20% (vol/vol) glycerol. These strains were cultured under microaerobic conditions at 37 °C on Brucella agar plate containing 7% horse serum (HS).

2.2. Isolation of outer membrane vesicle (OMV) structure

H. pylori strain TK1402 was grown on Brucella broth supplemented with 7% FCS for 1 day to 3 days. The cells were collected by centrifugation (10,000 g for 15 min), and the resulting supernatants were filtered (low protein binding Durapore membrane, 0.45 mm polyvinylidene fluoride, Millipore, Bedford, Mass.). The filtrates were centrifuged (40,000 g, 2 h at 4 °C), washed with PBS and re-centrifuged (40,000 g, 2 h at 4 °C). The pellets were next resuspended in PBS supplemented with 0.2 M NaCl. The OMV structures of other *H. pylori* strains cultured for 3 days in Brucella broth supplemented with 7% FCS were also isolated in a similar manner to that of strain TK1402.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The fractionated OMV from Brucella broth supplemented with 7% FCS in PBS were adjusted to an optical density of 2.0 at 280 nm. The adjusted OMV were treated with sodium dodecyl sulfate (SDS) loading buffer including 5% 2-mercaptoethanol at 100 °C for 5 min and separated by polyacrylamide gel electrophoresis (PAGE). The separated OMV proteins were stained with Coomassie brilliant blue (CBB).

2.4. Determination of N-terminal amino acid sequence

The OMV fraction was loaded onto gel and transferred to polyvinylidene difluoride membranes (Atto, Tokyo, Japan). After transfer, the membrane was stained with CBB. The band corresponding to approximately 22-kDa was excised from the gel. The N-terminal amino acid sequence was determined by ARPO science (Tokushima, Japan), based on the Edman degradation method.

3. Results

3.1. Evaluation of the OMV produced by TK1402

We previously demonstrated that strain TK1402 has a strong biofilm forming ability independent of its growth rate [12]. In addition, the OMV were located at the substratum–bacterium interface and extracellular space in the biofilm. We concluded that the OMV produced by strain TK1402 could play an important role for attachment to abiotic and cellular surfaces in the initial stages of biofilm formation.

Development of strain TK1402 from day 1 to day 6 demonstrated that the biofilm formation rapidly increased over 2 days and it took 3 days for biofilm maturation under these conditions. In order to examine the kinetics of OMV components before mature formation of the biofilm, the OMV were isolated from the cell cultures from day 1 to day 3 and characterized by SDS-PAGE (Fig. 1). The results indicated that the band profile of the OMV from day 1 displayed some remarkable differences compared to subsequent days. All OMV mostly shared similar bands, although the positions of the clear bands appear to be shifted downwards in the OMV from day 2 and day 3 compared to that from day 1. In addition, a 22-kDa band detected in the OMV from day 2 and day 3 was not detected in the OMV from day 1. There were no differences in the OMV between day 2 and day 3.

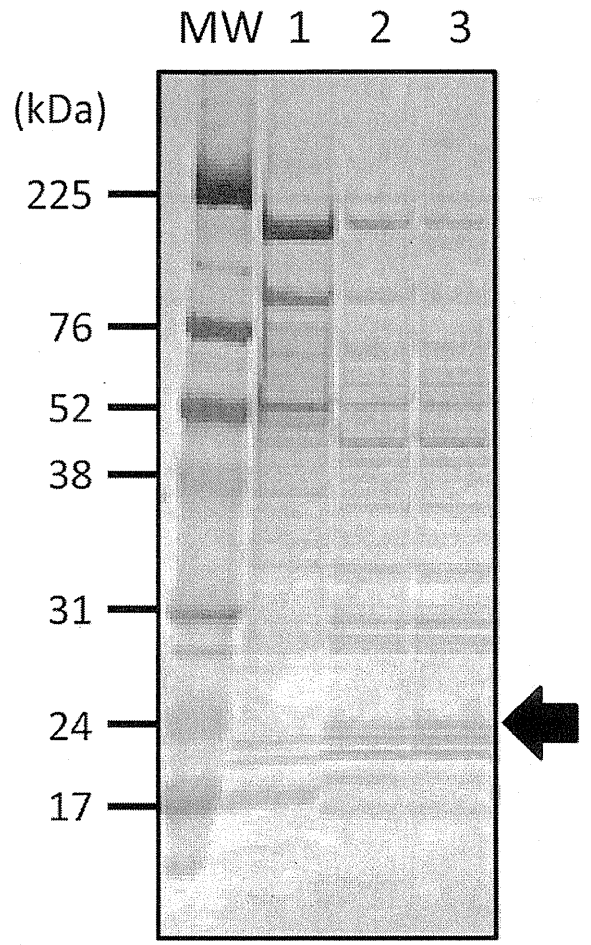

Fig. 1. The OMV were fractionated at day 1, 2 and 3 from TK1402 and the proteins were separated by SDS-PAGE (lane 1, 1-day culture; lane 2, 2-day culture; lane 3, 3-day culture). The approximate position of the 22-kDa band is shown by an arrow on the right side. Lane MW contains molecular weight markers. The data shown are representative of three independent experiments.

3.2. Differential OMV components in H. pylori strains

We attempted to compare the OMV components in the 8 strains of *H. pylori* with SDS-PAGE but all of the *H. pylori* strains except strain TK1402 formed very little biofilm biomass [12]. *H. pylori* strains were cultured for 3 days and the OMV were isolated. The band profile of the OMV seen by SDS-PAGE was variable but the 22-kDa band was detected only in the OMV from strain TK1402 (Fig. 2).

3.3. Amino acid sequences

Since the 22-kDa protein was only present in TK1402 OMV and this protein appeared before maturation of the TK1402 biofilm, we hypothesized that this protein might play an important role for biofilm formation in the strain. In order to characterize the 22-kDa protein, N-terminal amino acid sequencing was carried out. The five N-terminal residues that could be analyzed showed the amino acid sequence [VDFSK]. We attempted to search for a protein with a similar N-terminal sequence with Genbank but no other protein matched this N-terminus in the *H. pylori* database.

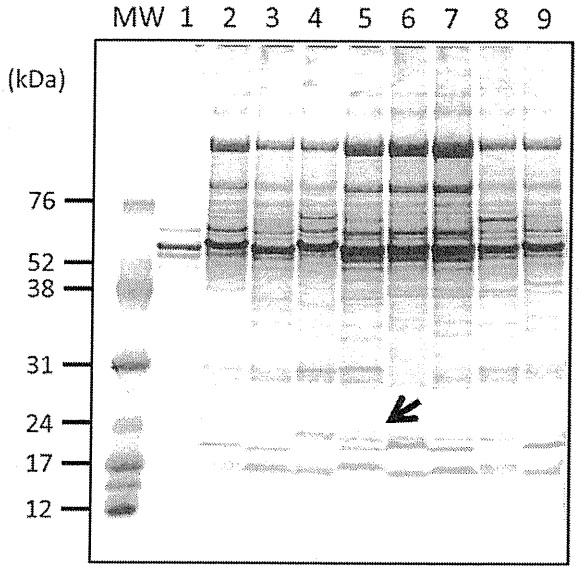

Fig. 2. The OMV were fractionated from 8 strains of *H. pylori* and the OMV were separated by SDS-PAGE (lane 1, medium control; lane 2, KR2003; lane 3, KR2005; lane 4, TK1029; lane 5, TK1402; lane 6, SS1; lane 7, NCTC11638; lane 8 ATCC 43503; lane 9, ATCC 43579). The approximate position of the 22-kDa band is shown by a black arrow. Lane MW contains molecular weight markers. The data shown are representative of three independent experiments.

4. Discussion

Recently, some of reports indicate that *H. pylori* has the ability to form biofilms on abiotic surfaces in-vitro as well as on the human gastric mucosa [11,18–21]. In our previous study, we demonstrated that strain TK1402, a clinical isolate derived from a Japanese peptic ulcer patient, has a strong biofilm forming ability [12] and that this property is associated with the production of OMV. In the present study, we analyzed the OMV with SDS-PAGE and found that the OMV from 1-day culture exhibited a different protein profile compared to those from 2-day and 3-day culture (Fig. 1). There were some bands present in the OMV from the 2- and 3-day cultures that were absent at day 1. This result indicated that the components of the OMV were different between 1 day culture and 2–3-day culture.

We further analyzed the OMV produced by 8 strains of *H. pylori* by SDS-PAGE with Coomassie blue staining and observed that there were several specific protein bands in the OMV from strain TK1402 which were absent in other strains of *H. pylori* (Fig. 2). It appears from our results that the clear 22-kDa band found in TK1402 OMV may be important in biofilm formation. The amino acid sequence determined by N-terminal sequencing was [VDFSK], but this amino acid sequence was not present in the *H. pylori* database. The reason for this may be that *H. pylori* strains exhibiting strong biofilm forming ability are relatively rare [12] and indeed a database strain, 26695, showed little biofilm formation (data not shown). These data suggest that 22-kDa protein may play a part of important role in biofilm formation of strain TK1402. However, the role of 22-kDa protein for biofilm formation still remains to be determined. Identification, purification, and chemical characterization will be required to clarify the 22-kDa protein. Additional investigations are now in progress to analyze it. Biofilm formation is critical for not only environmental survival but also successful infection in numerous pathogenic bacteria. Our present results provide useful information for understanding of biological significance of *H. pylori* biofilm formation.

5. Conclusions

We have demonstrated that strain TK1402 has a strong biofilm forming ability. In addition, the results suggested that this property might be dependent upon a 22-kDa protein obtained from the OMV of this strain. This may represent a potentially novel gastric cell colonization factor for this organism.

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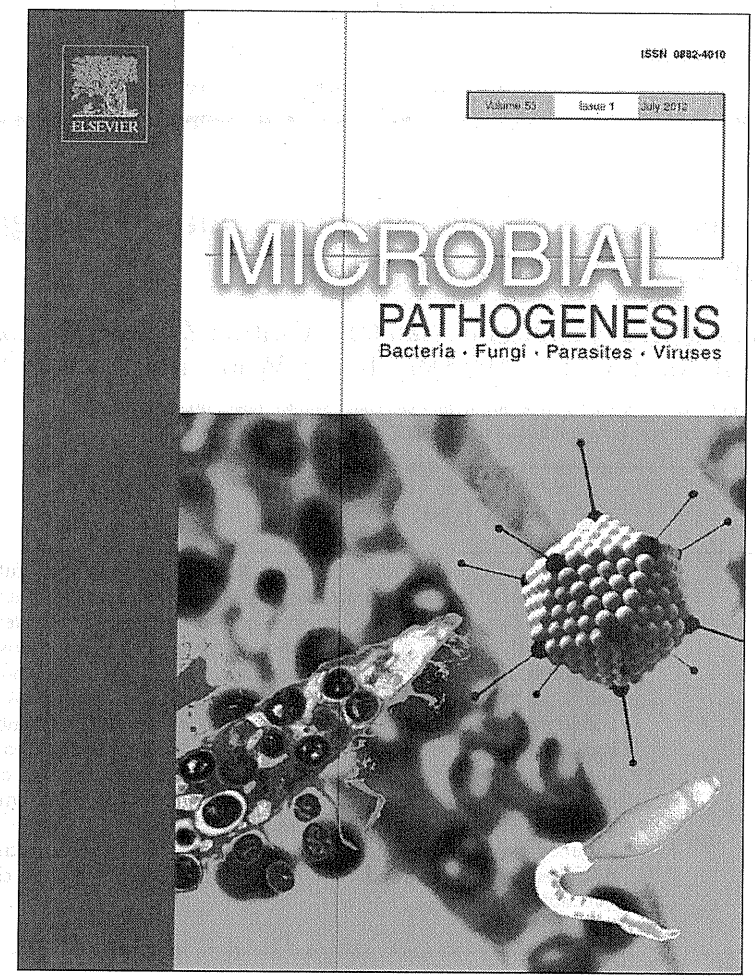

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Comparative analysis of gastric bacterial microbiota in Mongolian gerbils after long-term infection with *Helicobacter pylori*

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ABSTRACT

Quantitative (qt) real time PCR using 16SrDNA primers is useful for determination of the bacterial composition of the gastric microbiota in Mongolian gerbils. The aim of this study was to determine the change in the gastric microbiota after long-term infection with *Helicobacter pylori*. One year after inoculation with *H. pylori*, five gerbils were determined as *H. pylori*-positive and 6 gerbils *H. pylori*-negative by culture and real time qt PCR methods. The gastric microbiota of each group of gerbils was also compared with that of 6 gerbils uninfected with *H. pylori*. DNA from the *Atopobium* cluster, *Bifidobacterium* spp., *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Enterococcus* spp. and *Lactobacillus* spp. were detected in the gastric mucus of both infected and uninfected gerbils. In contrast, *Eubacterium cylindroides* group and *Prevotella* spp. were detected only in *H. pylori*-negative gerbils. The numbers of *C. leptum* subgroup, *C. coccoides* group and *Bifidobacterium* spp. in gastric mucus of *H. pylori*-negative Mongolian gerbils were significantly lower than those in non-infected gerbils. The results obtained suggest that 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.

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1. Introduction

Helicobacter pylori infection of the human gastric mucosa induces gastritis [1,2] and is related to many gastric diseases including gastric cancer [3]. Infection continues throughout the life of the patient and influences not only gastroduodenal diseases but also gastric environment and function [4]. In our previous study, persistent infection of the gastric mucosa of Mongolian gerbils with *H. pylori* could be detected by real-time reverse transcription (RT)-PCR for 6 months after infection, but *H. pylori* could not be isolated by culture methods after 4 months [5]. Also, it was noted that the change in the gastric environment caused by *H. pylori* infection may affect not only the number of *H. pylori* bacteria, but also the indigenous gastric bacterial composition.

Identification of bacterial strains by conventional culture-based methods provides an incomplete and biased picture of the biodiversity of intestinal microbiota, as many species cannot be cultivated *in vitro* [6]. Therefore, culture-independent molecular methods based on 16S rRNA genes such as fluorescent *in situ* hybridization [7,8], dot-blot hybridization with rRNA-targeted probes [9], denaturing gradient gel electrophoresis [10,11], temperature gradient gel electrophoresis [12], and cloning and sequencing of rDNA [6,13] have been introduced to obtain a better understanding of the gut microbiota. Recently, Matsuki et al. [14–16] reported a quantitative PCR method with 16S rRNA-gene-targeted species-specific primers to species including *Bacteroides fragilis* group, *Bifidobacterium*, *Clostridium coccoides* group and *Prevotella*, which were designed for analysis of human intestinal microbiota.

H. pylori is inhibited by a number of commensal bacterial species as well as opportunistic human pathogens [17]. The aim of the present study was to determine the differences in the gastric microbiota between *H. pylori*-positive and negative gerbils. We determined the composition of the gastric microbiota of Mongolian gerbils after long-term infection with *H. pylori* and compared infected and non-infected gerbils to elucidate any increasing or

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decreasing trends in the indigenous bacterial population. To analyze bacterial microbiota quantitatively, we used a real time PCR method with 16S rRNA-gene-targeted species-specific primers. Twelve primer sets for obligate anaerobes (*C. coccoides* group, *Clostridium leptum* subgroup, *B. fragilis* group, genus *Bifidobacterium, Atopobium* cluster, genus *Prevotella, Eubacterium cylindroides* group, *Clostridium ramosum* subgroup, genus *Veillonella,* genus *Fusobacteriumi),* 2 sets for facultative anaerobes (genus *Enterococcus* and genus *Lactobacillus*) [14–16,18] and 1 set for *H. pylori* were used.

2. Materials and methods

2.1. Bacterial strains and culture condition

H. pylori strain TK1402 was cultured on Brucella broth (Difco) containing 1.5% Bact agar (Difco) supplemented with 7% horse serum (BHS plates) as previously described [19]. *H. pylori* were grown under microaerobic conditions using Anaero Pack (A28, Mitsubishi-Gas Chemical Co. Inc, Tokyo, Japan) at 37 °C [20]. For the detection of *H. pylori* in gastric mucus samples, specimens was inoculated onto *H. pylori* selective medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and cultured at 37 °C for 5 days under microaerobic conditions. After the incubation period, purple colonies that exhibited urease activity were counted.

Standard strains of *Ruminococcus productus* YIT 6141^T, *Faecalibacterium prausnitzii* YIT 6174, *Bacteroides vulgatus* YIT 6159^T, *Bifidobacterium longum* YIT 4021^T, *Collinsella aerofaciens* ATCC 25986^T, *Prevotella melaninogenica* YIT 6039^T, *Clostridium innocuum* YIT1015 ^{T}, *C. ramosum* subgroup *ramosum* YIT10062 ^{T}, *Veillonella parvula* YIT 6072^T, *Fusobacterium varium* ATCC 8501^T, *Lactobacillus casei* ss. *casei* JCM 1134^T and *Enterococcus faecalis* ATCC 19433^T were used in this study. These microorganisms were obtained from the Culture Collection of the Yakult Central Institute (Tokyo, Japan) (YIT) or the Japan Collection of Microorganisms, RIKEN BioResource Center (Saitama, Japan) (JCM/ATCC). The strains were cultured anaerobically in GAM broth (Gifu Anaerobic Medium, Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 1% glucose at 37 °C for 18–48 h, and 1 ml samples of these were used for DNA extraction. The numbers of bacterial strains were counted microscopically by the DAPI (4′, 6′-diamidino-2-phenylindole) staining method as described previously [16]. Serial 10-fold dilutions of the cultures were also plated on nonselective GAM agar (Nissui Seiyaku, Tokyo, Japan). The plates were subsequently incubated at 37 °C for 3–5 days in an anaerobic chamber (HIRASAWA WORKS Inc., Tokyo, Japan), and cultural counts (in CFU) were determined in triplicate.

2.2. Animal experiments

Mongolian gerbils (MGS/sea; age, 8 weeks; male) were purchased from Kyudo, Ltd. (Fukuoka, Japan) and bred under specific-pathogen free conditions (room temperature, 23 ± 2 °C; relative humidity, 40–60%; 12-h light–dark cycle) in the animal facility of Kyorin University. A standard diet (CE-2; Clea Japan, Tokyo, Japan) and sterilized tap water were provided ad libitum in microisolator units. The experiments were approved by the Experimental Animal Ethics Committee at the Kyorin University School of Medicine.

Eleven gerbils were fasted for 2 days and orally inoculated with 1–2 × 10^9 cfu of *H. pylori* TK1402 strain suspended in 1 ml of Hanks' balanced salt solution (HBSS, Sigma) on two consecutive days. Five control gerbils were inoculated with HBSS only. One (infected) or two gerbils (control) were kept in one animal cage during the infection period. At 1 year after inoculation, the gerbils were sacrificed, and the mucus layer of half of the stomach was scraped off with a spatula and collected into 500 μl of HBSS [21,22]. Of this 500 μl suspension, 100 μl was used for the detection of viable *H. pylori* and another 400 μl was stored at −80 °C until use for DNA preparation.

2.3. DNA extraction from gastric samples and standard bacterial strains

For DNA extraction, 200 μl of the gastric mucus sample suspension was added to a solution containing 250 μl of extraction buffer (200 mmol/l Tris–HCl, 80 mmol/l EDTA; pH 9.0) and 50 μl of 10% sodium dodecyl sulfate. Then, 300 mg of glass beads (GB-01, TOMY, Tokyo; diameter = 0.1 mm) and 500 μl of buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 30 s using a FastPrep FP 120 (BIO 101, Vista, CA., USA) at a power level of 5.0. After centrifugation at 14,000 × *g* for 5 min, 400 μl of the supernatant was collected. Phenol-chloroform extractions were then performed and 250 μl of the supernatant was subjected to isopropanol precipitation. Finally, the DNA was suspended in 1 ml of 10 mmol/l Tris–HCl, 1 mmol/l EDTA buffer (pH 8.0).

Standard DNA samples were extracted using the same method from 1 ml aliquots of the cultures in GAM broth of *R. productus* YIT 6141^T, *F. prausnitzii* YIT 6174, *B. vulgatus* YIT 6159^T, *B. longum* YIT 4021^T, *C. aerofaciens* ATCC 25986^T, *P. melaninogenica* YIT 6039^T, *E. cylindroides* group, *C. innocuum* YIT1015 ^{T}, *C. ramosum* YIT10062 ^{T}, *V. parvula* YIT 6072^T, *F. varium* ATCC 8501^T, *Lactobacillus gasseri* YIT 0192^T and *E. faecalis* ATCC 19433^T. For the detection of *H. pylori,* standard DNA was extracted from cultures of *H. pylori* TK1402 using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). One ml of overnight bacterial broth culture containing 3–5 × 10^8 cfu quantified by microaerophilic cultivation was centrifuged (21,000 × *g* for 2 min) and the supernatant removed. The pellet was resuspended in 480 μL of 50 mmol/l EDTA, 120 μl of 400 μg/ml lysozyme was added, and the mixture was incubated at 37 °C for 60 min. Consequent steps were performed according to the documentation. Finally, 100 μl of nuclease-free water was used for elution of purified DNA.

2.4. Quantitative (qt) real-time PCR

qt-PCR amplification and detection was performed with an ABI PRISM 7900HT and 7500 sequence detection system (Applied Biosystems (ABI), Foster City, CA, USA) [15–17] as previously reported [21]. Each reaction mixture (10 μl) was composed of 10 mmol/l Tris–HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μmol/l, a 1:75,000 dilution of SYBR Green I (Molecular Probes, Eugene, Oreg.), 11 ng of TaqStart antibody (ClonTech, Palo Alto, Calif.) per μl, 0.05 U of *Taq* DNA polymerase (Takara, Tokyo, Japan) per μl, each of the specific primers at a concentration of 0.25 μmol/l, and 1 μl of 1x or 10x diluted template DNA. The amplification program consisted of one cycle at 94 °C for 5 min and followed by 40 cycles at 94 °C for 20 s, 60, 58, 55 or 50 °C for 20 s (Table 1) [14–16,21,23], and 72 °C for 50 s. The fluorescent products were detected at the last step of each cycle.

Bacterial numbers in gastric samples were calculated by comparing the PCR kinetics of target genes in the sample with standard curves made by amplifying serial dilutions of a known quantity of amplicon. With each primer set, PCR was performed in parallel with different amounts of standard strain chromosomal DNA. DNA extracted from *R. productus* YIT 6141^T (g-Ccoc), *F. prausnitzii* YIT 6174 (sg-Clept), *B. vulgatus* YIT 6159^T (g-Bfra), *B. longum* YIT 4021^T (g-Bifid), *C. aerofaciens* ATCC 25986^T (c-Atopo), *P. melaninogenica* YIT 6039^T (g-Prevo), *E. cylindroides* group (g-Ecylin), *C. innocuum* YIT1015 ^{T}, *C. ramosum* YIT10062 ^{T} (sg-Cram),

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Table 1
16S rRNA gene-targeted group-specific primers used in this study.

Target bacterial group	Primer	Sequence	Size (bp)[a]	Annealing temp (°C)	Reference
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTAA	440	50	14
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			
Clostridium leptum sub-group	sg-Clept-F	GCACAAGCAGTGGAGT	239	50	16
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA			
Bacteroides fragilis group	g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	495	50	14
	g-Bfra-R	CCAGTATCAACTGCAATTTTA			
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	550	55	14
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Atopobium cluster	c-Atopo-F	GGGTTGAGAGACCGACC	190	55	16
	c-Atopo-R	CGGRGCTTCTTCTGCAGG			
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	513	55	14
	g-Prevo-R	GGTCGGGTTGCAGACC			
Eubacterium cylindroides group	g-Ecylin-F	GTGAYGGTAKCTTACCAGA	416	87–89	12
	g-Ecylin-R	CTTGCGTGCATACTCCC			
Clostridium ramosum sub-group	sg-Cram-F	GACACTGCATGGTGACC	466	91.3	12
	sg-Cram-R	GGTTTCTATGGCTTACTG			
Veillonella	g-Veillo-F	GRAGAGCGATGGAAGCTT	459	89–90	12
	g-Veillo-R	CCGTGGCTTTCTATTCC			
Fusobacterium	g-Fuso-F	CWAACGCGATAAGTAATC	317	88.1	12
	g-Fuso-R	GCAGGCAGTATCGCAT			
Enterococcus	g-Ent-F	CCCTTATTGTTAGTTGCCATCATT	144	60	23
	g-Ent-R	ACTCGTTGTACTTCCCATTGT			
Lactobacillus	g-Lact-F	AGCAGTAGGGAATCTTCCA	341	58	23
	g-Lact-R	CACCGCTACACATGGAG			
Helicobacter pylori	16S2-F	CGCTAAGAGATCAGCCTATGTCC	139	58	21
	16SB2-R	CCGTGTCTCAGTTCCAGTGTGT			

[a] DNAs extracted from *Ruminococcus productus* YIT 6141^T, *Faecalibacterium prausnitzii* YIT 6174, *Bacteroides vulgatus* YIT 6159^T, *Bifidobacterium longum* YIT 4021^T, *Collinsella aerofaciens* ATCC 25986^T, *Prevotella melaninogenica* YIT 6039^T, *Clostridium innocuum* YIT1015 ^{T}, *Clostridium ramosum* subgroup *ramosum* YIT10062 ^{T}, *V. parvula* YIT 6072^T, *Fusobacterium nucreatum* ATCC25586, *Lactobacillus gasseri* YIT 0192^T or *L. casei* ss. *casei* JCM 1134 ^{T} and *Enterococcus faecalis* ATCC 19433^T were used as real-time PCR controls.

V. parvula YIT 6072^T (g-Veillo), *F. varium* ATCC 8501^T (g-Fuso), *L. gasseri* YIT 0192^T or JCM *L. casei* ss *casei* 1134^T (g-Lact) and *E. faecalis* ATCC 19433^T (g-Entc) were used as real-time PCR standards for the each group or genus-specific primer. Data were analyzed using the standard 7900 or 7500 quantification software (Applied Biosystems). To confirm the specificity of the PCR product, a melting curve analysis was done after amplification to distinguish the target product from non-specific annealing. The melting curves were obtained by slow heating at temperatures from 60 to 95 °C at a rate of 0.2 °C/s, with continuous fluorescence collection. The melting temperature (Tm) value of standard strains was used to confirm PCR specificity, with standard Tm ± 1 °C taken to be identical to the target product. *E. cylindroides* group and genus *Lactobacillus* exhibited a wider range of PCR products between species. In these cases, Tm ± 2 °C was used.

2.5. ELISA

Whole antigen samples of *H. pylori* for ELISA were prepared based on our previous report [5]. Three-day cultures of *H. pylori* TK1402 on BHS-agar at 37 °C were suspended in 0.01 M PBS (pH 7.4) and disrupted using an ultrasonic Sonifier 250 (Branson Ultrasonics) for 5 min at 20 kHz. The supernatant was separated from the insoluble cell fragments by centrifugation. Microtitre plates (Greiner Labortechnik Japan) were coated at 4 °C for 18 h with the whole *H. pylori* sonicate (3 μg per well) and then washed three times with PBS. Antigens were blocked with PBS containing 1% skim milk (PBS-S; Yukijirushi Nyugyo) at 37 °C for 1 h, washed with PBS. Serum samples from infected or uninfected gerbils were diluted 300-fold with PBS-S. Diluted serum samples (100 μl) were added to the plates and incubated at 37 °C for 2 h, washed three times with PBS, and then 100 μl horseradish peroxidase–protein G (Sigma) at 25 μg ml^{-1} in PBS-S was added to the plates and reacted with antigen–antibody complexes at 37 °C for 1 h. The plates were incubated with 0.1% o-phenylenediamine in developing buffer (0.1 M citric acid, 0.07 M sodium phosphate dibasic, 0.035% H_2O_2) at room temperature for 5 min and then the reaction was stopped by adding 50 μl 1 M H_2SO_4. The A_{490} was measured using a model iMark microplate reader (Bio-Rad).

2.6. Statistical analysis

The Student's *t*-test for unpaired values was used to compare the differences among the three groups.

3. Results

3.1. Long-term infection of Mongolian gerbils with H. pylori

The gerbils were divided into three groups according to the status of *H. pylori* infection. The gastric samples collected from the gerbils infected with *H. pylori* were analyzed by culture and PCR. Five gerbils were determined as *H. pylori*-positive by culture and 6 gerbils were negative by both methods (Table 2). The gastric samples from six uninfected gerbils bred for 1 year were also checked for the status of *H. pylori* infection. The organisms were not detected in these gerbils (control group) by either method. There was no significant association between *H. pylori* infection of individual gerbils and cage sharing. DNA of *Atopobium* cluster, *Bifidobacterium* spp., *C. coccoides* group, *C. leptum* subgroup, *Enterococcus* spp. and *Lactobacillus* spp. was detected from the gastric mucus of Mongolian gerbils in all three groups, but DNA of *C. ramosum* subgroup, *B. fragilis* group, *Fusobacterium* spp. and *Veillonella* spp. was not detected. DNA of *Prevotella* spp. and *E. cylindroides* group was detected from the gastric mucus in the *H. pylori*-negative group only (Table 2).

The results of the ELISA are shown in Fig. 1. Serum antibody titers against TK1402 sonicate of *H. pylori*-infected gerbils

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Table 2
Bacterial number determined by real time PCR of gastric flora in *H. pylori*-infected and uninfected gerbils.

Infection status	Average bacterial number/g gastric mucus (Log)				(Positive number/total animal number)				*Helicobacter pylori*[a]
	Atopobium cluster	*Bifidobacterium*	*Clostridium coccoides* group	*Clostridium leptum* sub-group	*Prevotella*	*Eubacterium cylindroides* group	*Enterococcus*	*Lactobacillus*	
Uninfected[b]	6.1 ± 0.5 (6/6)	5.9 ± 0.4 (6/6)	6.3[b] ± 0.7 (6/6)	6.7 ± 0.5 (3/6)	<5.0 (0/6)	<5.0 (0/6)	6.7 ± 0.5 (5/6)	7.3 ± 0.8 (6/6)	–
H. pylori-positive[c] gerbils	6.5 ± 0.4 (5/5)	5.6 ± 0.4 (4/5)	5.4 ± 0.3 (3/5)	6.1 (1/5)	<5.0 (0/5)	<5.0 (0/5)	6.4 ± 0.4 (5/5)	6.4 ± 1.0 (5/5)	4.2 ± 0.3 (5/5)
H. pylori-negative[d] gerbils	6.7 ± 0.3 (6/6)	4.2 ± 0.2 (4/6)	5.4 ± 0.5 (6/6)	5.2 ± 0.0 (2/6)	5.6 ± 0.0 (6/6)	5.1 ± 0.5 (6/6)	7.3 ± 0.2 (6/6)	7.2 ± 0.7 (6/6)	<2.5 (0/6)

[a] Number of *H. pylori* was determined by culture method.
[b] Uninfected Mongolian gerbil bred for 12 months.
[c] *H. pylori* was isolated from gastric mucus of infected gerbils by culture.
[d] *H. pylori* was not detected from gastric mucus of infected gerbils by culture and qt-PCR.

(*H. pylori*-positive ($n = 5$) and negative groups ($n = 6$)) were significantly higher than those of the uninfected gerbils ($n = 6$) ($P < 0.01$). Furthermore, the antibody titer in the *H. pylori*-positive gerbils ($n = 5$, $OD_{490} = 0.940 \pm 0.248$) was significantly higher ($P < 0.01$) than that of *H. pylori*-negative gerbils ($n = 6$, $OD_{490} = 0.335 \pm 0.169$).

3.2. Gastric microbiota in Mongolian gerbils

Bacterial numbers determined by real time PCR of gastric microbiota in the three groups of gerbils are shown in Fig. 2. The dominant bacterial genera in the gastric mucus of Mongolian gerbils were *Enterococcus* spp. ($10^{6.4-7.3}$/g), *Lactobacillus* spp. ($10^{6.4-7.3}$/g) and *Atopobium* cluster ($10^{6.1-6.7}$/g).

By the comparison of the average bacterial numbers of these genera between the three groups, there were several significant differences. The average number of *Atopobium* cluster in the gastric mucus of *H. pylori*-negative gerbils ($n = 6$) was significantly higher than that in control group ($n = 6$) (Fig. 2A). The numbers of *Bifidobacterium* spp. ($n = 4$, $p = 0.0004$), *C. coccoides* group ($n = 6$, $p = 0.046$) and *C. leptum* sub-group ($n = 2$, $p = 0.035$) in *H. pylori*-negative gerbils were significantly lower than those in control gerbils (*Bifidobacterium* spp. $n = 6$, *C. coccoides* group $n = 6$ and *C. leptum* subgroup $n = 3$) (Fig. 2B–D). Furthermore, the number of *Bifidobacterium* spp. ($n = 4$) in *H. pylori*-positive gerbils was significantly higher ($n = 4$, $p = 0.003$) than that in the *H. pylori*-negative gerbils ($n = 4$) (Fig. 2B) and the number of *C. coccoides* group in the *H. pylori*-positive group was significantly lower ($n = 3$, $p = 0.023$) than that in control group ($n = 5$) (Fig. 2C). On the other hand, there was no difference in the number of *C. coccoides* group between *H. pylori*-positive and negative gerbils (Fig. 2C). Similarly, there was no difference in the number of *Enterococcus* and *Lactobacillus* spp. among the three groups (Fig. 2E and G). *E. cylindroides* group and *Prevotella* spp. were detected only in *H. pylori*-negative gerbils (Fig. 2F and H).

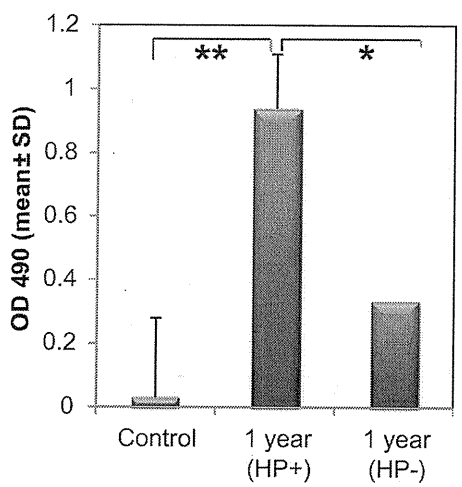

Fig. 1. Serum antibody titers to *H. pylori* sonicated antigens in Mongolian gerbils after long-term infection with *H. pylori*. Significant differences between among groups are shown. (*, $p < 0.05$; **, $p < 0.01$).

4. Discussion

Mongolian gerbils are used to study *H. pylori*-induced gastritis and its consequences [24,25]. In our previous study on long-term infection with *H. pylori* in the gastric mucosa of Mongolian gerbils, *H. pylori* was detected by real-time RT-PCR for 6 months after infection, but no *H. pylori* could be isolated after 4 months by cultivation [5]. This implies that comparative analysis of gastric microbiota between *H. pylori*-positive and negative gerbils may be of use. In this study, we have divided infected gerbils into two groups according to the *H. pylori* status 1 year after infection together with an uninfected control. The gastric microbiota was then analyzed by a culture-independent molecular method based on 16S rRNA genes using primer sets detecting obligate and facultative anaerobes. DNA of *Atopobium* cluster, *Bifidobacterium* spp., *C. coccoides* group, *C. leptum* subgroup *Enterococcus* spp., and *Lactobacillus* spp. were detected in all three groups (Fig. 2A–E and G). These bacterial genera were also components in human [14] and mouse feces [26], but conversely the *B. fragilis* group, *C. ramosum* subgroup, *Fusobacterium* spp. and *Veillonella* spp. were not detected. It is likely that this particular gastric environment is not suitable for these obligate anaerobes.

We have already reported that marked atrophy of the mucosa and multiple cysts in the submucosa were observed in the glandular stomachs of gerbils after long term infection with *H. pylori* TK1402 strain [5]. As a result of this, the gastric mucus layer becomes thinner and the number of *H. pylori* was reduced. These hallmarks of chronic infection with *H. pylori* are also often observed in the human stomach [27]. As chronic inflammation induces gastric atrophy and reduces the number of acid secreting cells, the following gastric pH elevation influences the gastric localization of *H. pylori* colonization [28]. Furthermore, in an acidic gastric environment, Gram negative microorganisms undergo growth suppression, and therefore the effect of *H. pylori* colonization may cause the numbers of Gram negative bacteria such as *Prevotella* spp. to increase, with reciprocal changes in other Gram positive bacteria

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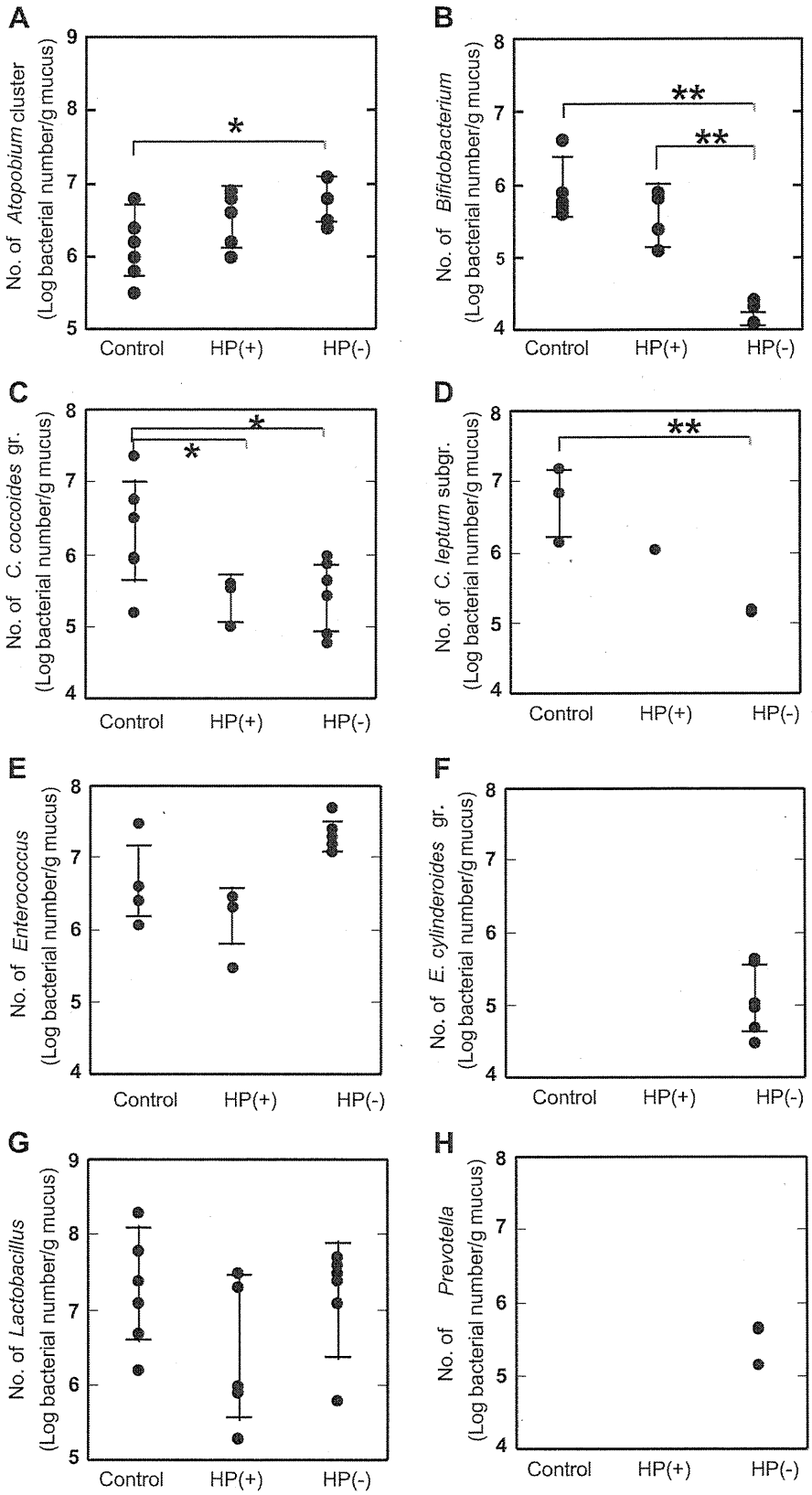

Fig. 2. Number of gastric bacteria in Mongolian gerbils with or without *H. pylori* infection. A, *Atopobium* cluster, B, *Bifidobacterium*, C, *C. coccoides* gr.,, D, *C. leptum* subgr., E. *Enterococcus*, F, *E. cylindroides* gr, G, *Lactobacillus*, H, *Prevotella*. Each gastric mucosa sample was collected from either *H. pylori*-positive(HP(+)), -negative (HP(−)) or uninfected gerbils (Control). The total DNA was extracted from each sample and analyzed by quantitative-real time PCR using genus or sub-group specific primers for the determination of bacterial number. The SD value of bacterial number in each group is shown as a bar. Significant differences between groups were shown. (*, $p < 0.05$; **, $p < 0.01$).

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including *Bifidobacterium* and clostridia. Dicksved et al. [29] have reported the differences in stomach microbiota in patients with gastric cancer compared to controls. They showed that the microbiota in gastric cancer was dominated by different genera such as *Streptococcus, Lactobacillus, Veillonella* and *Prevotella.*

Suzuki et al. [30] have reported that the prevalence of *Prevotella intermedia* in the saliva was significantly greater in *H. pylori*-positive than *H. pylori*-negative patients, suggesting that co-existence of *H. pylori* with periodontopathic bacteria including *Prevotella* spp. may be indirectly associated with oral pathological halitosis. On the other hand, it was shown in the present study that *Prevotella* spp. were detected only in *H. pylori*-negative gerbils (Fig. 2H), suggesting a negative correlation between *Prevotella* spp. and *H. pylori* in gastric mucosa. It is possible that *Prevotella* spp. may colonize the gastric mucosa following *H. pylori* infection, as *Prevotella* spp. were not detected in uninfected control gerbils and *Prevotella* spp. may in fact affect *H. pylori* colonization in long-term persistent infection. *E. cylindroides* group were also detected only in *H. pylori*-negative gerbils (Fig. 2F), although the reason for this remains to be determined.

In the *H. pylori*-negative group, it was not possible to infer the time when *H. pylori* was eradicated, and lower serum antibody levels were still detected in this group compared to *H. pylori* positive animals. Cao et al. [31] have reported that the higher antibody titer was continued for 25 weeks and lower antibody levels were detected 50 weeks after eradication in *H. pylori* infected gerbils. This implies that the *H. pylori*-negative gerbils in our study cleared the infection early after inoculation. It is possible that *H. pylori* persistence in a gastric ecosystem decreases gastric acidity and causes loss of the acid barrier to colonization by environmental, oral or intestinal microbiota [32]. It is also possible that the alteration in gastric microbiota in itself inhibits *H. pylori* persistence.

There were several significant differences in the numbers of microbiota between the three groups. The average numbers of *Bifidobacterium* spp., *C. coccoides* group and *C. leptum* subgroup in the gastric mucus of *H. pylori*-negative gerbils were significantly lower than those in control gerbils. *C. coccoides* group is a member of the genus *Clostridium*, and is a non-motile, spore-forming, Gram-positive obligate anaerobe. Kibe et al. [33] have reported that *C. coccoides* spp. were detected as the dominant bacteria in the intestinal microbiota of mice [18], and it seems that they are also one of the main constituents of the intestinal microbiota of the Mongolian gerbil. However, long-term infection with *H. pylori* reduced the number of *C. coccoides* group compared to that in control gerbils (Fig. 2C), though there was no difference between *H. pylori*-positive and -negative gerbils, suggesting that *C. coccoides* group did not affect the number of *H. pylori.*

Collado et al. [34] reported that *Bifidobacterium* species produced antimicrobial peptides against *H. pylori* and that their effects were promoted by organic acids. It is possible that the production of antimicrobial peptides by bifidobacteria in the gastrointestinal tract of Mongolian gerbils may inhibit the growth of *H. pylori.* In this study, however, we did not observe a negative correlation between *H. pylori* and bifidobacteria, and the number of *Bifidobacterium* in *H. pylori*-negative gerbils was in fact significantly lower than those in *H. pylori*-positive and control gerbils (Fig. 2B). This is probably due to the fluctuations in gastric flora after long-term infection with *H. pylori.*

The number of *Atopobium* cluster bacteria in *H. pylori*-negative gerbils was significantly higher than in control gerbils (Fig. 2A), suggesting that *Atopobium* cluster may be associated with persistent *H. pylori* infection, though the reason for this was not clear. Many reports have shown that several strains of *Lactobacillus* inhibit the growth of *H. pylori* [35–39]. These effects are mainly based on the production of lactic acid and low pH in *in-vitro* studies. However, Ryan et al. [40] reported that growth inhibition of *H. pylori* by *Lactobacillus salivarius* was strain-dependent, and showed that 9 out of 28 *L. salivarius* strains and 3 out of 12 other *Lactobacillus* species tested inhibited the growth of *H. pylori.* In the present study, however, there was no significant correlation between the numbers of *H. pylori* and *Lactobacillus* spp. (Fig. 2G). It is possible that the dominant *Lactobacillus* spp. in the stomachs of Mongolian gerbils does not exert an inhibitory effect on *H. pylori.* Sun et al. [41] have also reported that there were no inhibitory microbiota against *H. pylori* infection using a Mongolian gerbil infection model.

The influence of *H. pylori* colonization on human gastric microbiota has previously been reported by Bik et al. [42]. In their study, bacterial diversity within the human gastric mucosa was characterized using a small subunit 16S rDNA clone library. However, they did not find any significant differences in gastric microbiota between *H. pylori* positive and negative patients. It was also shown that many oral bacteria were found in gastric biopsies. However, in this study we did not detect any oral bacteria such as *Fusobacterium* spp. or *Veillonella* spp., and the dominant bacteria in the gastric mucosa were *Lactobacillus* spp. (Fig. 2G) and *Enterococcus* spp (Fig. 2E). As gerbils have a habit of eating their own feces, bacteria in the gerbil stomach may originate chiefly from intestinal microbiota.

In summary, *Lactobacillus* spp. and *Enterococcus* spp. were detected in all three groups, but lower numbers of *Bifidobacterium* spp., *C. coccoides* group and *C. leptum* subgroup and higher numbers of *Atopobium* cluster were detected in *H. pylori*-negative gerbils. This implies that these changes seen in gerbils that end up *H. pylori*-negative may in fact be key in inhibiting *H. pylori* infection and that there is a significant interaction between the indigenous gastric microbiota and *H. pylori* in Mongolian gerbils.

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Destructive effects of butyrate on the cell envelope of *Helicobacter pylori*

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Helicobacter pylori can be found in the oral cavity and is mostly detected by the use of PCR techniques. Growth of *H. pylori* is influenced by various factors in the mouth, such as the oral microflora, saliva and other antimicrobial substances, all of which make colonization of the oral cavity by *H. pylori* difficult. In the present study, we analysed the effect of the cell supernatant of a representative periodontal bacterium *Porphyromonas gingivalis* on *H. pylori* and found that the cell supernatant destroyed the *H. pylori* cell envelope. As *P. gingivalis* produces butyric acid, we focused our research on the effects of butyrate and found that it significantly inhibited the growth of *H. pylori*. *H. pylori* cytoplasmic proteins and DNA were detected in the extracellular environment after treatment with butyrate, suggesting that the integrity of the cell envelope was compromised and indicating that butyrate has a bactericidal effect on *H. pylori*. In addition, levels of extracellular *H. pylori* DNA increased following treatment with the cell supernatant of butyric acid-producing bacteria, indicating that the cell supernatant also has a bactericidal effect and that this may be due to its butyric acid content. In conclusion, butyric acid-producing bacteria may play a role in affecting *H. pylori* colonization of the oral cavity.

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INTRODUCTION

Helicobacter pylori is a spiral-shaped, microaerophilic, non-invasive, Gram-negative bacterium that colonizes the human gastrointestinal tract, primarily the stomach (Marshall & Warren, 1984). A number of factors such as production of VacA cytotoxin, CagA, the *cag* pathogenicity island, motility and urease production are known to be involved in the virulence of this organism (Akada *et al.*, 2000; Bijlsma *et al.*, 1999; Censini *et al.*, 1996). *H. pylori* has been identified as an aetiological agent of chronic active gastritis, peptic ulcer disease (Blaser, 1992; Graham, 1989), gastric adenocarcinoma (Parsonnet *et al.*, 1991) and mucosa-associated lymphoid tissue lymphoma (Wotherspoon *et al.*, 1993).

The mode of *H. pylori* transmission is not fully understood despite considerable research; however, it is very likely that *H. pylori* is transmitted via the oral cavity. Detection of *H. pylori* in the human oral cavity and faeces is consistent with this transmission pathway and there are many reports of colonization by this micro-organism of the stomach and oral cavity, particularly in patients with gingivitis or periodontal disease (Berroteran *et al.*, 2002; Khandaker *et al.*, 1993; Hardo *et al.*, 1995; Tursi *et al.*, 1996; Ferguson *et al.*, 1993). These reports suggest that the oral cavity is a primary extragastric reservoir for *H. pylori*. However, most of these studies detected *H. pylori* with PCR and therefore the numbers of viable bacteria could not be assessed.

The oral cavity is colonized by a variety of micro-organisms (Kolenbrander & London, 1993). Periodontal diseases are initiated by bacteria such as *Porphyromonas gingivalis*, which is a Gram-negative anaerobic bacterium isolated frequently from subgingival lesions in adult patients with periodontitis (Loster *et al.*, 2006). A number of virulence factors, such as fimbriae, LPS, and cysteine proteinases have been implicated in this organism (Slots & Genco, 1984; Socransky & Haffajee, 1991; Yonezawa *et al.*, 2001). Moreover, the butyric acid produced by *P. gingivalis* is thought to play a pathogenic role in human periodontal

Abbreviations: FCS, fetal calf serum; SCFA, short-chain fatty acid; SEM, scanning electron microscopy.

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disease (Kurita-Ochiai *et al.*, 2006). Conversely, butyric acid has been shown to exhibit an antimicrobial effect on *Campylobacter* species, *Escherichia coli* and *Staphylococcus aureus* (Van Deun *et al.*, 2008; Weber & Kerr, 2008; Ochoa-Zarzosa *et al.*, 2009). In addition, our previous study demonstrated that butyric acid has the specific property of inhibiting the survival of *H. pylori* independent of low pH (Takahashi *et al.*, 2000). Furthermore, the cell-free supernatant of *Clostridium butyricum* MIYAIRI 588, a butyric acid-producing probiotic bacterium commonly used for treating and preventing both non-antimicrobial-induced and antimicrobial-associated diarrhoea in humans and animals (Sato & Tanaka, 1997; Kamiya *et al.*, 1997), also exhibited antibacterial properties independent of the decrease in pH (Takahashi *et al.*, 2000). These observations suggest that the butyric acid produced by *P. gingivalis* could be an antibacterial agent against *H. pylori* in the oral cavity.

The purpose of this study was to evaluate the possibility that *H. pylori* can survive in the oral cavity, especially in periodontal pockets. It is important to clarify the colonization mechanism of *H. pylori* in the oral cavity and the potential ecological roles of the oral bacterial flora in this process, and so we examined the effects of the culture filtrate of *P. gingivalis* on *H. pylori*. Taking into account the results from our previous studies, we also assessed the antibacterial effects of sodium butyrate on *H. pylori in vitro*. Based on these results, it was suggested that the butyrate in the culture supernatant of *P. gingivalis* plays a role in its antibacterial activity against *H. pylori*.

METHODS

Bacterial strains and culture conditions. *H. pylori* strains ATCC 49503 and TK1402 were used in this study. The latter strain is a clinical isolate from a Japanese patient, which has been used in previous studies (Yamaguchi *et al.*, 2003; Yonezawa *et al.*, 2009). All strains were stored at −70 °C in Brucella broth (Becton Dickinson) with 20 % (v/v) glycerol. These strains were cultured under microaerobic conditions at 37 °C on Brucella medium agar plates containing 7 % horse serum. *C. butyricum* strain MIYAIRI 588 was grown under anaerobic conditions (85 % N_2, 10 % H_2, 5 % CO_2) at 37 °C in brain heart infusion medium (Becton Dickinson). *P. gingivalis* strain ATCC 33277 was grown under anaerobic conditions at 37 °C in brain heart infusion medium supplemented with 5 μg haemin ml^{-1} and 0.5 μg menadione ml^{-1}.

SEM analysis. For SEM analysis, *H. pylori* strain TK1402 was treated with various amounts of *C. butyricum* or *P. gingivalis* cell supernatant, or various concentrations of sodium butyrate. The cell supernatants were prepared as described below. After anaerobic cultivation of *C. butyricum* MIYAIRI 588 or *P. gingivalis* ATCC 33277 at 37 °C for 24 h, the cells were collected by centrifugation (10 000 ***g*** for 10 min) and the resulting supernatants were filtered (low-protein-binding Durapore membrane, 0.22 mm polyvinylidene fluoride, Millipore). The filtrate (1.5 ml) was added to 0.5 ml of fresh *H. pylori* medium. Similarly, sodium butyrate (Sigma) was added to the medium at a final concentration of 20 mM. Approximately 5×10^6 c.f.u. of pre-cultured *H. pylori* were then added to the medium. After 24 h incubation at 37 °C, *H. pylori* cells were collected and attached to poly-L-lysine-coated coverslips (IWAKI) by centrifugation (1500 ***g***, 5 min). Cells on the coverslips were washed twice in PBS and fixed with 2.5 % glutaraldehyde for 1 h at 4 °C. The samples were observed using a JSM-6330F electron microscope (JEOL).

Effect of sodium butyrate or cell supernatants on the growth of *H. pylori in vitro*. Growth of *H. pylori* strains was examined under microaerobic cultivation in Brucella broth supplemented with 7 % fetal calf serum (FCS) with or without the addition of sodium butyrate at final concentrations of 20, 10 or 5 mM. Approximately 5×10^6 c.f.u. pre-cultured *H. pylori* was added to fresh Brucella broth supplemented with 7 % FCS with or without various concentrations of sodium butyrate. After 24 h or 48 h cultivation at 37 °C, cell growth was determined by measuring OD_{600}.

Western blot analysis. For Western blotting, *H. pylori* strain TK1402 was grown under microaerobic conditions at 37 °C with shaking for 24 h in Brucella broth supplemented with 7 % FCS and various concentrations of sodium butyrate (20, 10, 5 or 0 mM). After cultivation, the OD_{600} of the cell cultures was adjusted to 0.5 with each respective medium. The cells were collected by centrifugation (100 000 ***g*** for 15 min) and the resulting supernatants were filtered (0.22 μm, Millipore). Total proteins in cell-free supernatants were obtained by precipitating 1.0 ml of the culture with 7.5 % trichloroacetic acid. The precipitates were washed with ice-cold acetone and solubilized with 100 μl 0.5 M Tris/HCl (pH 8.0). The soluble protein was treated with SDS loading buffer including 5 % 2-mercaptoethanol at 100 °C for 5 min and separated by PAGE. The separated proteins were transferred to polyvinylidene difluoride membranes (Atto), after which the membrane was blocked with 3 % BSA in PBS for 60 min at room temperature and incubated with anti-UreB MAb, termed L2 (Hirota *et al.*, 2001) (1 : 200) at 37 °C for 60 min. After washing with PBS containing 0.05 % Tween 20 (PBS-T), peroxidase-labelled rabbit anti-mouse immunoglobulin (Dako A/S) was used at 1 : 2000 dilution as a secondary antibody. After washing with PBS-T, the blot was developed using the ECL Plus Detection system (GE Healthcare).

Extraction of *H. pylori* extracellular DNA from culture supernatant. Pre-cultured *H. pylori* cells were harvested by centrifugation (10 000 ***g*** for 5 min) and then washed twice with PBS. The cells were suspended in fresh Brucella broth supplemented with 7 % FCS or PBS with or without various concentrations of sodium butyrate at an OD_{600} of 1.0. After 24 h microaerobic incubation at room temperature, the culture samples were centrifuged and the supernatants were filtered (0.22 μm, Millipore). Extracellular *H. pylori* genomic DNA was extracted from 500 μl volumes of the filtrates using phenol/chloroform, precipitated with ethanol and resuspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Extracted DNA was used for real-time quantitative PCR using SYBR Premix Ex *Taq* (TAKARA) using an *H. pylori* 16S rDNA specific primer pair [forward: 5′-GAAGATAATGACGGTATCTAAC-3′; reverse: 5′-ATTTCACACCTGACTGACTAT-3′ (Rinttilä *et al.*, 2004)] in an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). Quantitative data were calculated from a standard curve generated by amplifying serial dilutions of a known quantity of amplicon. For this approach, the specificity of the PCR product was confirmed by dissociation curve analysis (7500 Fast Real-Time PCR quantification program, Applied Biosystems).

The effect of supernatants from *C. butyricum* and *P. gingivalis* on *H. pylori* was examined by a similar method with slight modifications. The preparation of these bacterial filtrates was performed as described above. Various amounts of the filtrates were added to PBS along with *H. pylori* cells to a final OD_{600} of 1.0 and the amounts of extracellular DNA were examined in a similar manner as described above.

Statistical analysis. Statistical analysis was performed using the Mann-Whiney U test. *P*-values $\leqslant 0.05$ were considered significant.

RESULTS

Antimicrobial effects of cell supernatants of *C. butyricum* and *P. gingivalis* on *H. pylori*

We previously demonstrated that cell supernatants of *C. butyricum* MIYAIRI 588 inhibited *H. pylori* survival independent of acidic conditions (Takahashi *et al.*, 2000) and that the butyric acid produced by this micro-organism contributed to the antibacterial action. In order to investigate the effects of cell supernatants of *C. butyricum* MIYAIRI 588 on *H. pylori* in detail, morphological changes after treatment with the cell supernatant were observed by scanning electron microscopy (SEM) analysis. A clear bacillary morphology was observed in *H. pylori* control cells (without treatment) (Fig. 1a), whereas, *H. pylori* cells treated with the cell supernatant exhibited morphological changes. Swelling of some parts of the cell body and bleb-like structures on the cell surface were detected following treatment with the cell supernatant of *C. butyricum* (Fig. 1b). Although only a few cells are depicted in the figure, these pictures represented typical morphological changes observed in almost all bacterial cells analysed, and these changes were not detected in controls. These results suggest that the cell envelope of *H. pylori* may have been damaged by treatment with the supernatant. Next, we examined the effects of the cell supernatant of *P. gingivalis* ATCC 33277 on *H. pylori* by SEM analysis (Fig. 1c) and the changes observed were similar to the effects of the supernatant of *C. butyricum*.

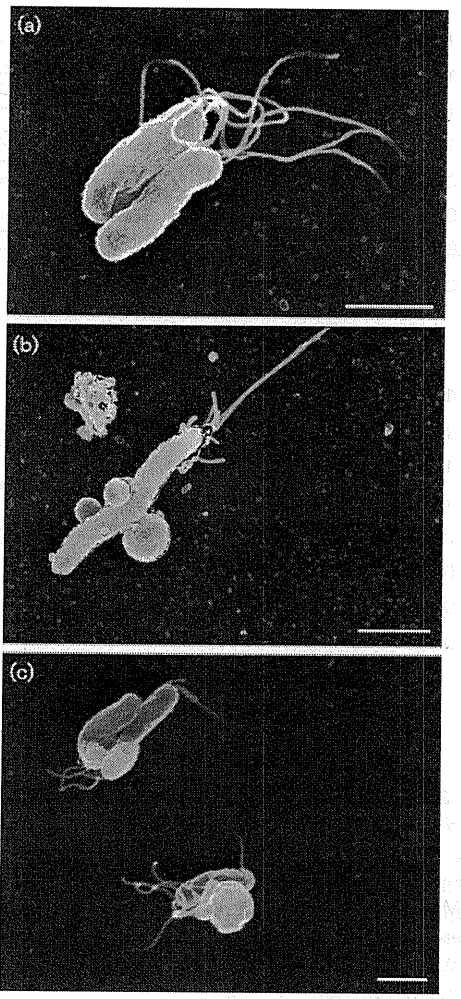

Fig. 1. Scanning electron micrographs of untreated *H. pylori* cells (a) and *H. pylori* cells treated with cell supernatants of *C. butyricum* (b) and *P. gingivalis* (c). Bars, 1 μm.

Inhibitory effect of sodium butyrate on the growth of *H. pylori in vitro*

The results from our previous study and from the SEM analysis suggested that both *P. gingivalis* and *C. butyricum* culture supernatants could destroy the cell envelope of *H. pylori*, and that this action could be independent of acidic conditions. In order to confirm whether this action was due to butyrate, we investigated the growth of *H. pylori* strains in various concentrations of sodium butyrate. We had already established that the pH of the medium was not changed after addition of sodium butyrate (data not shown). Addition of 20 or 10 mM sodium butyrate to the growth medium inhibited the growth of *H. pylori* strain TK1402 (Fig. 2a). Moreover, even the addition of 5 mM sodium butyrate tended to inhibit growth compared to the control, although this difference was not statistically significant. When another *H. pylori* strain, ATCC 49503, was used, similar results were observed (Fig. 2b).

Regarding the growth inhibition of *H. pylori* by sodium butyrate, the growth yield of these strains increased relative to the initial inoculum, even though growth was inhibited compared to controls. Therefore, in order to confirm that *H. pylori* TK1402 cells were viable after treatment with 20 mM sodium butyrate, a measurement of viable cells was carried out. The c.f.u. value of the inoculum was approximately 5×10^5 and the c.f.u. values in 1 day, 2 day and 3 day-old cultures were $3.17 \pm 1.81 \times 10^8$, $1.85 \pm 0.32 \times 10^9$ and $2.25 \pm 1.17 \times 10^9$, respectively. However, the c.f.u. values of sodium butyrate-treated cells in 1 day, 2 day and 3 day-old cultures were markedly decreased at $1.07 \pm 0.81 \times 10^8$, $3.53 \pm 0.91 \times 10^5$ and undetectable, respectively.

Morphological analysis of *H. pylori* after treatment with sodium butyrate

In order to investigate the effects of sodium butyrate on *H. pylori* cells, morphological changes following treatment with 20 mM sodium butyrate were observed by SEM analysis. The results indicated that *H. pylori* cells treated with sodium butyrate exhibited similar morphological changes to those seen in *H. pylori* cells treated with bacterial supernatants (*C. butyricum* and *P. gingivalis*) (Fig. 1). Swelling of some parts of the cell body was observed (Fig. 3a) and the cell envelope was significantly altered by

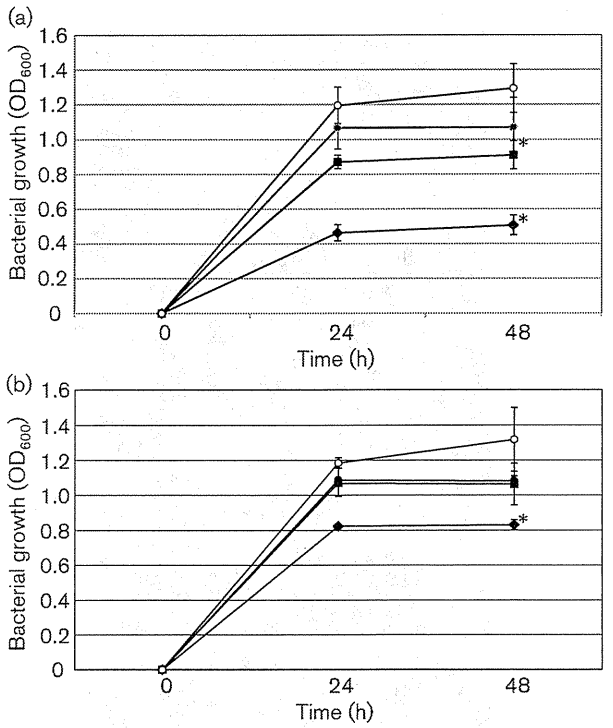

Fig. 2. Inhibitory effect of sodium butyrate on the growth of *H. pylori* strains TK1402 (a) and ATCC 49503 (b). *H. pylori* strains were grown in Brucella broth supplemented with 7% FCS and sodium butyrate at concentrations of 20 mM (◆), 10 mM (■), 5 mM (●) and 0 mM (○). Results are expressed as means ± SD of at least three independent experiments. *Significantly different ($P<0.05$) relative levels of growth (OD_{600}) compared to control without sodium butyrate (○).

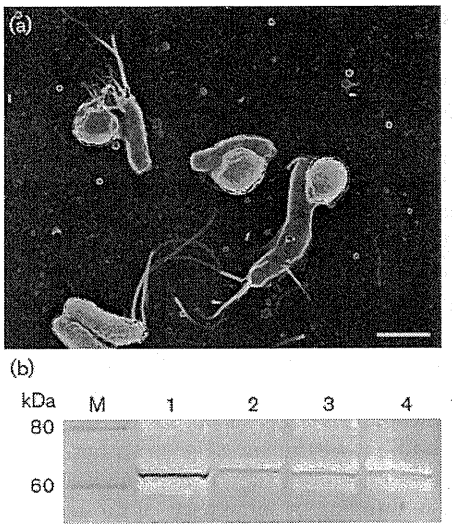

Fig. 3. (a) Scanning electron micrograph of *H. pylori* TK1402 cells treated with sodium butyrate. *H. pylori* cells were treated with 20 mM of sodium butyrate and morphological changes were analysed by SEM. Bar, 1 μm. (b) Western blot analysis of extracellular proteins derived from various concentrations of sodium butyrate-treated *H. pylori* TK1402 cells with anti-UreB MAb. Lanes: M, molecular mass marker; 1, 20 mM sodium butyrate; 2, 10 mM; 3, 5 mM; 4, 0 mM.

treatment with sodium butyrate. These results indicated that butyrate alone could damage the cell envelope of *H. pylori* and also implied that butyrate plays a role in the antibacterial action of the culture supernatants.

Urease, one of the important virulence factors of *H. pylori*, is a cytoplasmic protein. As the cell envelope was damaged by treatment with butyrate, we hypothesized that extracellular urease levels would increase. Therefore, we compared the amount of urease in the supernatant by using Western blotting with UreB MAb. As we expected, the amount of urease in the supernatant after treatment with 20 mM sodium butyrate (Fig. 3b, lane 1) was significantly increased compared to control (Fig. 3b, lane 4). The intensity of the bands tended to correlate with the dose of sodium butyrate added.

Detection of extracellular *H. pylori* DNA after treatment with sodium butyrate

The results of SEM analysis and Western blotting suggested that the alteration of the cell envelope may also lead to the release of bacterial DNA into the external environment, as with the cytoplasmic urease. Moreover, we hypothesized that the amount of DNA released into the extracellular environment might increase depending upon the degree of destruction of the cell envelope. In order to investigate the extent of cell envelope damage in *H. pylori* after treatment with different concentrations of sodium butyrate, the amount of extracellular DNA was analysed by real-time PCR. First, we established that *H. pylori* could not grow under these experimental conditions (data not shown). Treatment with 20 mM sodium butyrate exhibited significantly higher amounts of extracellular DNA ($P<0.05$, compared to treatment with 10, 5 and 0 mM sodium butyrate). The mean extracellular DNA levels after treatment with 20 mM sodium butyrate were 1.340 ± 0.131 μg ml^{-1} and 1.345 ± 0.078 μg ml^{-1} for *H. pylori* strains ATCC 49503 (Fig. 4a) and TK1402 (Fig. 4b), respectively. The amounts of extracellular DNA were not significantly different between the two strains but tended to correlate with the concentration of sodium butyrate added. As a comparison, we also determined the antibacterial effect of sodium acetate on *H. pylori* using a similar method and showed that treatment with 20 mM sodium acetate caused no increase in extracellular DNA compared to the control (data not shown).

The experiments described here were carried out by adding sodium butyrate to Brucella broth. There was a possibility that the growth of *H. pylori* may have affected the increase of extracellular DNA, even if the cultures were incubated at room temperature. Therefore, we measured the quantity of

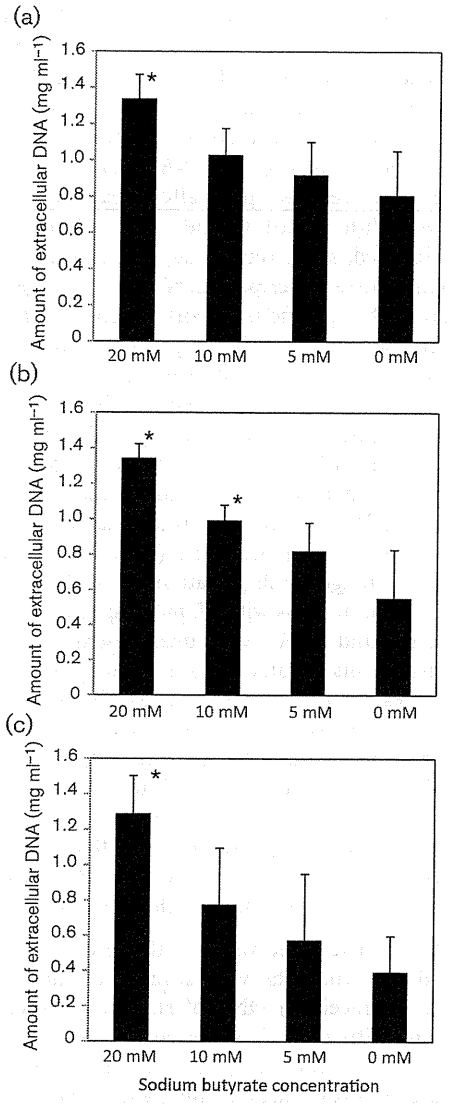

Fig. 4. Amounts of *H. pylori* extracellular DNA following treatment with sodium butyrate. The sodium butyrate was added to *H. pylori* culture medium or PBS. After incubation, extracellular DNA was detected with quantitative PCR. (a) *H. pylori* ATCC 49503 cultured with growth medium; (b) *H. pylori* TK1402 cultured with growth medium; and (c) *H. pylori* TK1402 suspended in PBS. *Significantly different ($P<0.05$) relative levels of extracellular DNA compared to control without sodium butyrate. Results are expressed as means ± SD of at least three independent experiments.

extracellular DNA of *H. pylori* in PBS with or without sodium butyrate. The results were similar to those when medium was used (Fig. 4c).

Detection of extracellular *H. pylori* DNA after treatment with bacterial cell supernatant

To analyse the extent of *H. pylori* cell envelope damage after treatment with cell supernatants of *C. butyricum* and *P. gingivalis*, the amount of extracellular DNA in samples was analysed by real-time PCR. Various doses of the cell supernatants were added to *H. pylori* in PBS and incubated at 37 °C for 24 h. It was found that the amount of extracellular DNA detected when cell supernatants of *C. butyricum* (1.5, 1.0 and 0.5 ml) and *P. gingivalis* (1.5 and 1.0 ml) were added was significantly increased compared to the PBS control (Fig. 5a and b) and was dependent upon the dose of cell supernatant added.

DISCUSSION

H. pylori is detected in the human oral cavity as well as in the gastrointestinal tract (Marshall & Warren, 1984; Berroteran *et al.*, 2002; Khandaker *et al.*, 1993; Hardo *et al.*, 1995; Tursi *et al.*, 1996; Ferguson *et al.*, 1993). In addition to the stomach, the oral cavity is proposed as a potential reservoir of *H. pylori* and many research groups have detected the bacterium, by using PCR, in dental plaque and saliva of

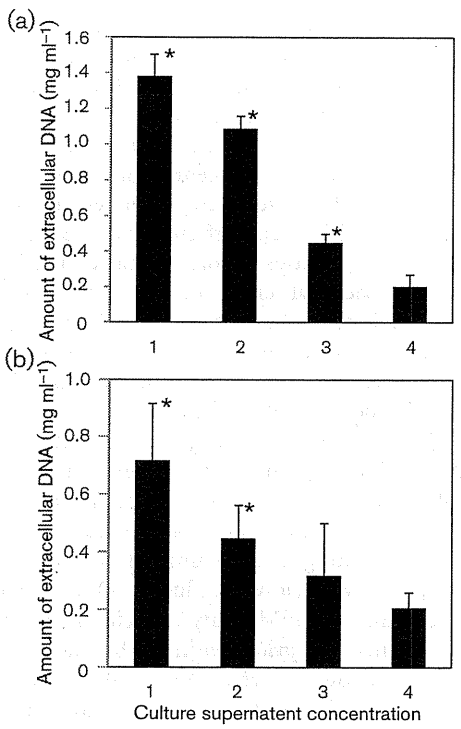

Fig. 5. Effects of cell supernatants derived from *C. butyricum* (a) or *P. gingivalis* (b) on *H. pylori* cells. Various amounts of cell-free supernatant were added to *H. pylori* strain TK1402 cultures and the levels of extracellular DNA were measured with quantitative PCR. *x*-axis: 1, 1.5 ml of culture supernatant added to 0.5 ml PBS; 2, 1.0 ml of culture supernatant was added to 1.0 ml PBS; 3, 0.5 ml of culture supernatant was added to 1.5 ml PBS; 4, 2.0 ml PBS only (control). *Significantly different ($P<0.05$) relative levels of extracellular DNA (compared to PBS control). Results are expressed as means ± SD of at least three independent experiments.

patients with periodontal disease. On the other hand, few studies have successfully cultured this bacterium from oral specimens. It has also been observed that the periodontal-disease-associated bacteria *Porphyromonas*, *Prevotella* and *Fusobacterium* can produce butyric acid (Kurita-Ochiai *et al.*, 2006) and, recently, Imai *et al.* (2009) indicated that butyric acid is the major short-chain fatty acid (SCFA) produced by *P. gingivalis*. Our previous study demonstrated that the cell supernatants of *C. butyricum* strain MIYAIRI 588 inhibited survival of *H. pylori* (Takahashi *et al.*, 2000). This antibacterial activity is, in part, due to the production of butyric acid by this micro-organism. We demonstrated in the present study that the culture supernatants of *C. butyricum* strain MIYAIRI 588 and *P. gingivalis* strain ATCC 33277 exhibited antibacterial activity against *H. pylori* (Fig. 1b and c). In fact, these bacteria produce various other SCFAs in smaller quantities, such as formic, acetic, propionic and lactic acids, and therefore the butyric acid in the supernatant may not be solely responsible for the bactericidal activity against *H. pylori*.

The antimicrobial effects of these SCFAs have been well characterized. Chaveerach *et al.* (2002) reported that organic acids, such as formic acid, propionic acid and acetic acid have a strong bactericidal effect on *Campylobacter jejuni* and *Campylobacter coli* culturability at low pH. Midolo *et al.* (1995) reported the inhibition of the growth of *H. pylori* by lactic acid, acetic acid and hydrochloric acid in a concentration-dependent manner. These antimicrobial effects are dependent upon acidic pH conditions, which were affected by the organic acids. However, our previous report indicated that butyric acid has an antibacterial effect on *H. pylori* and that this property is independent of acidic pH (Takahashi *et al.*, 2000). In order to clarify this antibacterial action, we used sodium butyrate in the present study, since it does not change the medium pH. After treatment of the *H. pylori* strains with sodium butyrate, growth inhibition of *H. pylori* was detected, correlating with the concentration of sodium butyrate added (Fig. 2). In addition, the c.f.u. value decreased after 48 h of culture and treatment with sodium butyrate, suggesting that the antibacterial effect of butyrate on *H. pylori* was slowly induced. Our previous study indicated that 50 mM butyric acid had antibacterial activity against *H. pylori* within 5 h, but also indicated that *H. pylori* could survive after treatment with 12.5 mM butyric acid (Takahashi *et al.*, 2000). SEM analysis indicated that this antibacterial action causes damage of the cell envelope of *H. pylori* (Fig. 3a). The bacterial cell envelope is responsible for many essential functions such as transport, biosynthesis and cross-linking of peptidoglycans, and the synthesis of lipids, and envelope integrity is absolutely necessary for all of these functions. Disturbance of the cell envelope, directly or indirectly, causes a significant increase in permeability leading to a destabilization of the cell membrane and finally cell death. We analysed the amount of urease in the extracellular environment and the results indicated that the extracellular urease was elevated following treatment with sodium butyrate in a dose-dependent manner (Fig. 3b). Urease is a cytoplasmic protein and becomes associated with cell-surface proteins as well as extracellular proteins in the external environment following bacterial autolysis (Cao *et al.*, 1998). Similarly, the DNA of *H. pylori* was released into the extracellular space after treatment with sodium butyrate. With regard to the origin of the extracellular DNA detected, some reports suggest that cell lysis may be the main source, whereas others observed that DNA could be secreted by specific transport systems in *H. pylori* or in the presence of specific reagents in oral streptococci (Grande *et al.*, 2011; Kreth *et al.*, 2009). We analysed the DNA of *H. pylori* treated with sodium butyrate using random amplification of polymorphic DNA (RAPD)-PCR analysis as described previously (Grande *et al.*, 2011). The pattern and intensity of the bands in the <1 kb size range were identical between the extracellular DNA and control DNA extracted from the cells (data not shown). In the >1 kb size range, multiple attempts at detecting conventional PCR amplicons with *H. pylori* specific primers (*ureA*, *ureB*, *vacA* and *oipA*) were unsuccessful, suggesting that DNA fragments greater than 1 kb in size were digested by DNases in the medium. Nevertheless, these results suggested that the extracellular DNA was derived from disintegration of the cells. In addition, we demonstrated that there was a positive correlation between the amount of extracellular DNA and sodium butyrate concentration (Fig. 4). These findings strongly indicated that the antibacterial properties of butyrate on *H. pylori* are bactericidal and act through disintegration of the cell envelope.

In order to determine whether the antibacterial action of the cell supernatants was similar to that of sodium butyrate, extracellular DNA of *H. pylori* was analysed after treatment with the cell supernatants (Fig. 5) and it was found that the amounts of extracellular DNA detected also increased in a dose-dependent manner. These results were similar to the action of sodium butyrate, suggesting that the butyrate produced by these strains may be the principal product responsible for their antibacterial activity. However, we did not obtain any direct evidence of this, so purification and chemical characterization of the butyric acid in these supernatants will be required to resolve this issue. In addition, the antibacterial effects of other SCFAs produced by *P. gingivalis* or *C. butyricum* on *H. pylori* still remain to be determined.

Ishihara *et al.* (1997) found that *P. gingivalis* and *F. nucleatum* strongly coaggregated with *H. pylori*. This finding suggested that these resident oral bacteria might be effectively trapping newly transiting bacteria such as *H. pylori* in periodontal pockets of the oral cavity. Indeed, some reports indicated that *H. pylori* is a transient member of the oral microflora, since the growth of *H. pylori* in the oral cavity is influenced by various factors such as temperature, pH, oxidation–reduction potential, the availability of nutrients, flow of saliva and antimicrobial substances (Song *et al.*, 2000; Silva Rossi-Aguiar *et al.*, 2009;

Okuda *et al.*, 2000, 2003). Furthermore, Imai *et al.* (2009) indicated that *P. gingivalis* culture supernatant contained butyric acid in high concentrations, from 6.7 to 14.7 mM. Moreover, Margolis *et al.* (1988) indicated that the concentration of butyric acid in dental plaque was in the range of 14.4 to 20.0 mM. On the basis of our previous and present results, we have shown that 12.5 mM butyrate is sufficient to exhibit a bactericidal effect on *H. pylori*. Therefore, survival of *H. pylori* in the oral cavity, especially within the periodontal pocket which harbours butyrate-producing bacteria such as *P. gingivalis*, may be difficult based upon this observation. *H. pylori* DNA released from dying cells into the environment would therefore be increased in concentration and might contribute to periodontal bacterial biofilm formation, as it has been shown that bacterial DNA is a major component of the extracellular matrix. This would then explain the ease of detection of *H. pylori* DNA in the human oral cavity by PCR, and the difficulty of detection by culture methods in patients with periodontal disease.

In summary, we have characterized the antimicrobial effects of butyrate on *H. pylori*. This molecule exhibited bactericidal effects and reacted with the cell envelope of *H. pylori*. Similar properties were also detected with the culture supernatants of butyrate-producing bacteria, which suggests that *H. pylori* may have difficulty in colonizing sites which harbour butyric acid-producing inhabitants such as *P. gingivalis* in subgingival plaque. It is possible that *H. pylori* previously detected in the oral cavity may have originated from the stomach following regurgitation and as we have shown, the presence of *H. pylori* DNA in the oral cavity, as observed in other studies, may not represent the true viability of the organism in this site.

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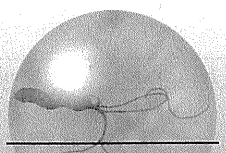

特集

Helicobacter Year Book 2010〜2011
—*Helicobacter* はここまで解明された—

Helicobacter 研究の年間レビュー
小児領域の新知見をみる

奥田真珠美* 前川講平* 大崎慶子* 福田能啓**

先進国において小児の *Helicobacter pylori*（*H. pylori*）感染率は急速に低下しており，論文が非常に少なくなっているのが現状である．途上国でも小児の感染率が低下してきているが小児の報告はブラジルやイラン，イスラエルなどからのものが多い．*H. pylori* 感染経路としては同胞間に注目したものが，疾患との関連では鉄欠乏性貧血との関連に関するものが多かったが，小児のクローン病と *Helicobacter* 属の関連を示唆する興味深い報告もあった．

KEY WORDS
Helicobacter pylori（*H. pylori*），小児，感染経路，鉄欠乏性貧血

はじめに

先進国における小児の *Helicobacter pylori*（*H. pylori*）感染率は低下しており，感染率の高い途上国からの論文が多くなっている．本稿では小児期の *H. pylori*，その他の *Helicobacter* 属に関して感染率，感染経路，診断法，疾患との関連などについて 2010 年に掲載されたものを中心に知見を述べる．

1．感染率・感染経路

1）各国から報告された小児の感染率（表❶）

ブラジルにおける検討[1]であるが，公立学校に通う 10〜16 歳の青年 400 名のうち 194 名に対して尿素呼気試験（UBT）をおこない，79 名が陽性で感染率 40.7%であった．性別では男児 50%，女児 32.7%と男児で感染率が高かった．

アフリカ難民の小児 163 名（2〜16 歳）における *H. pylori* 感染率は 84%であった[2]．その他，**表❶**に示したが，これまで感染率が高いと考えられていた国々でも小児の感染率は低下してきている．2006 年以降にわれわれが日本の小児で便中抗原を用いた検討では 5%以下の陽性率と急激に低下している．

2）感染経路

小児でも感染率が高い国からの報告がほとんどであり，同胞間感染を示唆する論文が多かった．

ブラジルの小児における *H. pylori* 感染率（血清抗体保有率）と感染に関与する危険因子の検討である[3]．ブラ

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