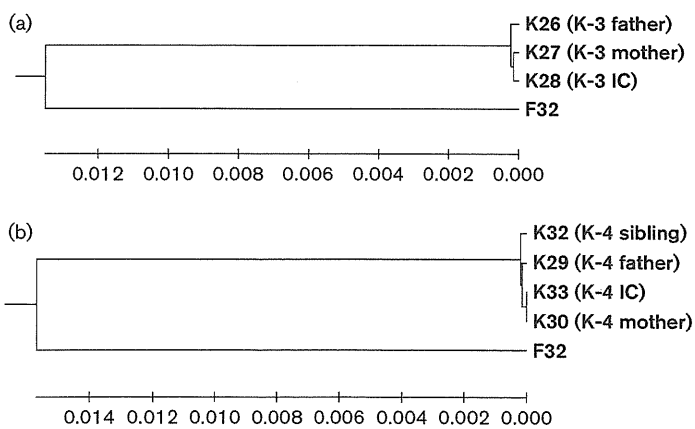


**Fig. 3.** Phylogenetic trees showing the correlation of the isolates from family K-3 (a) and family K-4 (b) based on sequence arrangements of seven loci. Paternal isolates are K26 and K29, displayed as K-3 father and K-4 father. Maternal isolates are K27 and K30 displayed as K-3 mother and K-4 mother. The K-4 sibling isolate is K32 displayed as K-4 sibling. The index child isolates (IC) are K28 and K33 displayed as K-3 IC and K-4 IC. The genome sequence of Japanese *H. pylori* strain F32 was used for the comparison as a control and displayed as F32. The scale bar indicates distance between the strains tested (substitutions/site).

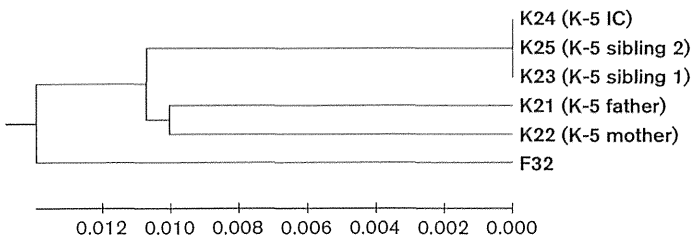


**Fig. 4.** Phylogenetic tree showing the correlation of isolates from family K-5 based on sequence arrangements of seven loci. Paternal isolate is K21 displayed as K-5 father. Maternal isolate is K22 displayed as K-5 mother. The K-5 sibling isolates are K23 and K25 displayed as K5 sibling 1 and K5 sibling 2. The index child isolate is K24 displayed as K-5 IC. The genome sequence of Japanese *H. pylori* strain F32 was used for the comparison as a control and displayed as F32. The scale bar indicates distance between the strains tested (substitutions/site).

independently infected with the same *H. pylori* strain. It is well known that recombination occurs frequently in the *H. pylori* genome (Suerbaum *et al.*, 1998). If the couple were infected with same *H. pylori* strain before marriage, their *H. pylori* strains would not keep the same genotype and therefore it is reasonable to assume the transmission of *H. pylori* between parents occurred recently.

Sibling-to-sibling(s) transmission of *H. pylori* was found in family K-5, but parent-to-child transmission was not. In this case, it is suggested that the isolates from the children originated from outside the family. There are several hypotheses as to the sources, including a study in which *H. pylori* DNA was detected in drinking water (Fujimura *et al.*, 2008). In this family, the origin of infection and the reason for the lack of parent-to-child transmission were not clear.

In Japan, oral transmission through saliva and gastric juice is thought to be the main cause of *H. pylori* infection. Japanese families have customarily fed their children pre-chewed food. It has been reported that use of soothers or bottle teats is closely related with *H. pylori* transmission in Canadian children (Sinha *et al.*, 2004). In contrast, it was reported that feeding infants food first chewed by a parent did not affect *H. pylori* status (Kurosawa *et al.*, 2000). On the other hand, children vomit more frequently and *H. pylori* strains from a young child with vomiting can be transmitted to other family members. Siblings may therefore play an important role in *H. pylori* transmission among children (Fialho *et al.*, 2010). Faecal–oral transmission is another route of infection related to sanitary conditions; water supply and sewage are considered important factors for *H. pylori* infection (Goh *et al.*, 2011). After the Second World War, sanitary conditions improved in Japan and the prevalence of *H. pylori* infection decreased (Shiota *et al.*, 2010).

In this study, sibling-to-sibling transmission of *H. pylori* without maternal or paternal infection was detected. The type of transmission of *H. pylori* has been reported in several articles (Garg *et al.*, 2006; Miehlke *et al.*, 1999). However, mother-to-child transmission was also found in these cases, and it was therefore difficult to clarify the transmission route of *H. pylori* in these families. Households with many children have been shown to be one of the risk factors for *H. pylori* infection (Fiedorek *et al.*, 1991). In Japan, the average number of children per household is 1.70 in 2010, and the number of children in family K-5 was higher than the average. Although *H. pylori* infection from the elder-to-younger sibling was most likely, the origin of the strain isolated from the index child was unclear.

Several SNPs (single nucleotide polymorphisms) were found by comparing all MLST gene sequences. Deletion and transformation of other sequences was not detected. It was shown that the SNPs were derived from the original strain during a long infection period after transmission of *H. pylori* (Raymond *et al.*, 2004). According to Graphical review of Japanese households-from comprehensive survey of living conditions, 2010, edt. Japanese Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/tokei/list/dl/20-21-01.pdf).

It is well known that clinical strains of *H. pylori* have numeric diversity. Although the genotype of intra-familial transmitted strains were closely related each other, it was shown that K16 (K-1 family), K34 (K-2 family), K21 and K22 (K-5 family) had different allele types in the seven loci tested, compared to the isolates from other family members. These results showed that similar molecular type strains were detected in the cases of intra-familial transmission than in cases with other infection routes. In the phylogenetic study, we found similar strains were present in each family (Figs 2, 3, 4 and S2). These results support the notion that the same original strain was transmitted to family members.

Another possibility is that family members were infected with two or more strains at the same time. There have been several reports of multiple infections (Hirschl *et al.*, 1994; Fiedorek *et al.*, 1991; Miehlke *et al.*, 1999; Raymond *et al.*, 2004) with different types of *H. pylori* strains in a single individual. On study reported the examination of clonal diversity by RAPD fingerprinting method (Toita *et al.*, 2013); the isolates obtained from several patients at 5 to 9 year intervals showed identical or very similar RAPD patterns. It was concluded that each Japanese individual of an urban population is predominantly infected with a

single *H. pylori* clone (Toita *et al.*, 2013). As we used single colony isolates of *H. pylori* from each family member and MLST analysis was performed for this isolate, mixed infection of *H. pylori* could not be detected. Two different strains of *H. pylori* were isolated from the same individuals at different sampling times, but all isolates derived from a single individual showed the same MLST in this study (data not shown). The dominant population of *H. pylori* may be determined by microbiota environmental factors. In our previous study, the composition of gastric indigenous microbiota in Mongolian gerbils may be disturbed by long-term infection with *H. pylori*, and that these changes may in fact inhibit *H. pylori* infection (Osaki, *et al.*, 2012). Further study is necessary to clarify the mechanism of intra-familial infection.

In conclusion, person-to-person transmission between family members was detected frequently in this study, with mother-to-child, parent-to-child, intra-spousal and sibling(s)-to-sibling transmissions being demonstrated. This may be the predominant mode of *H. pylori* transmission in Japan.

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# 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 uninfectivity 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 (Krausse *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 % $N_2$, 10 % $CO_2$ and 5 % $O_2$.

**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 ($1 \times 10^9$ 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 $1 \times 10^9$ 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 Beader (Wakenyaku). 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′-CGCTAAGAGATCAGCCTATGTCC-3′) and HP-16-R (5′-CCGTGTCTCAGTTCCAGTGTGT-3′), for the detection of *H. pylori* (Osaki *et al.*, 2006), and g-Lact-F (5′-ACCA-CAGTCCATGCCATCAC-3′) and g-Lact-R (5′-TCCACCACCCT-GTTGCTGTA-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 $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 µM, a 1 : 75 000 dilution of SYBR Green I, 11 ng *Taq* Start antibody (Clontech) $\mu l^{-1}$, 0.005 U *Taq* DNA polymerase (Takara) $\mu l^{-1}$, 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 20 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$^T$. 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 16SrRNA 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 anaero 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 (bioMérieux), 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 % $N_2$, 10 % $H_2$ and 10 % $CO_2$ 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 cycler (Gene Amp PCR system 9600-R; PerkinElmer). We used two universal primers (27F, 5′-AGAGTTTGATCMTGGC-TCAG-3′; 1492R, 5′-TACGGYTACCTTGTTACGACTT-3′) (DeLong, 1992) and two primers (518F, 5′-CCAGCAGCCGCGGTAATACG-3′; 800R, 5′-TACCAGGGTATCTAATCC-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 800R). 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 % glycerine (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 anaero incubator (Hirasawa) for 48 h. Liquid culture of lactobacilli was performed using MRS broth (Oxoid) at 37 °C in 5 % $CO_2$ 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 precultured *H. pylori* was inoculated in 100 μl of diluted samples into 96-well plates after setting the final $OD_{595}$ as 0.05. Shaking culture using the culture filtrates of the *H. pylori*-negative gerbils was done for 48 h. After the incubation, $OD_{595}$ 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 *Brucella* Broth containing 7 % horse serum. MRS broth was used as control to compare the inhibitory effect of this broth and it was also diluted with *Brucella* Broth containing 7 % horse serum. An aliquot of 50 μl of suspension of the precultured *H. pylori* was inoculated in 100 μl of various diluted samples into 96-well plates after setting the final $OD_{595}$ as 0.050. Shaking culture using the culture filtrates of five lactobacilli was performed for 48 h. After incubation, $OD_{595}$ 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 $\chi^2$ 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

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

| | Group 1 | | | | | Group 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1-1 | 1-2 | 1-3 | 1-4 | 1-5 | 2-1 | 2-2 | 2-3 | 2-4 | 2-5 | 2-6 |
| Body weight (g) | 52.8 | 59.3 | 53.0 | 58.5 | 52.9 | 64.5 | 59.0 | 62.0 | 63.5 | 63.5 | 58.5 |
| Stomach weight (g) | 1.9 | 2.0 | 1.9 | 1.7 | 1.8 | 2.8 | 2.3 | 2.7 | 2.6 | 2.3 | 2.5 |
| Gastric pH | 2.5 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 4.5 | 4.5 | 4.5 | 3.0 |
| No. ($log_{10}$) *H. pylori* (real-time PCR) | 5.31 | ND | 4.31 | ND | ND | ND | 9.56 | 4.42 | 4.54 | ND | 4.79 |
| No. ($log_{10}$) of *Lactobacillus* spp. $g^{-1}$ mucus (real-time PCR) | 10.58 | 12.18 | 10.76 | ND | 10.80 | 8.78 | 10.65 | 11.40 | 10.97 | 11.18 | 9.77 |

ND, Not detected.

*H. pylori* by real-time PCR (Table 1). 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 micro-organisms 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.

## 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

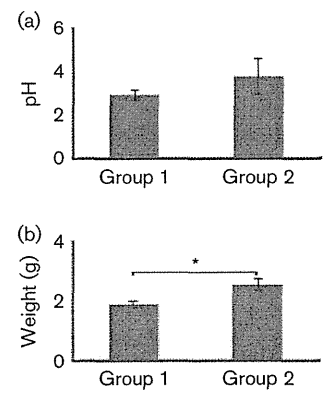


**Fig. 1.** Measurement of (a) pH and (b) stomach weight of Group 1 and 2 Mongolian gerbils after inoculation with gastric mucosa samples.

**Table 2.** Identification of facultative anaerobes isolated by aerobic culture from the gastric mucosa samples of Mongolian gerbils

| Group 1 | | | | | Group 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1-1 | 1-2 | 1-3 | 1-4 | 1-5 | 2-1 | 2-2 | 2-3 | 2-4 | 2-5 | 2-6 |
| *E. coli* *Kluyvera* spp. | *E. coli* *Kluyvera* spp. | *E. coli* | *E. coli* *Kluyvera* spp. | *E. coli* *Kluyvera* spp. | *E. coli* | *E. coli* *Kluyvera* spp. | *E. coli* *Kluyvera* spp. | *E. coli* *Kluyvera* spp. | *Kluyvera* spp. | *E. coli* *Kluyvera* spp. |

filtration (Mishra & Panigrahi, 2011) and edematous changes, resulting in the weight variations.

## 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

| Group | Gerbil | Isolated lactobacilli | | | |
|---|---|---|---|---|---|
| Group 1 | 1-1 | *L. reuteri*1*,‡ | *L. johnsonii* | Undetermined | – |
| | 1-2 | *L. reuteri*1 | *L. johnsonii* | – | – |
| | 1-3 | *L. reuteri*1 | *L. johnsonii* | – | *L. reuteri*2†,‡ |
| | 1-4 | *L. reuteri*1 | *L. johnsonii* | Undetermined | *L. reuteri*2 |
| | 1-5 | *L. reuteri*1 | *L. johnsonii* | Undetermined | *L. murinus* |
| Group 2 | 2-1 | *L. reuteri*1 | *L. johnsonii* | Undetermined | – |
| | 2-2 | – | *L. johnsonii* | Undetermined | *L. reuteri*2 |
| | 2-3 | – | *L. johnsonii* | Undetermined | *L. reuteri*2 |
| | 2-4 | *L. reuteri*1 | *L. johnsonii* | Undetermined | – |
| | 2-5 | – | *L. johnsonii* | *Lactobacillus* spp. | *L. reuteri*2 |
| | 2-6 | *L. reuteri*1 | *L. johnsonii* | – | – |

*Subtype 1 according to genomic sequence.
†Subtype 2 according to genomic sequence.
‡Difference in genomic sequence was detected between *L. reuteri*1 and *L. reuteri*2.

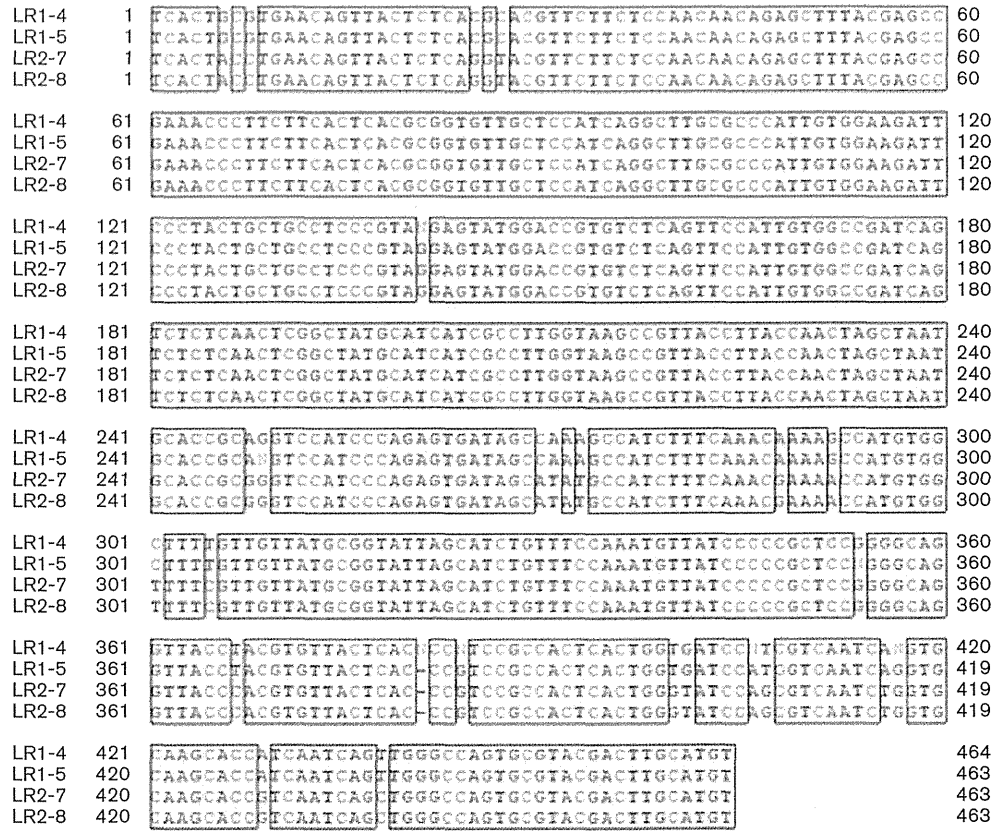

**Fig. 2.** Sequencing analysis of *L. reuteri*1 (LR1) and *L. reuteri*2 (LR2) isolated from different Mongolian gerbils. LR1-4, LR1 isolated from gerbil 1-4; LR1-5, LR1 isolated from gerbil 1-5; LR2-7, LR2 isolated from gerbil 2-2; LR2-8, LR2 isolated from gerbil 2-3.

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.

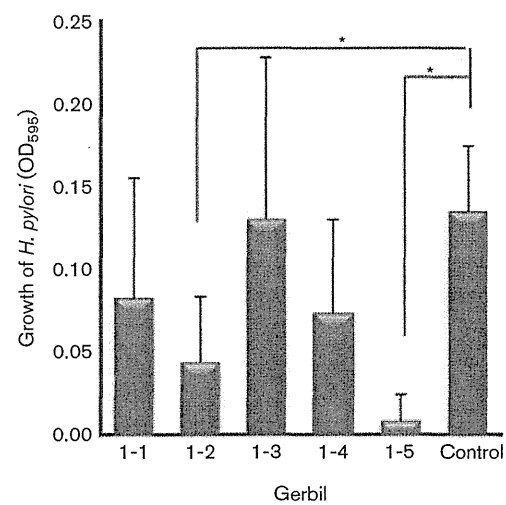


**Fig. 3.** Growth inhibition of *H. pylori* by culture filtrates (BHI broth) of Group 1 Mongolian gerbils.

## 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

lactobacilli was inhibited compared with the control (MRS broth only) on the basis of the inhibition of bacterial growth ($OD_{595}$). 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*

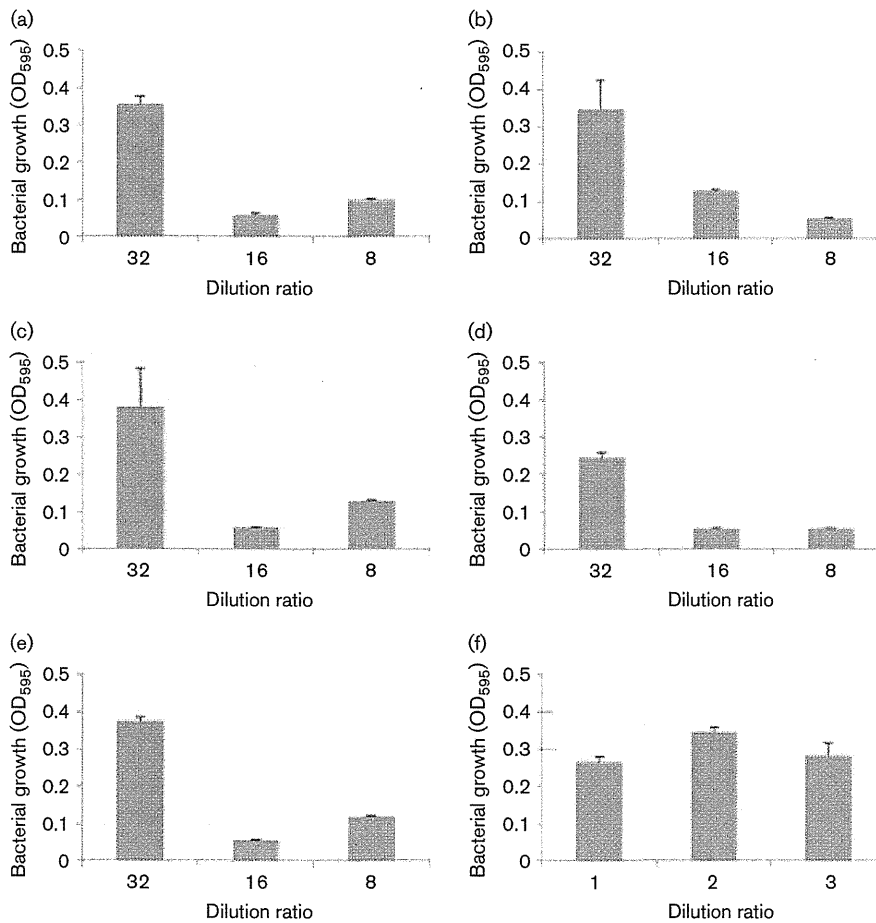


**Fig. 4.** Growth inhibition assay of *H. pylori* TK1402 by culture filtrates (MRS broth) of five lactobacilli at various dilutions: (a) *L. murinus* isolated from gerbil 1-5, (b) *L. reuteri* isolated from gerbil 1-5, (c) *L. johnsonii* isolated from gerbil 1-5, (d) *L. reuteri* isolated from gerbil 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 illness (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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# Development of New PCR Primers by Comparative Genomics for the Detection of *Helicobacter suis* in Gastric Biopsy Specimens

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**Keywords**
*Helicobacter suis*, next-generation sequencing, comparative genomics, gastric biopsy specimen, mouse infection model, PCR diagnosis.

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## Abstract

**Background:** Although the infection rate of *Helicobacter suis* is significantly lower than that of *Helicobacter pylori*, the *H. suis* infection is associated with a high rate of gastric mucosa-associated lymphoid tissue (MALT) lymphoma. In addition, in vitro cultivation of *H. suis* remains difficult, and some *H. suis*-infected patients show negative results on the urea breath test (UBT).

**Materials and Methods:** Female C57BL/6J mice were orally inoculated with mouse gastric mucosal homogenates containing *H. suis* strains TKY or SNTW101 isolated from a cynomolgus monkey or a patient suffering from nodular gastritis, respectively. The high-purity chromosomal DNA samples of *H. suis* strains TKY and SNTW101 were prepared from the infected mouse gastric mucosa. The SOLiD sequencing of two *H. suis* genomes enabled comparative genomics of 20 *Helicobacter* and 11 *Campylobacter* strains for the identification of the *H. suis*-specific nucleotide sequences.

**Results:** Oral inoculation with mouse gastric mucosal homogenates containing *H. suis* strains TKY and SNTW101 induced gastric MALT lymphoma and the formation of gastric lymphoid follicles, respectively, in C57BL/6J mice. Two conserved nucleotide sequences among six *H. suis* strains were identified and were used to design diagnostic PCR primers for the detection of *H. suis*.

**Conclusions:** There was a strong association between the *H. suis* infection and gastric diseases in the C57BL/6 mouse model. PCR diagnosis using an *H. suis*-specific primer pair is a valuable method for detecting *H. suis* in gastric biopsy specimens.

*Helicobacter pylori* infection is involved in the pathogenesis of several gastric diseases in humans, such as gastric and peptic ulceration, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and stomach cancer [1]. It is presumed that the gastric *H. pylori* infection is acquired primarily in infancy, as *H. pylori* is strictly transmitted from human to human. In contrast, the infection with *Helicobacter heilmannii* types 1 and 2 and its phylogenetically related *Helicobacter* species (*H. heilmannii*-like organisms, HHLOs; non-*H. pylori Helicobacter* species, NHPHS) is strongly associated with contact at any age with wild, domestic, or companion animals, as these bacteria have a large number of known mammalian host species [2–4]. *Helicobacter heilmannii* type 1



 

represents a single *Helicobacter* species, namely *H. suis*, whereas *H. heilmannii* type 2 represents a group of species, including *H. felis*, *H. bizzozeronii*, *H. salmonis*, and *H. heilmannii* [4,5]. Accordingly, to avoid confusion in the nomenclature, the terms *H. heilmannii* sensu lato (*H. heilmannii* s.l.) and *H. heilmannii* sensu stricto (*H. heilmannii* s.s.) were proposed to refer to the HHLOs (NHPHS) and *H. heilmannii*, respectively [6]. Therefore, *H. heilmannii* s.l. currently includes a total of at least seven species, that is, *H. felis*, *H. bizzozeronii*, *H. salmonis*, *H. cynogastricus*, *Helicobacter baculiformis*, *H. suis*, and *H. heilmannii* s.s. Among *H. heilmannii* s.l. species, only *H. bizzozeronii* has been successfully cultivated from human gastric biopsy specimens so far [4].

Epidemiological studies have revealed that although the infection rate of *H. heilmannii* s.l. (0.1–6%) [7–14] is significantly lower than that of *H. pylori* (about 50% lower), the infection rate of *H. heilmannii* s.l. seems to mostly depend on the geographical, national, or socioeconomic status of the patients in addition to the detection technology [7,9,13,15–22]. Even so, the infection with *H. heilmannii* s.l. induces a high prevalence of gastric MALT lymphoma [12,23]. Meanwhile, co-infection with *H. pylori* and *H. heilmannii* s.l. seems to be present in the gastric mucosa of a large percentage of patients [20,24].

It is noteworthy that an oral inoculation with gastric mucosal homogenates containing *H. suis* TKY (isolated from a cynomolgus monkey) induced gastric B-cell MALT lymphoma with nearly 100% probability in the C57BL/6J mouse model [25–27]. Further histochemical studies of this gastric MALT lymphoma revealed that an enhanced immunoreactivity of vascular endothelial growth factor-A (VEGF-A) and VEGF-C was observed in areas encircled by increased parietal cell apoptosis, indicating the pathophysiological relevance of both angiogenesis and apoptosis in the gastric MALT lymphoma formation [28]. It has been a subject of great interest whether *H. suis* isolated from the gastric biopsy specimen of a patient with nodular gastritis is capable of inducing gastric injuries in a mouse model, although this question remains unanswered. The results obtained from the present experiments may therefore lead to a better understanding of the relationship between *H. suis* infection and gastric diseases.

The problem in investigating this issue has been that the rapid diagnosis of *H. suis* infections in patients is a difficult task, because most Japanese patients infected with *H. suis* have shown negative results on the urea breath test (UBT) [9]. In this study, we aimed to create a new PCR assay system based on a whole-genome analysis for the specific detection of *H. suis* in gastric biopsy specimens.

## Methods

### Bacteria

*Helicobacter suis* strains TKY (EMBL/GenBank/DDBJ database accession nos. AB252066 (16S rRNA gene) and AB252065 (*ureA* and *ureB*)) and SNTW101 (EMBL/GenBank/DDBJ database accession no. AB498800 (16S rRNA gene)) isolated from a cynomolgus monkey [26] and a 33-year-old Japanese woman suffering from nodular gastritis (UBT-negative), respectively, were used for the whole-genome sequencing. *H. suis* strains SH8 and SH10 isolated from Japanese men suffering from gastric MALT lymphoma and chronic gastritis, respectively, were used as the reference strains of human origin [9]. These four *H. suis* strains were individually maintained in the stomachs of female C57BL/6J mice, because they have not yet been successfully grown in vitro. The repeated inoculations with gastric mucosal homogenates from infected mice to uninfected mice have been performed at the intervals of 3–6 months. *H. felis* strain ATCC 49179; *Helicobacter mustelae* strain NCTC 12032; and *H. pylori* strains Sydney 1 (SS1), TN2GF4, NCTC 11637, ATCC 43579, and RC-1 were grown in *Brucella* broth (Oxoid Ltd., Hampshire, UK) supplemented with 7% (vol/vol) heat-inactivated fetal calf serum (FCS) under the conditions of humidified 5% $O_2$, 10% $CO_2$, and 85% $N_2$ at 37 °C [29–32]. *Helicobacter suis* strain HS5, *H. heilmannii* s.s. strain ASB1.4, *H. baculiformis* strain M50, *H. bizzozeronii* stain R1051, *H. cynogastricus* strain JKM4, and *Helicobacter salomonis* strain R1053 were individually grown under each culture condition that was previously described [5,33–37].

### *Helicobacter suis* SNTW101 Infection in Mice

Five-week-old female C57BL/6J mice were inoculated with gastric mucosal homogenates from mice originally infected with gastric biopsy specimens containing *H. heilmannii* s.s. SNTW101. At 20 months post-inoculation, the mice were sacrificed and examined histologically. A portion of each tissue sample was fixed with 4% (wt/vol) paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2) and embedded in paraffin. Tissue sections approximately 5 μm thick were prepared and mounted on glass slides. The slides were stained with hematoxylin–eosin (H&E) and scanned with a microscope (Axiovert 135; Carl Zeiss, Oberkochen, Germany). All mice were bred in the animal facility at the Kitasato Institute, and all mouse experiments were performed in accordance with institutional ethical guidelines under an approved study protocol.

### Preparation of *Helicobacter suis* Chromosomal DNA for Whole-Genome Sequencing

The gastric mucosa of mice inoculated with mouse gastric mucosal homogenates containing *H. suis* TKY or SNTW101 was scraped off using a glass slide and homogenized with ice-cold phosphate-buffered saline (PBS; pH 7.4) containing 0.05% (vol/vol) Tween 20 using two glass slides. The mucosal homogenates were then filtered through a cell strainer (40-μm nylon; BD Falcon, Franklin Lakes, NJ, USA) to remove any clumps. To avoid nonspecific adsorption, the filtrates were treated with magnetic beads coated with nonimmune rabbit IgG (Sepa-Max Rabbit IgG; Wako, Osaka, Japan). The nonadsorbed suspensions were then treated with magnetic beads coated with rabbit anti-*H. pylori* antibody (Sepa-Max Hp; Wako) for the separation of *Helicobacter* bacilli. The obtained bacterial cells were suspended with distilled water and lysed by heating for 5 minutes at 95 °C. Finally, the chromosomal DNA was purified by RNase A digestion (final concentration of 10 μg/mL, for 1 hour at 37 °C), followed by phenol/chloroform extraction and ethanol precipitation. The determination of DNA purity was performed by quantitative real-time PCR [26,38] with a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using iQ SYBR green supermix (Bio-Rad) with the following three primer pairs: 341F/519R for the 16S rRNA gene of enteric bacteria [39], HeilF/HeilR for the 16S rRNA gene of *H. suis* [13], and MactinF/MactinR for mouse β-actin [40], as shown in Table 1. The CFX96 run protocol was as follows: a denaturation and hot-start enzyme activation program (95 °C for 3 minutes), an amplification and quantification program repeated 50 times (95 °C for 10 seconds and 60 °C for 15 seconds with a single fluorescence measurement), and a melting curve program (65–95 °C with a heating rate of 0.5 °C/second and continuous fluorescence measurement). The standard dilution series were used on each experimental plate, and semiquantification of 16S rRNA or β-actin gene normalization was based on the averaged results. The amount of the target gene was calculated using standard curves generated from purified *Streptococcus pyogenes* strain GAS472 chromosomal DNA [41]. The yield of bacterial genomic DNA was about 1 μg from a single mouse infected with *H. suis* TKY or SNTW101.

**Table 1** PCR primers used in the present study

| Primer | Sequence (5′–3′) | Gene/Region | References |
|---|---|---|---|
| 341F | ctacgggaggcagcagtggg | Bacterial | [39] |
| 519R | attaccgcggckgctg | 16S rRNA | |
| HeilF | aagtcgaacgatgaagccta | *Helicobacter heilmannii* s.l. | [13] |
| HeilR | atttggtattaatcaccatttc | 16S rRNA | |
| MactinF | ggcaccacacyttctacaatg | mouse | [40] |
| MactinR | ggggtgttgaaggtctcaaac | β-Actin | |
| VAC3624F | gagcgagctatggttatgac | *Helicobacter pylori* | [13] |
| VAC4041R | cattcctaaattggaagcgaa | *vacA* | |
| HH-F5 | tgtcatctaccatgggcttag | *Helicobacter suis* | This study |
| HH-R4 | ggccaaccaaaaccttcagc | Noncoding region | |
| carR2F | tagctctagggtgcagggaata | *H. suis* | This study |
| carR2R | ctcacccacacctgctagtatttt | *carR* | |
| HH292F1 | gatttacccttatgcgatcc | *H. suis* | This study |
| HH292R1 | gatgcattgctctcttagttc | Noncoding region | |
| carRUP | gttttcatctacttgtgcaccca | *H. suis* | This study |
| carRDW | cgtgcccaagaggggtgtatt | *carR* | |

### Whole-Genome Sequencing

Whole-genome sequencing was performed using cycled ligation sequencing on a SOLiD sequencer (Life Technologies, Carlsbad, CA, USA) at the Functional Genomic Facility, National Institute for Basic Biology (NIBB), Okazaki, Japan. Approximately 3–5 μg of the purified bacterial genomic DNA was sheared into 100–150 bp with a Covaris S2 system (Covaris Inc., Woburn, MA, USA) in 120 μL of 10 mmol/L TE buffer in a Covaris microTube using a program consisting of a 20% duty cycle with an intensity of 5 and 200 cycles per burst for 60 seconds at 5 °C. The fragments were arranged into SOLiD barcoded fragment libraries using a SOLiD Fragment Library Construction Kit (Life Technologies). The fragment libraries were then amplified by emulsion PCR (ePCR) at a library concentration of 0.5 pmol/L following the manufacturer's instructions. Template-enriched beads were modified at the 3′ terminus and deposited onto a SOLiD sequencer slide. The slide was then loaded onto a SOLiD four instrument, and paired-end reads of 50 + 35 bp were obtained.

### Sequence Assembly, Gene Identification, and Comparative Analysis

The color-space reads from the SOLiD sequencer were assembled using the SOLiD de novo accessory tools v.2.0 (Denovo 2), which internally use Velvet as an assembly engine combined with pre- and post-processes including error collection, gap filling, and translation from color space to base space. The assembly with the default parameter set gave only very short contigs. We therefore tested combinations of several assembly parameters and picked the one that generated the


 

longest total contig size. The parameter set used here was hsize = 23, cov cutoff = 0.3, maxcov = 1000, and min_pair_count = 3.

The assembled contig sequences were then compared with the published contig sequences of *H. suis* strains HS1 (GenBank accession no. ADGY00000000) and HS5 (GenBank accession no. ADHO00000000) [33] using FASTA. To identify genes, the assembled sequences were subjected to BLASTX searches against 20 *Helicobacter* strains (*H. pylori* 26695, B38, B8, G27, HPAG1, J99, P12, PeCan4, SJM180, and Shi470; *Helicobacter acinonychis* SHeeba; *H. felis* ATCC 49179; *Helicobacter hepaticus* ATCC 51449; *H. mustelae* 12198; *H. bizzozeronii* CIII-1; *H. heilmannii* s.s. ASB1.4; and *H. suis* HS1, HS5, TKY, and SNTW101) and 11 *Campylobacter* strains (*C. jejuni* RM1221, 269.97, 81–176, NCTC 11828, NCTC 11168, and ICDCCJ07001; *C. concisus* 13826; *C. curvus* 525.92; *C. fetus* 82-40; *C. hominis* ATCC BAA-381; and *C. lari* RM2100), and then orthology analysis was conducted using the research environment for comparative genomics (RECOG) system (http://mbgd.genome.ad.jp/RECOG/).

### Preparation of Template DNA for PCR Diagnosis

The chromosomal DNA from each of the isolates *H. suis* HS5, *H. heilmannii* s.s., *H. felis*, *H. baculiformis*, *H. bizzozeronii*, *H. cynogastricus*, *H. mustelae*, *H. salomonis*, or *H. pylori* cultured in the broth; cellular DNA from the gastric mucosa of mice infected with each of *H. suis* TKY, SNTW101, SH8, or SH10; and cellular DNA from each gastric biopsy specimen (14 patients with nodular gastritis including four UBT-negative and 10 UBT-positive cases) were extracted using a DNeasy Blood & Tissue kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's protocol.

### PCR Diagnosis

PCR was performed with a Mastercycler ep (Eppendorf AG, Hamburg, Germany) using *Tfi* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and the following two or three primer pairs: VAC3624F/VAC4041R for the *vacA* gene of *H. pylori* [13]; HH-F5/HH-R4 for the noncoding region; and/or carR2F/carR2R for the *carR* gene of *H. suis* (Table 1). The assay mixture contained, in a final volume of 25 μL, 1× PCR reaction buffer, 200 μM of each dNTP, 1.5 mmol/L $MgCl_2$, 0.2 μM of each primer pair, 10 ng of chromosomal DNA (*H. suis* HS5, *H. heilmannii* s.s., *H. felis*, *H. baculiformis*, *H. bizzozeronii*, *H. cynogastricus*, *H. mustelae*, *H. salomonis*, and *H. pylori*) or 100 ng of cellular DNA (from mouse gastric mucosa infected with *H. suis* or from gastric biopsy specimens), and 1 unit of *Tfi* DNA polymerase. The PCR run protocol was as follows: a denaturation and hot-start enzyme activation program (94 °C for 1 minute), an amplification program repeated 35 times (94 °C for 15 seconds, 57 °C for 30 seconds, and 72 °C for 1 minute), and a final extension (72 °C for 10 minutes). The 5 μL of PCR products was mixed with 1 μL of 6× loading buffer, separated on 2% (wt/vol) agarose gels, and stained with ethidium bromide. This study was approved by the research ethics committees of the Kitasato Institute, Shinshu University School of Medicine, Kyorin University School of Medicine, and Tokyo Women's Medical University.

## Results

### *Helicobacter suis* SNTW101 Infection Induced the Formation of Gastric Lymphoid Follicles in a Mouse Model

We have reported that *H. suis* TKY isolated from a cynomolgus monkey induces gastric MALT lymphoma associated with protrusive lesions in the fundic regions in C57BL/6J mice [3,25–28,42,43]. Similarly, *H. suis* SNTW101 isolated from a patient suffering from nodular gastritis induced a gastric disorder in C57BL/6J mice. As shown in Fig. 1, 20 months after inoculation with gastric mucosal homogenates containing *H. suis* SNTW101, several round protrusive lesions in the fundic

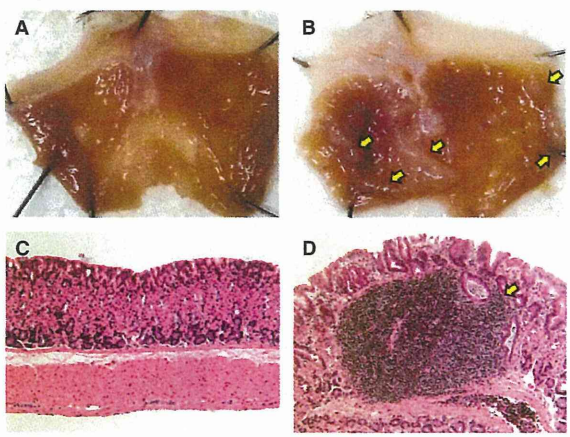


**Figure 1** Representative macroscopic view of the stomach of an age-matched control (*A*) and a gastric homogenate-containing *Helicobacter suis* SNTW101-inoculated mouse (*B*) at 20 months post-inoculation. The representative microscopic appearance of the H&E-stained stomach sections of an age-matched control (*C*) and an *H. suis* SNTW101-infected mouse (*D*) at 20 months post-inoculation is also shown (original magnification, ×100). Arrows indicate the round protrusive lesions (*B*) and lymphoid follicle (*D*).

area of the stomach were visible to the naked eye in all the infected mice (n = 8; Fig. 1B). Indeed, the gastric lymphoid follicles were clearly detected in the gastric mucus layer of infected mice (Fig. 1D). In contrast, no lesions were detected in any of the uninfected mice (n = 6; Fig. 1A,C). This suggests that like *H. suis* TKY, *H. suis* SNTW101 was able to provoke gastric diseases.

### *Helicobacter suis* Chromosomal DNA was Successfully Isolated from the Mouse Gastric Mucosa by Treatment with Immunomagnetic Beads

We found in preliminary experiments that indigenous *Lactobacillus* species (Gram-positive facultative anaerobic or microaerobic bacteria) appeared in the gastric mucosa of mice in unexpectedly large amounts after inoculation with gastric mucosal homogenates containing *H. suis* present in gastric biopsy specimens. The gastric mucosal homogenates prepared from infected mice contained *H. suis* (about $10^4$ copies of 16S rRNA genes) and *Lactobacillus* species ($10^5$ copies of 16S rRNA genes). Under these conditions, we attempted to use in vitro cultivation of *H. suis* strains to isolate them from mouse gastric mucosal homogenates according to the method reported by Baele et al. [5]. Unfortunately, we detected only the *Lactobacillus* colonies on the plates after 7 days of culture. Meanwhile, instead of the culture procedure, the treatment with anti-*H. pylori* antibody-coated magnetic beads (Sepa-Max Hp) effectively reduced the ratios of contamination (mouse gastric cells and enteric bacteria). The *H. suis* purity of the final sample was calculated as follows: *H. suis*/(bacteria + β-actin). A DNA purity of as much as 30–60% was obtained, which was suitable for sequencing purposes (Fig. 2).

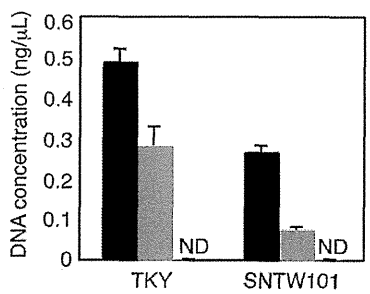


**Figure 2** Purity of DNA samples. One microliter of final DNA solutions prepared from the *Helicobacter suis* TKY or SNTW101-infected mouse gastric mucosa was examined to determine the concentrations of the 16S rRNA gene of bacteria (including *H. suis*; the left columns), the 16S rRNA gene of *H. suis* (the center columns), and mouse β-actin (the right columns) by quantitative real-time PCR using the primer pairs 16S341F/16S519R, HeilF/HeilR, and MactinF/MactinR, respectively. ND, not detected.

### Comparative Genome Analysis of *Helicobacter* and *Campylobacter* Strains was Used to Identify the *Helicobacter suis*-Specific Nucleotide Sequences

The reads from the SOLiD sequencer were assembled with the Denovo 2 program to create contigs. The total lengths of the resulting contigs were 2.12 Mb for *H. suis* TKY and 1.62 Mb for *H. suis* SNTW101. However, the length of each contig was rather short: The N50 values of the *H. suis* TKY and SNTW101 assemblies were 639 and 241 bp, respectively. Moreover, when we aligned the assembled contig sequences with the previously published contig sequences of *H. suis* HS1 and HS5, we found in the alignments several blocks of mismatches whose lengths were tens of base pairs, which appeared to be introduced originally as a few errors in a color-space sequence but which were expanded through the conversion from color space to base space. Therefore, the quality of the resulting sequence was not high enough for detailed sequence analysis. Nonetheless, when these contig sequences were subjected to BLASTX searches against the nonredundant protein sequence database, in a large majority of cases (89.9% in total), the top hit sequences were *H. suis*, other *Helicobacter* species, or *Campylobacter* species. Thus, most of the assembled contig sequences can be considered to be derived from the *H. suis* strains. Then, we found two conserved nucleotide sequences among the *H. suis* strains by comparative genome analysis of 20 *Helicobacter* and 11 *Campylobacter* strains. Although one sequence was conserved in the *H. suis* TKY, SNTW101, SH8, SH10, HS1, and HS5; the *H. felis* ATCC 49179; and the *H. bizzozeronii* CIII-1, this sequence did not code for any open reading frame (ORF; Fig. 3). The other sequence included the *carR* gene, which did not reside on the chromosome of any *Helicobacter* species except for *H. suis* (Fig. 4).

### The Target DNA of *Helicobacter suis* was Specifically Amplified by PCR

To determine whether the above sequences were actually specific to *H. suis*, we carried out conventional PCR using the primer pairs designated above (HH-F5/HH-R4 and carR2F/carR2R). The PCR results indicated that although both the HH-F5/HH-R4 and caR2F/carR2R primer pairs specifically amplified the target DNA fragments of *H. suis* strains (TKY, SNTW101, SH8, SH10, and HS5), there were no such amplified DNA fragments of *H. heilmannii* s.s., *H. felis*, *H. baculiformis*, *H. bizzozeronii*, *H. cynogastricus*, or *H. mustelae* in addition to the absence of amplified fragments of the *H. pylori* strains


 

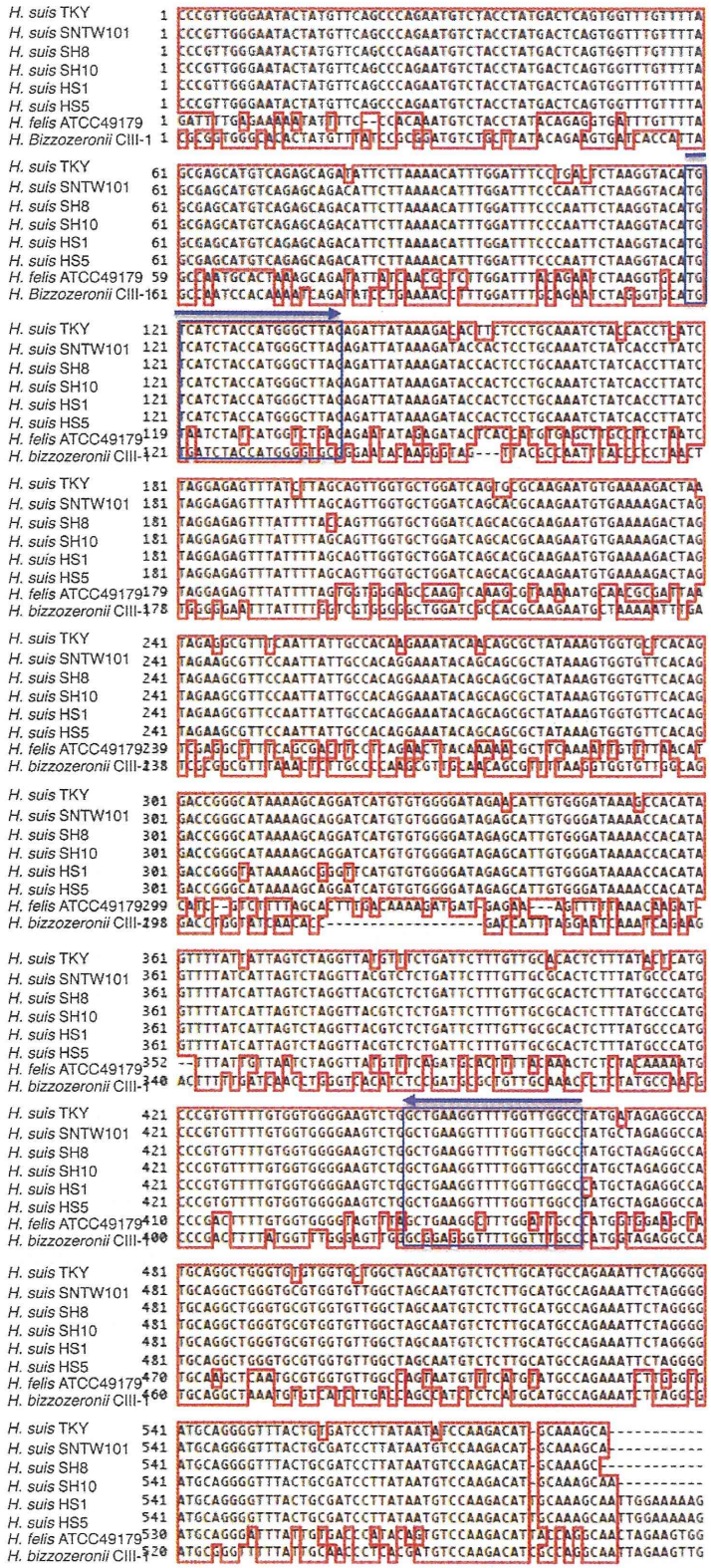
H. suis TKY
H. suis SNTW101
H. suis SH8
H. suis SH10
H. suis HS1
H. suis HS5
H. felis ATCC49179
H. Bizzozeronii CIII-1
H. bizzozeronii CIII-1

**Figure 3** Comparison of *Helicobacter suis*-specific arrangements in the noncoding region. The results of a parallel DNA sequencing of the conserved noncoding region among *H. suis* TKY, *H. suis* SNTW101, *H. suis* SH8, *H. suis* SH10, *H. suis* HS1 (EMBL/GenBank/DDBJ accession no. ADGY01000090.1, contig00090), *H. suis* HS5 (EMBL/GenBank/DDBJ accession no. ADHO01000292.1, contig00292), *Helicobacter felis* ATCC 49179 (EMBL/GenBank/DDBJ accession no. FQ670179.2), and *Helicobacter bizzozeronii* CIII-1 (EMBL/GenBank/DDBJ accession no. FR871757) are shown. The DNA sequences of *H. suis* TKY, SNTW101, SH8, and SH10 were determined after the PCR amplification using the primer pair HH292F1/HH292R1 (Table 1). For the diagnostic PCR, the primer pair HH-F5/HH-R4 was enclosed and indicated.

(Fig. 5A, lanes 2–18). In contrast, the VAC3624F/VAC4041R primer pair specifically amplified the target DNA fragment of *H. pylori* strains (Fig. 5A, lanes 14–18). Moreover, when 100 ng of chromosomal DNA of *H. heilmannii* s.s. was used as the template, no DNA fragments were amplified by PCR using the HH-F5/HH-R4, caR2F/carR2R, or VAC3624F/VAC4041R primer pairs (data not shown). In turn, even at 20 months after inoculation, the target DNA fragments of *H. suis* SNTW101 were clearly amplified and detected in the infected mouse gastric mucosa by PCR using both the HH-F5/HH-R4 and carR2F/carR2R primer pairs (Fig. 5B, lanes 9–16). In contrast, no amplified DNA bands were detected in the age-matched uninfected mouse gastric mucosa (Fig. 5B, lanes 3–8).

### PCR Detected *Helicobacter suis* in Gastric Biopsy Specimens

As shown in Fig. 6, lanes 3–5 and 16 are the samples from UBT-negative patients, and lanes 6–15 are the samples from UBT-positive patients. The diagnostic PCR using the specific primer pairs of VAC3624F/VAC4041R and carR2F/carR2R clearly identified the *H. pylori*-infected (lanes 6–15), *H. suis*-infected (lane 16), and uninfected (lanes 3–5) samples. This finding indicates that the diagnostic PCR using the carR2F/car2R primer pair is useful to identify the *H. suis* infection in gastric biopsy specimens.

## Discussion

In the C57BL/6 mouse model of *H. suis* infection, a single oral inoculation with gastric mucosal homogenates from the *H. suis* TKY-infected mice induced gastric MALT lymphoma, whereas the inoculation with gastric mucosal homogenates from uninfected mice did not induce any macroscopic or microscopic gastric lesions [26]. This result supports the conclusion that even if other bacteria existed in the inoculated gastric homogenates, *H. suis* SNTW101 but not other bacteria induced the formation of gastric lymphoid follicles (Fig. 1).

We attempted to identify the colony morphology of *H. suis* on an agar plate according to the previously reported method [33]. The mouse stomach involving bacteria was subjected to acid treatment, and the mucus was then scraped off using a glass slide and collected in a sterile tube. The mucus was slightly liquefied with *Brucella* broth supplemented with 20% FCS and inoculated on *Brucella* agar containing 20% FCS, 5 mg/L amphotericin B, *Campylobacter*-selective supplement, Vitox supplement, 0.1% activated charcoal, and hydrochloride to obtain a pH of 5. The plates were incubated for 7 days with the lids uppermost at 37 °C under a humidified atmosphere (5% $O_2$, 10% $CO_2$, and 85% $N_2$). The plates were checked every 2 days, and *Brucella* broth supplemented with 20% FCS was added to the agar surface to ensure that they would not dry out. Light microscopic examination, Gram staining, and PCR revealed that *H. suis* were spread on the agar surface with no apparent growth. Based on 16S rRNA gene sequencing, the bacterial colonies growing on the solid agar media were identified as belonging to either *Lactobacillus reuteri* or *Lactobacillus murinus*. Pena et al. [44] have previously reported that *L. reuteri* or *L. murinus* is the most dominant *Lactobacillus* colonizing the C57BL/6 mouse intestine. We are currently continuing these cultivation studies. Meanwhile, magnetic particles have been used widely in both biotechnological and medical fields, including for immunoassay, enzyme immobilization, drug transport, and immunological diagnosis. Particles with bioactive molecules such as antibodies and streptavidin are particularly useful tools for cell separation [45]. In this study, small amounts of the bacterial cells of *H. suis* TKY and SNTW101 (<1%) were successfully separated from large amounts of the other cells, including enteric bacteria and host cells, in the gastric mucosal homogenates by the treatment of magnetic nanoparticles (<100 nm particle size) coupled with rabbit anti-*H. pylori* antibody (Fig. 2). The present method has potential for avoiding the main difficulties in isolating and culturing non-pylori *Helicobacter* from human colonic tissues. That is, a method that combines immunomagnetic separation and PCR would be useful for the detection of unculturable and non-pylori *Helicobacter* colonization in human or animal tissues.

*Helicobacter suis* SNTW101 lacks urease activity, and the *ureAB* genes were not amplified by PCR using the general primer pair of U430F and U1735R or U430F and


 