Sensitivity of *Helicobacter pylori* to an innate defence mechanism, the lactoperoxidase system, in buffer and in human whole saliva

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**INTRODUCTION**

*Helicobacter pylori* is a Gram-negative, microaerophilic bacterium that causes chronic active gastritis and is also associated with gastric and duodenal ulcers and gastric carcinoma. This species has been detected in human dental plaque and in whole saliva in numerous studies by culture, PCR and other techniques (Namavar *et al.*, 1995; Song *et al.*, 2000a, b; Young *et al.*, 2001; Allaker *et al.*, 2002), but there are also opposite findings (Asikainen *et al.*, 1994; Wahlfors *et al.*, 1995; Hardo *et al.*, 1995; Luman *et al.*, 1996). This discrepancy can be explained by differences between human populations as well as methodological differences. The mode of transmission of *H. pylori* is still partly unclear, but faecal–oral– or oral–oral–transmissions are generally accepted as the most probable ones (Dowsett & Kowolik, 2003).

Human saliva contains peroxidase enzymes and lysozyme among many other host innate defence systems (for a review see Tenovuo, 1989). The complete peroxidase system in saliva comprises three components: the peroxidase enzymes – salivary peroxidase and myeloperoxidase, hydrogen peroxide (H₂O₂) and an oxidizable substrate such as a pseudo-halide thiocyanate (SCN⁻). The typical concentration of peroxidases in whole saliva is roughly 5 μg ml⁻¹, the myeloperoxidase concentration being approximately twice as high as that of salivary peroxidase (Thomas & Aune, 1978a, b; Courtois *et al.*, 1992; Shin *et al.*, 2002). To extend the currently limited knowledge, we investigated the effects of the LP system on several *H. pylori* strains, including clinical

Abbreviation: LP, lactoperoxidase.

Helicobacter pylori has frequently been isolated from human dental plaque, and oral spread via saliva is thought to be one of its principal modes of transmission. Among other innate defence systems human saliva contains peroxidase enzymes and lysozyme. The sensitivity of *H. pylori* to physiological concentrations of lactoperoxidase and its salivary substrate thiocyanate, and different amounts of hydrogen peroxide (H₂O₂) was investigated in buffer and in human whole saliva. The effect of lysozyme was also studied in saliva. All tested *H. pylori* strains, ATCC 43504T and five clinical isolates, were efficiently inhibited by the peroxidase system with high concentrations of H₂O₂ in buffer. The inhibition was stronger at lower pH. However, in human saliva these high concentrations of H₂O₂ generated less hypothiocyanite, the antibacterial product of the peroxidase system and the effects of the peroxidase system were weaker. Physiological concentration of lysozyme was not bactericidal against *H. pylori*, nor did it enhance the effect of the peroxidase system in saliva. Thus, further studies are needed to enhance the efficacy of peroxidase systems in human saliva to make it more beneficial not only against dental but also against gastric pathogens.
isolates, both in buffer solution and in sterilized human whole saliva. The experiments in whole saliva will more reliably mimic the conditions in vivo than in any previous study.

METHODS

Bacteria and growth conditions. H. pylori ATCC 43504T and five clinical isolates (A–E) were studied. The clinical strains of H. pylori used in this study were from patients attending for an upper gastrointestinal endoscopy at the Helsinki university hospital during 1997. Stock cultures were stored at −70 °C in brain heart infusion (BHI) broth supplemented with 20% (v/v) glycerol. Bacteria were cultured on Brucella agar plates supplemented with 7% (v/v) horse blood and 1% (v/v) IsoVitalex-supplement (BBL) in a microaerophilic atmosphere generated with Anaerocult C (Merck). For each experiment the bacteria stored at −70 °C were cultured for 3 or 4 days, collected with BHI and subcultured on the above described Brucella-agar plates for 2 days. Bacteria were harvested with buffer, centrifuged and the pellet was resuspended in buffer to give an OD600 of 10 (approx. 10^9 c.f.u. ml⁻¹).

Buffer solution and saliva. Solution I (Inabin et al., 1998) was used as the buffer in all assays. It contained 9 mM Na2HPO4, 24 mM KH2PO4, 1.5 mM MgSO4 and 67 mM Na2SO4 at pH 6.5 and 5.0. The buffer was adjusted to pH 5.0 by adding 0.1 M acetic acid.

Paraffin-stimulated whole saliva was collected from 11 non-smoking female donors between 8 and 10 am. The donors were asked not to eat or drink and to avoid oral hygiene for 1 h before collection. Salivas were pooled, centrifuged (17 300 g, 20 min), filtered (0.45 μm filter) and stored in aliquots at −20 °C. This saliva pool was used in experiments to measure OSCN⁻ formation in saliva as well as to investigate the effects of the LP system and lysozyme in saliva. Applying for permission for saliva collection from the ethical committee was considered unnecessary, since the collection of oral fluid was non-invasive, and pooled saliva, where no individual parameters could be determined, was used. In addition the study protocol was a non-clinical in vitro design.

Chemical assays. Peroxidase activity was measured at room temperature by following the rate of oxidation of coloured (5,5)-dithiobis-2-nitrobenzoic acid (Nbs) to colourless (Nbs), by OSCN⁻ ions, generated during the oxidation of SCN⁻ by peroxidases in saliva or by bovine lactoperoxidase (assays in buffer) (Wever et al., 1982), as modified by Månnsson-Rahemtulla et al. (1986). The amount of OSCN⁻ generated from KSCN and H2O2 by peroxidases in buffer or saliva was quantified by reaction with (Nbs)2 as described by Aune & Thomas (1977) and Pruitt et al. (1983).

The salivary SCN⁻ concentration was measured using the ferric nitrate method (Bets & Dainton, 1953) and the protein concentration using Folin phenol reagent (Lowry et al., 1951). Lysozyme activity in saliva was quantified by measuring the decrease of absorbance at 540 nm of commercial non-viable Micrococcus lysodeikticus culture (Bacto lysozyme substrate, Difco) in 66 mM Na2HPO4 and NaH2PO4, pH 6.2, using hen egg white lysozyme as a standard (Difco manual, 1984).

Effect of the LP system on the viability of H. pylori in buffer. For test reactions 100 μl bacterial suspension was added to 900 μl buffer to which the components of LP system (5 μg LP ml⁻¹, 1 mM KSCN and 10, 100, 300 or 500 μM H2O2) had been added. As a control 100 μl bacterial suspension was added to 900 μl buffer without any components of the LP system. After 1 h incubation at 37 °C in a candle jar, 5 μl DTT was added to the final concentration of 1 mM to stop the oxidation reactions. Aliquots of 100 μl were withdrawn before incubation from the control tube and after incubation from every tube. Tenfold dilutions were made in buffer and plated. The colonies were counted after 3 days incubation in a microaerophilic atmosphere. Experiments were repeated at least three times with each bacterial strain at pH 6.5. At pH 5.0 the experiments were made with strain ATCC 43504T. Since H. pylori is a catalase-positive organism, we used strain ATCC 43504T to ascertain that the results were not different when the bacterial suspension was added before the addition of H2O2.

Effect of the LP system on the viability of H. pylori ATCC 43504T in saliva. The effects of saliva and saliva supplemented with H2O2, with lysozyme or with lysozyme and H2O2 on H. pylori ATCC 43504T were investigated at pH 7.1 and 5.0. Bacterial suspension (made as described above) was added to 800 μl sterilized saliva supplemented with 100 μl buffer, lysozyme, H2O2 or both lysozyme and H2O2 (final concentrations 100 μM H2O2 and 50 μg lysozyme ml⁻¹). After 1 h incubation at 37 °C, DTT was added, aliquots of 100 μl were taken and diluted and plated as with the experiments made in buffer. The colonies were counted after 3 days of incubation. The pH of the test tubes was measured before and after incubation.

The pH of the saliva was above 8 after thawing, but when H2O2 in buffer (or buffer alone) as well as the bacterial suspension in buffer (pH 6.5) were added, the pH was 7.1. For the reactions at pH 5.0, the pH of the saliva was first adjusted with acetic acid, and pH 5.0 buffer was used for the bacterial suspensions and H2O2.

Statistics. Nonparametric tests were used to compare the differences between different strains. The samples with the LP system were grouped together and groups were compared with the Mann–Whitney test. The Joncheere–Terpstra test was used to test the significance of the trend.

With strain ATCC 43504T the effectiveness of the LP system with different amounts of H2O2 was studied in buffer. These data were first subjected to a one-way ANOVA. Subsequent pair-wise comparisons (each H2O2 concentration with 1 h control) were made with Dunnett’s two-sided t-test. Differences between pHs were compared with Student’s two-tailed t-test. Statistical analyses were made for original data (c.f.u. ml⁻¹) although log10 of the c.f.u. ml⁻¹ are represented in figures and tables.

The effects of the LP system and lysozyme were studied in saliva. The data (log10 c.f.u. ml⁻¹) was first subjected to one-way ANOVA and further pair-wise comparisons (reactions with H2O2 or H2O2 and lysozyme with saliva and buffer controls at same pH) were based on contrast corrected with Bonferroni correction.

RESULTS AND DISCUSSION

Effect of the LP system on viability of H. pylori in buffer. All strains tested were sensitive to the LP system and the effects on viability were dose-dependent with respect to H2O2 (Fig. 1, Joncheere–Terpstra test, P < 0.01 in all cases). The inhibition of the clinical isolates did not differ from each other or strain ATCC 43504T when compared using the Mann–Whitney test. The differences in c.f.u. ml⁻¹ in different experiments within the same strain were probably due to the different growth phase: on plate bacteria are never in exactly the same growth phase, and growth phase is known to affect the susceptibility of bacteria to antimicrobial agents (Purdy et al., 1983; Bortolussi et al., 1987). None of the strains were sensitive to H2O2 or KSCN alone (data not shown). These results are in accordance with those reported by Shin et al. (2002).
The concentrations of H₂O₂ needed to inhibit *H. pylori* cells by the LP system were relatively high when compared to previous studies in the same buffer at pH 6.5 with other Gram-negative anaerobic oral bacteria. As little as 10 μM H₂O₂ caused a significant, over two log, reduction in c.f.u. ml⁻¹ of *Actinobacillus actinomycetemcomitans* (Ihalin et al., 1998) and a slight reduction was seen with 1.25 μM H₂O₂ on *Porphyromonas gingivalis* (Ihalin et al., 2001). Since *H. pylori* is a catalase-positive organism, in our study the components of the peroxidase system were mixed before the addition of the bacterial suspension. In addition there was no difference in c.f.u. ml⁻¹ when the bacterial suspension was added before H₂O₂ (data not shown). Thus, it seems unlikely that catalase activity would have affected the activity of the peroxidase system. Furthermore, it has been shown that the catalase activity of *A. actinomycetemcomitans* does not affect the production of hypo- or pseudohalides in Solution I (Ihalin et al., 1998).

The effect of the LP system on *H. pylori* ATCC 43504ᵀ at pH 6.5 and 5.0 in buffer is shown in Table 1. The LP system inhibited the viability of *H. pylori* significantly at pH 6.5 at H₂O₂ concentrations of 100 μM and above. The inhibition was significantly stronger at pH 5.0 than at pH 6.5 with 10 μM H₂O₂. The major oxidation product of SCN⁻ is OSCN⁻ which is in equilibrium with HOSCN, having a pKₐ value of...

**Table 1.** The effect of the LP system on *H. pylori* ATCC 43504ᵀ in buffer (pH 6.5 and 5.0) after 1 h incubation

Results are shown as the mean (± SD) log₁₀ c.f.u. ml⁻¹ of at least three different experiments. The tubes containing the LP system were compared with the 1 h control (Dunnett’s two-sided t-test) and the difference between pHs with Student’s two-tailed t-test.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH 6.5</th>
<th></th>
<th>pH 5.0</th>
<th></th>
<th>P (pH 6.5 compared to pH 5.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean log₁₀ c.f.u. ml⁻¹ (± SD)</td>
<td>P</td>
<td>Mean log₁₀ c.f.u. ml⁻¹ (± SD)</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>Control 0 h</td>
<td>8.14 (0.36)</td>
<td>0.277</td>
<td>8.40 (0.39)</td>
<td>0.507</td>
<td></td>
</tr>
<tr>
<td>Control 1 h</td>
<td>8.02 (0.22)</td>
<td></td>
<td>8.07 (0.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP system 10 μM H₂O₂</td>
<td>7.95 (0.12)</td>
<td>0.376</td>
<td>6.98 (0.57)</td>
<td>0.005</td>
<td>0.020</td>
</tr>
<tr>
<td>+ 100 μM H₂O₂</td>
<td>7.39 (0.96)</td>
<td></td>
<td>6.71 (2.97)</td>
<td></td>
<td>0.119</td>
</tr>
<tr>
<td>+ 300 μM H₂O₂</td>
<td>5.23 (1.82)</td>
<td></td>
<td>0.85 (1.70)</td>
<td></td>
<td>0.167</td>
</tr>
<tr>
<td>+ 500 μM H₂O₂</td>
<td>0.63 (1.10)</td>
<td></td>
<td>1.6 (1.86)</td>
<td></td>
<td>0.427</td>
</tr>
</tbody>
</table>
5-3 (Thomas, 1981). The antimicrobial effects are enhanced in acidic pH, i.e. the protonated form is more efficient because it can diffuse inside the cell and oxidize intracellular components (Thomas, 1981). This theory is supported by our results as well as by previous studies with other bacteria (Thomas et al., 1983; Månsson-Rahemtulla et al., 1987; Lumikari et al., 1991).

**Generation of OSCN⁻ in saliva and buffer**

Preliminary experiments showed that individual differences between salivas were significant (Fig. 2) and therefore we decided to use pooled saliva in further experiments. Since pooled salivas differ from day to day, we chose to use frozen saliva. The saliva was used after thawing, and its pH, peroxidase and lysozyme activities, and the concentrations of SCN⁻ and protein were measured. Peroxidase activity was 0-75 μmol min⁻¹ in 1 ml saliva, concentration of SCN⁻ was 1 mM and protein concentration was 940 μg ml⁻¹. Lysozyme activity was completely lost during sterilization and freezing, in accordance with previous observations (Lenander-Lumikari et al., 1992). In saliva the highest amount of OSCN⁻ was generated from 100 μM H₂O₂ (Table 2) within 15 min. In buffer the added H₂O₂ concentration was proportional to the amount of OSCN⁻/HOSCN generated (not shown), and corresponded to the antibacterial activity. In saliva, on the other hand, there was a limit after which the amount of OSCN⁻/HOSCN did not increase but decreased by the addition of H₂O₂ (Table 2). Different behaviour of OSCN⁻ generation in saliva and buffer is well known (Pruitt et al., 1983; Månsson-Rahemtulla et al., 1983). This is most likely due to further reactions, *in vivo*, of OSCN⁻ with endogenous SCN⁻ and peroxidase in saliva (Pruitt et al., 1986). In addition, OSCN⁻ reacts with e.g. thiol groups of proteins (Thomas & Aune, 1978a).

**Effects of the LP system and lysozyme on the viability of *H. pylori* ATCC 43504ᵀ in saliva**

One hour incubation in human whole saliva with or without added lysozyme had no, or only minor, influence on the viability of *H. pylori* ATCC 43504ᵀ (Fig. 3). In contrast, when 100 μM H₂O₂ was added, the c.f.u. counts were lower when compared with the saliva control (Fig. 3). The effect was stronger at pH 5-0 than at pH 7-1. The highest concentration of OSCN⁻/HOSCN was formed in saliva from 100 μM H₂O₂ (Table 2) and the effect of the H₂O₂ in saliva with 1 mM SCN⁻ was strongest at this concentration (Fig. 2). Therefore, higher H₂O₂ concentrations were not studied in the experiment.

**Table 2. Amount of OSCN⁻ (μM) formed from different amounts of H₂O₂ in human whole saliva, pH 7.1, mean (± SD) of three different experiments**

<table>
<thead>
<tr>
<th>Added H₂O₂ (μM)</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10 (1-8)</td>
<td>9.8 (0.2)</td>
<td>8.3 (1-8)</td>
<td>9.2 (1-9)</td>
<td>12 (0-5)</td>
<td>10 (0-4)</td>
</tr>
<tr>
<td>100</td>
<td>21 (5-6)</td>
<td>35 (0-6)</td>
<td>47 (6-6)</td>
<td>51 (8-0)</td>
<td>50 (3-1)</td>
<td>46 (3-4)</td>
</tr>
<tr>
<td>300</td>
<td>7.6 (0-6)</td>
<td>8.2 (4-4)</td>
<td>13 (0-5)</td>
<td>20 (2-7)</td>
<td>30 (5-0)</td>
<td>28 (3-0)</td>
</tr>
<tr>
<td>500</td>
<td>2.5 (3-8)</td>
<td>4.0 (1-0)</td>
<td>5.3 (3-9)</td>
<td>11 (4-2)</td>
<td>26 (4-2)</td>
<td>24 (2-2)</td>
</tr>
</tbody>
</table>

**Fig. 2.** Effect of different concentrations of H₂O₂ on *H. pylori* ATCC 43504ᵀ after 1 h incubation at 37 °C in saliva of two different persons (I, open bars and II, filled bars). Salivary SCN⁻ concentrations were 2-6 and 1-0 mM, respectively.

**Fig. 3.** Effects of H₂O₂ (100 μM) and lysozyme (50 μg ml⁻¹) on *H. pylori* ATCC 43504ᵀ after 1 h incubation at 37 °C in pooled human whole saliva at pH 5.0 (open bars) or 7.1 (filled bars). Saliva (S) was supplemented with buffer, H₂O₂ in buffer, lysozyme (Lz) or both H₂O₂ and lysozyme. The SCN⁻ concentration was 1-0 mM, the same as the SCN⁻ concentration of person II in Fig. 2. Results represent mean ± SD of two independent experiments. *, P = 0-048; **, P = 0-008 and ***, P < 0-008 compared to buffer control at same pH; #, P = 0-024 compared to saliva control at pH 5.
ment presented in Fig. 3. The reactions containing H$_2$O$_2$ differed significantly ($P < 0.05$) from buffer controls at the same pH, and at pH 5.0 there was a significant difference between the reactions with saliva and H$_2$O$_2$ and the saliva control. Lysozyme did not enhance the effect of H$_2$O$_2$ although it has been reported to have a modest inhibitory effect on growth of $H.$ pylori (Dial et al., 1998).

During 1 h incubation in saliva, the pH of both control and all the reaction tubes, originally at pH 7.1, elevated to 7.5. However, at pH 5.0 the pH did not change during incubation. Urease is an enzyme that catalyses the hydrolysis of urea to ammonia and bicarbonate. It enables $H.$ pylori to survive in acidic pH, since ammonia increases the gastric pH levels in the microenvironment of the bacterial cells. The LP system has been shown to reduce the urease activity of $H.$ pylori (Shin et al., 2002), but in our experiment the constant pH was more likely to have been due to the citrate in reaction mixtures at pH 5. Urease production of $H.$ pylori is dramatically reduced in citrate buffer at pH 5 (Bauerfeind et al., 1997).

Dental plaque has been estimated to harbour over 300 different bacterial species, one of which can be $H.$ pylori. Although there is no absolute evidence for the possibility of $H.$ pylori recolonizing the stomach after eradication therapy, quite convincing studies have been made: deep (≥4 mm) periodontal pockets and supra- and/or subgingival calculus together with poor oral hygiene (indicated by soft debris covering the exposed tooth surface) are both associated with a greater number of $H.$ pylori in the oral cavity (Avcu et al., 2001; Umeda et al., 2003) and more frequent recurrence of gastric $H.$ pylori after treatment when compared to patients with better oral health (Avcu et al., 2001). Butt et al. (2001) reported that after completion of triple therapy all patients who did not receive local dental treatment in addition to the triple therapy were still $H.$ pylori-positive in their dental plaque. Saliva is a potential medium in person to person transmission as well. Therefore, it is of interest to find clinically applicable and safe ways to diminish the number of living $H.$ pylori-cells in the oral cavity, especially in whole saliva.

To conclude, although $H.$ pylori can be killed by the LP system in buffer with high concentrations of H$_2$O$_2$ as shown in this study and earlier by Shin et al. (2002), our results show that, in contrast to buffer, in human whole saliva these high concentrations do not seem to generate enough antibacterial OSCN$^-$/HOSCN to effectively kill $H.$ pylori. Therefore, further studies will be done to enhance the efficacy of salivary peroxidase systems in vivo, as successfully performed against some other Gram-negatives (Ihalin et al., 2003).

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