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.

**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
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 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% N2, 10% H2, 5% CO2) 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 × 10⁵ 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% FCS and various concentrations of sodium butyrate (20, 10, 5 or 0 mM). After cultivation, the OD₆₀₀ 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 OD₆₀₀ 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'-GAAGAATATGGCAGTCTTACAC-3'; reverse: 5'-ATTTCACACCTGACTGACTAT-3'] (Rintilla 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 OD₆₀₀ 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 < 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.

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 strain TK1402 could be 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 $5 \times 10^5$ 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 $2.25 \pm 1.17 \times 10^8$, 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
treatment with sodium butyrate. These results indicated that butyrate alone could damage the cell envelope of \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 ng ml$^{-1}$ and 1.345 ± 0.078 ng ml$^{-1}$ for \textit{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 \textit{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 \textit{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
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...
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 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; Kretz 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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