Antagonism of *Helicobacter pylori* by Bacteriocins of Lactic Acid Bacteria

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ABSTRACT

Antimicrobial activity of seven bacteriocins produced by lactic acid bacteria against *Helicobacter pylori* strains (ATCC 43504, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSM] 4867, DSM 9691, and DSM 10242) was investigated in vitro using a broth microdilution assay. The bacteriocins chosen for the study were nisin A, lacticins A164, BH5, JW3, and NK24; pediocin PO2; and leucocin K. Antimicrobial activity of the bacteriocins varied among the *H. pylori* strains tested, of which strain ATCC 43504 was the most tolerant. Among the bacteriocins tested, lacticins A164 and BH5 produced by *Lactococcus lactis* subsp. *lactis* A164 and *L. lactis* BH5, respectively, showed the strongest antibacterial activity against *H. pylori* strains. MICs of the lacticins against *H. pylori* strains, when assessed by the critical dilution micromethod, ranged from 0.097 to 0.390 mg/liter (DSM strains) or from 12.5 to 25 mg/liter (ATCC 43504), supporting the strain-dependent sensitivity of the pathogen. Pediocin PO2 was less active than the lacticins against four strains of *H. pylori*, and leucocin K was the least active peptide, with no inhibition toward *H. pylori* ATCC 43504. Anti-*Helicobacter* activity of lactacin A164 was dependent on initial inoculum size as well as concentration of the bacteriocin added.

*Helicobacter pylori*, inhabiting the gastric mucosa, is the most important etiologic factor of gastritis and peptic ulcer in humans (6, 36, 46, 49, 51). The eradication of *H. pylori* is critical in the treatment and prevention of the relapse of peptic ulcers (21, 26). A few antibiotics, such as tetracycline, amoxicillin, metronidazole, and bismuth, are effective against *H. pylori* (14, 17, 23, 33, 37). Clinical treatments for the patients having peptic ulcer or gastritis have included mixtures of antibiotics, such as amoxicillin, ampicillin, erythromycin, clarithromycin, or metronidazole, for eradicating the causative agent, *H. pylori* (2, 26, 33, 37). These antibiotic treatments, however, have caused significant adverse effects, jeopardizing their continued effectiveness (18, 48, 50). In addition, the emergence of antibiotic-resistant microflora and pH instability of antibiotics have further limited the use of antibiotic therapy (18, 39, 50). These limitations of antibiotics as clinical treatments promote the search for effective alternatives, such as natural antimicrobial proteins and peptides.

Probiotics have been proposed as alternatives to the antibiotic therapy for eradicating *H. pylori*. It was demonstrated that lactic acid bacteria (LAB) exhibit an antagonistic activity against human pathogens including *H. pylori* (1, 4, 12, 13, 34, 38). Coconnier et al. (13) reported that the spent supernatant fluid from cultures of *Lactobacillus acidophilus* strain La1 was active against *H. pylori*. However, the scientific basis of their action has not been clearly established.

Antibiotics, such as nisin A, are resistant to an acidic pH, making them good candidates as the potential antibacterial agents of choice against *H. pylori* (44). Nisin, in comparison to other bacteriocinogenic antimicrobial agents, is generally thought to be ineffective against gram-negative bacteria other than in the presence of nonbacterial enhancers such as chelating agents and surfactants. However, Edwards and Morwood (20) claimed in their patent that nisin was efficacious in the in vitro eradication of the gram-negative organism *H. pylori*. Similarly, it was reported that nisin A and some mutacins inhibited *H. pylori* in vitro (42, 44). These substances could thus be considered for their development as a peptic ulcer treatment. The objective of this study was to examine the anti-*Helicobacter* activity of bacteriocins produced by LAB. We studied the comparative antagonistic activity of LAB bacteriocins against *H. pylori* on the basis of growth inhibition and MICs.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* strains used in this study included an American Type Culture Collection reference strain (ATCC 43504) and three Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) reference strains (DSM 4867, DSM 9691, and DSM 10242). All strains were examined by reverse transcription-polymerase chain reaction to confirm the strain identity. *H. pylori* strains were cultivated in brain heart infusion (BHI) broth supplemented with 5% inacti-
vated fetal horse serum (JRH Biosciences, Lenexa, Kans.) and Skirrow’s supplements (0.016 mg/ml polymyxin B, 0.5 mg/ml vancomycin, and 0.25 mg/ml trimethoprim). If a cryopreserved H. pylori stock was used, the cells were quickly thawed at 37°C, and the cells (10^9 CFU) were suspended in 10 ml of the selective broth medium (BHI broth supplemented with 5% fetal horse serum) in a 25-cm² tissue culture flask (Corning Inc., Acton, Maine) with a loosely fitted cap; this flask was then incubated microaerobically at 37°C with shaking at 120 rpm in a CO₂ incubator (NAPCO 5410, Precision Scientific Co. Ltd., Winchester, Va.) with 10% CO₂ (2). H. pylori was verified each time before use by Gram stain and tests for urease, catalase, or oxidase as described (24, 47). Viable cell counts were assessed by serial 10-fold dilution using the broth as diluents, which was followed by plating on the BHI agar. The morphology of H. pylori cells, either in bacillary or coccoid form, was observed microscopically using a Zeiss microscope (Model JENAMED2, ×1,000; Zeiss, Thornwood, N.Y.). H. pylori was maintained in 20% (wt/vol) glycerol-containing BHI broth with the above supplements as frozen stocks at −80°C. Before use, H. pylori strains were subcultured at appropriate culture conditions.

LAB and their bacteriocins used in this study are listed in Table 1 (9–11, 22, 27, 28, 32). Nisin A, the most extensively studied bacteriocin, is included as a reference. The nisin A-producing strain was obtained from the Department of Biotechnology and the Bio-products Research Center, Yonsei University, Seoul, Korea. Micrococcus flavus ATCC 10240 was used as an indicator strain in the antimicrobial assay. M. flavus was grown in BHI broth (Difco Laboratories, Detroit, Mich.) at 30°C. All cultures were maintained as frozen stocks stored at −80°C in appropriate broth containing 20% glycerol (wt/vol). Before use, strains were subcultured at appropriate culture conditions.

Preliminary tests of supernatant fluids from cultures of LAB. Composition and concentration of organic acids in supernatant fluids from cultures were determined using an assay kit and gas chromatography. Lactate was enzymatically determined using Sigma Diagnostics lactate reagents (Sigma Chemical Co., St. Louis, Mo.), whereas acetate was quantitatively analyzed with a gas chromatograph (SRI Instrument, Inc., Torrance, Calif.). Prior to the assay for the inhibitory activity, supernatant fluids were neutralized by adjusting the pH of supernatant fluids to 7.0 using a 1.0 N NaOH solution. To determine the effect of proteolysis on the antimicrobial activity of supernatant fluids, pronase E or chymotrypsin was used at a final concentration of 1 mg/ml (4 or 40 U/ml, respectively, for each enzyme). Reaction mixtures were incubated under optimum conditions for each protease, and the remaining inhibitory activity was measured. Heat stability of inhibitory agents was determined by treating the supernatant fluids at 100°C for 15 min, cooling to room temperature, and assaying for the antibacterial activity. Antibacterial activity was determined by the spot-on-the-lawn method.

Bacteriocin production and partial purification. Bacteriocin production was carried out as described (10, 22). Briefly, bacteriocin production was carried out in a 5-liter jar fermenter (3.0-liter working volume; Korea Fermenter Co., Incheon, Korea) in the appropriate culture broth. An individual producer strain was inoculated into 250 ml of sterile medium (1% [vol/vol]), and this seed culture was transferred into the jar fermenter containing 3 liters of medium. The temperature was tightly controlled to the respective optimum growth temperature, and the pH of the culture was maintained at 6.0 ± 0.1 by the addition of 3 N HCl and 3 N NaOH. Agitation speed was set at 200 rpm in the fermenter, and no aeration was provided.

The bacteriocin was partially purified as described below (16). Briefly, solid ammonium sulfate was slowly added to the supernatant fluid to achieve 75% saturation at 4°C with constant stirring, over a period of 5 h. Slow stirring was continued for an additional 1 h at 4°C. Precipitated proteins and peptides were collected by centrifugation at 12,000 × g for 20 min at 4°C, resuspended in 100 mM phosphate buffer (pH 7.0), and extensively dialyzed against 2 liters of the same buffer for 12 to 24 h in dialysis tubing (molecular-weight cutoff = 1,000; Spectrum Medical Industries, Los Angeles, Calif.). The dialyzed samples were stored at −80°C until use. Protein concentrations of the partially purified bacteriocin were determined using the method of Lowry et al. (35). Bovine serum albumin was used as a standard protein.

Antimicrobial susceptibility tests. Antimicrobial susceptibility tests were determined by a broth microdilution technique (2), with slight modifications. Briefly, after a 48-h cultivation in the BHI broth supplemented with 5% fetal horse serum as described above, H. pylori culture was adjusted to 2 × 10⁶ CFU/ml with the culture medium, and 100 μl of the H. pylori culture was inoculated in each well of the 96-well flat-bottom microplate (BBL, Becton Dickinson, Cockeysville, Md.) that had been filled with 100 μl of medium containing anti-Helicobacter agents. The microplate was placed immediately under microaerobic conditions at 37°C, and the bacterial growth was monitored with a spectrophotometer (UV-1201, Shimadzu, Tokyo, Japan) or a microplate reader (THERMOMax, Molecular Devices, Sunnyvale, Calif.) at an absorption wavelength of 550 nm (15). MIC (milligrams per liter) was determined from the lowest concentration showing inhibition.

TABLE 1. Characteristics of bacteriocins of lactic acid bacteria used in this study

<table>
<thead>
<tr>
<th>LAB strains</th>
<th>Bacteriocins</th>
<th>Molecular weight (kDa)</th>
<th>Cell-free culture broth</th>
<th>Purified bacteriocin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus lactis subsp. lactis ATCC 11454</td>
<td>Nisin A</td>
<td>3.5</td>
<td>20,480</td>
<td>328,000</td>
</tr>
<tr>
<td>L. lactis subsp. lactis A164</td>
<td>Lacticin A164</td>
<td>3.5</td>
<td>81,920</td>
<td>1,310,000</td>
</tr>
<tr>
<td>L. lactis BH5</td>
<td>Lacticin BH5</td>
<td>3–3.5</td>
<td>40,960</td>
<td>655,000</td>
</tr>
<tr>
<td>L. lactis JW3</td>
<td>Lacticin JW3</td>
<td>3–3.5</td>
<td>40,960</td>
<td>655,000</td>
</tr>
<tr>
<td>L. lactis NK24</td>
<td>Lacticin NK24</td>
<td>3–3.5</td>
<td>40,960</td>
<td>655,000</td>
</tr>
<tr>
<td>Pediococcus acidilactici PO2</td>
<td>Pediocin PO2</td>
<td>4.6</td>
<td>10,240</td>
<td>164,000</td>
</tr>
<tr>
<td>Leuconostoc sp. LAB145-3A</td>
<td>Leucocin K</td>
<td>4.4</td>
<td>640</td>
<td>20,000</td>
</tr>
</tbody>
</table>

* Antimicrobial activity was assayed by the critical dilution micromethod against Micrococcus flavus and expressed as an arbitrary unit (AU/ml), which is the reciprocal of the highest dilution showing inhibition.
TABLE 2. Antagonism of Helicobacter pylori by supernatant fluid from cultures of lactic acid bacteria

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH of supernatant fluid</th>
<th>Acid concentrations of supernatant fluids (mM)</th>
<th>Inhibitory effect of neutralized supernatant fluidsa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lactic acid</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>Lactobacillus acidophilus P38</td>
<td>3.9</td>
<td>83</td>
<td>3</td>
</tr>
<tr>
<td>Bifidobacterium longum P29</td>
<td>4.1</td>
<td>55</td>
<td>32</td>
</tr>
<tr>
<td>Lactococcus lactis M92</td>
<td>4.8</td>
<td>57</td>
<td>5</td>
</tr>
<tr>
<td>L. lactis subsp. lactis A164</td>
<td>5.2</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td>L. lactis BH5</td>
<td>4.9</td>
<td>55</td>
<td>3</td>
</tr>
</tbody>
</table>

a Acid was quantitated using an assay kit and gas chromatograph.
b Antibacterial activity was determined by the spot-on-the-lawn method. Inhibition was expressed as follows: --, no inhibition; +, halos <5 mm; ++, halos >5 mm.

Antimicrobial activity of lacticin A164 at different growth phases. Lacticin A164 was added at a final concentration of 1 X MIC, after filtration through a 0.22-μm sterile membrane, into the H. pylori broth culture (ATCC 43504) seeded with 2 x 10^8 CFU/ml at lag, early exponential, or early stationary phase. The cultures were further incubated microaerobically with shaking, and the growth of the pathogen was monitored spectrophotometrically as described above.

Sensitivity of the bacillary or coccoid form of H. pylori to lacticin A164. The coccoid form of H. pylori ATCC 43504 was induced by exposing the culture at the midexponential growth phase to air for 6 h with periodic monitoring with the Zeiss microscope as described (13). After adjusting the cell density of the bacillary or coccoid cell cultures to 3 x 10^8 or 2 x 10^8 CFU/ml, sterile lacticin A164 was added to yield a final concentration of 1 X MIC. Total viable cells were examined as described above at 12- and 24-h incubations.

Scanning electron microscopy of lacticin A164-treated H. pylori ATCC 43504. The morphology of H. pylori cells with or without lacticin A164 treatment was examined by scanning electron microscopy (SEM) as described (13). H. pylori ATCC 43504 was cultivated to late exponential phase, and then lacticin A164 was added to yield a final concentration of 4 X MIC. The bacteriocin-treated H. pylori culture was further incubated microaerobically at 37°C for 2 h. After centrifugation at 10,000 x g for 15 min, both control and bacteriocin-treated cells were collected, washed with phosphate-buffered saline, and fixed with 2% paraformaldehyde and 2% glutaraldehyde. The fixed H. pylori cells were dried with 100% hexamethyldisilazane, followed by gold coating. SEM of the treated cells was carried out with a model JSM-5410LV SEM (JEOL, Tokyo, Japan).

RESULTS AND DISCUSSION

Antimicrobial activity of LAB and their respective bacteriocins against H. pylori. LAB strains produce a wide range of antagonistic factors that include metabolic products, antibiotic-like substances, hydrogen peroxide, and bactericidal proteins, which are collectively termed “bacteriocins.” Bacteriocins of LAB are a heterogeneous group of antimicrobial peptides that vary in spectrum of activity, mode of action, molecular weight, genetic origin, and biochemical properties (45). Before examining whether the bacteriocin family produces antimicrobial activity against H. pylori, seven strains of bacteriocin-producing LAB were screened for antimicrobial activity using M. flavus ATCC 10240 as an indicator strain (Table 1). Among the seven LAB strains tested, Lactococcus lactis subsp. lactis A164, a strain isolated from kimchi, Korean fermented vegetables, showed strong inhibitory activity against M. flavus. Lacticins A164, BH5, JW3, and NK24 showed nearly identical inhibitory spectra to that of nisin A as an experimental control (10, 22, 28, 32). Compared to the lactocin family, pediocin PO2 and leucocin K had relatively narrow bactericidal spectra (29).

Organic acids, such as lactic acid and acetic acid, produced by probiotics play important roles in the antagonism of microflora in environments as well as in fermented foods. Lactic and acetic acids account for more than 90% of the acids produced. Other acids produced in small quantities include citric, hippuric, orotic, and uric acids (31). The lowering of pH due to lactic or acetic acid produced by these bacteria has a bactericidal or bacteriostatic effect. Midolo et al. (40) reported that lactic, acetic, and hydrochloric acids demonstrated an inhibition of H. pylori growth in a concentration-dependent manner, with lactic acid resulting in the highest inhibition. The antibacterial activity of the supernatant fluid of selected LAB strains against H. pylori ATCC 43504 was examined to determine the effect of organic acids and other components in the antagonism of the pathogen (Table 2). Concentrations of lactic acid produced by the strains ranged from 52 to 83 mM, and small amounts of acetic acid were produced by the LAB strains but not by bifidobacteria. Bifidobacterium longum P29 produced 55 mM lactic acid and 32 mM acetic acid. All supernatant fluids without neutralization showed inhibitory activity against H. pylori. When the pH values of supernatant fluids were adjusted to pH 7.0, however, two strains of L. lactis subsp. lactis A164 and L. lactis BH5 inhibited H. pylori, indicating that the inhibition mediated by these bacterial supernatant fluids might include a nonacidic sub-
FIGURE 1. Inhibition profiles of cell-free culture supernatant of lactic acid bacteria against Helicobacter pylori strains (A) ATCC 43504, (B) DSM 4867, (C) DSM 9691, and (D) DSM 10242. Legends in each figure are as follows, with the relative arbitrary unit per milliliter (AU/ml) of each bacteriocin stated in parenthesis: ●, control; ○, nisin A (ATCC 11454, 20,480 AU/ml); ▼, lacticin A164 (81,920 AU/ml); △, lacticin BH5 (40,960 AU/ml); ■, lacticin JW3 (40,960 AU/ml); □, lacticin NK24 (40,960 AU/ml); ●, leucocin K (640 AU/ml); ○, pediocin PO2 (10,240 AU/ml).

stance (or substances). On the other hand, three probiotic strains of L. acidophilus P38, B. longum P29, and L. lactis M92 did not inhibit H. pylori, which is suggestive of acid-mediated antagonism for these organisms (Table 2). To determine whether the lactic acid produced by the LAB strains participated in the inhibition of H. pylori, we examined the in vitro effects of both pH and concentrations of lactic acid on H. pylori (data not shown; 29). It was found that a range of concentrations from 20 to 200 mM DL-lactic acid totally inhibited the H. pylori, whereas when the pH of the lactic acid solution was adjusted to 7.0, it failed to inhibit the viability of H. pylori. These results demonstrated that lactic acid participated in the antibacterial activity of L. acidophilus P38, B. longum P29, and L. lactis M92, whereas the other two strains secreted antibacterial substances other than organic acids. To elucidate whether the nonacidic antibacterial substance belongs to the bacteriocin family, the supernatant fluids of the latter two strains were treated with heat or proteases (Table 2). The activity of the supernatant fluids remained stable after heat treatment at 100°C for 15 min, but they showed no antibacterial activity after proteolytic enzyme treatment such as pronase E or chymotrypsin at 4 or 40 U/ml, respectively (Table 2).

The results suggest that the substance (or substances) produced by the strains L. lactis subsp. lactis A164 and L. lactis BH5, which demonstrate antagonistic activity against H. pylori, may be a proteinaceous compound.

The growth inhibition of H. pylori (four strains: ATCC 43504, DSM 4867, DSM 9691, and DSM 10242) by the supernatant fluids of the bacteriocin-producing LAB strains was examined using the BHI broth supplemented with 5% fetal horse serum, Skirrow’s reagents, and individual supernatant fluid to a final concentration of 10% (Fig. 1). The pH of supernatant fluids was adjusted to neutral to avoid any inhibition of H. pylori strains by acids in the supernatant fluids. The type strain of H. pylori ATCC 43504 was the most resistant to the bacteriocins tested, showing a maximum 1-log cycle reduction in viable cells after a 3-h incubation (Fig. 1A). On the other hand, the viability of three H. pylori DSM strains (inocula, 10⁷ CFU/ml) decreased markedly as a function of time, resulting in 4-log reductions by the lacticins—namely, A164, BH5, JW3, and NK24—whereas nisin A (ATCC 11454) showed far less activity than the lacticins (Fig. 1B through 1D). The other two bacteriocins, leucocin K and pediocin PO2, showed bacterio-
ATCC 11454 showed 2- to 32-fold higher MICs for the tested strains of *H. pylori* which is consistent with the clinical observations on genetic variations of the pathogen. MICs of antibiotics, including amoxicillin, clarithromycin, and kanamycin, against bacteriocins of LAB against *Helicobacter pylori* strains under the test conditions. Antimicrobial activity of the bacteriocins was dependent on the *H. pylori* strains tested. *H. pylori* ATCC 43504 was the most resistant strain, whereas all three DSM cultures were sensitive to the lacticins. The strain-dependent anti-*Helicobacter* activity of the bacteriocins was also observed with clinical strains (data not shown; 29). This result suggested the strain variability among *H. pylori*, which is consistent with the clinical observations on genetic variations of the pathogen. MICs of antibiotics, including amoxicillin, clarithromycin, and kanamycin, against *H. pylori* varied from 2-fold to 2,000-fold, depending on the strain tested (2, 3, 23). It was therefore critical to accurately assess the antimicrobial activity of each bacteriocin to each *H. pylori* strain.

**MICs of bacteriocins of LAB against *H. pylori*.** The average MICs of the partially purified bacteriocins toward *H. pylori* are presented in Table 3. Against *H. pylori* ATCC 43504, the bacteriocin-tolerant strain, the MICs of both lacticin A164 and lacticin BH5 were 12.5 mg/liter, which was the lowest value among the bacteriocins tested. Three DSM strains were very sensitive toward lacticins A164 and BH5 (MIC range = 0.097 to 0.195 mg/liter). This result was consistent with the observation from the growth inhibition study, supporting the notion that the two bacteriocins lacticin A164 and lacticin BH5 exert the strongest antimicrobial activity against *H. pylori*. Nisin A produced by *L. lactis* ATCC 11454 showed 2- to 32-fold higher MICs for the tested strains of *H. pylori* than those of lacticins A164 and BH5. Pediocin PO2 was less active, with MICs of 6.25 to 50 mg/liter higher than those of the lacticin family, whereas leucocin K was weakly inhibitory against the three DSM strains but not against *H. pylori* ATCC 43504. As observed from the anti-*Helicobacter* activity, the MICs of the bacteriocins varied greatly, depending on the *H. pylori* strains. Among the *H. pylori* strains tested, DSM 4867 and DSM 10242 appeared to be the most sensitive to all of the bacteriocins tested, with MICs ranging from 0.097 to 6.25 mg/liter, except for leucocin K (MIC = 25 mg/liter). On the basis of these results, *H. pylori* ATCC 43504 was designated a bacteriocin-tolerant strain, and *H. pylori* DSM 10242 was designated a bacteriocin-sensitive strain. These two strains were used for the rest of the study.

Three antibiotics—amoxicillin, ampicillin, and erythromycin—were highly effective in killing *H. pylori*, as expected (data not shown). The MICs of amoxicillin, ampicillin, or erythromycin ranging from 0.015 to 2.44 mg/liter were consistent with those reported in the literature (2, 26, 33, 37). Among the *H. pylori* strains tested, ATCC 43504 was tolerant to all three antibiotics, with MICs approximately two- to four-fold higher than those against other *H. pylori* strains. This observation further supported *H. pylori* type strain ATCC 43504 as the most resistant strain to the antimicrobial agents tested, including bacteriocins and antibiotics.

**Concentration-dependent inhibition profiles of lacticin A164 against *H. pylori*.** Dose-dependent activity of lacticin A164 was determined using two strains of *H. pylori*, ATCC 43504 and DSM 10242, in the growth medium (Fig. 2). After a 12-h incubation at 37°C without lacticin A164 (control), approximately 2-log increases in viable *H. pylori* cells were observed for both strains. When lacticin A164 was added at a final concentration of 1× MIC, no growth of *H. pylori* was observed during the incubation period. The viability of *H. pylori* DSM 10242 decreased rapidly within 3 h and reached dose-dependent final reduction values of 1 to 5 log cycles as the amount of lacticin A164 increased from 4 to 64× MIC (Fig. 2A). With the addition of 128× MIC, the viable cells decreased to less than 10 cells, even within a 2-h incubation. When the bacteriocin-tolerant strain ATCC 43504 was tested, the inhibitory effect of lacticin A164 resulting from the addition of 4 to 64× MIC was far less, with only a 0.5- to 2-log reduction in viability (Fig. 2B). Other bacteriocins of LAB, except for leucocin K, showed a bactericidal mode of action against *H. pylori* but to a lesser degree than lacticin A164 (data not shown).

Previous observations of the concentration-dependent inhibition of lacticin A164 in the later stages of incubation (Fig. 2) suggested that the inoculum size of *H. pylori* could be a critical parameter. The antimicrobial activity of lacticin A164 was assessed in the growth medium containing 1× MIC and in various inoculum sizes of *H. pylori* ranging from 10⁷ to 10⁹ CFU/ml (Fig. 3). The antimicrobial activity of lacticin A164 against *H. pylori* DSM 10242 increased as the initial cell population decreased from 8 × 10⁶ CFU/ml to 1 × 10³ CFU/ml (Fig. 3A). MICs are influenced by...
FIGURE 2. Concentration-dependent inhibition profiles of partially purified lacticin A164 against Helicobacter pylori DSM 10242 (A) and ATCC 43504 (B) suspended in the growth medium. ●, control; ○, 1× MIC; ▼, 4× MIC; ▽, 16× MIC; ■, 64× MIC; □, 128× MIC.

FIGURE 3. Inhibitory effect of partially purified lacticin A164 against Helicobacter pylori DSM 10242 (A) and ATCC 43504 (B) at different initial cell concentrations. ■, control; ▼, 8×10^6 CFU/ml; ○, 1×10^6 CFU/ml; ●, 6×10^3 CFU/ml.

the cell density of indicators as well as pH, cell morphology, and strains tested (45). In this study, MICs for H. pylori strains were determined with the initial cell density of 1×10^6 CFU/ml. When the initial cell density of H. pylori DSM 10242 was over 1×10^5 CFU/ml in the presence of 1× MIC, a 2-log reduction in its viability was observed after an 8-h incubation, whereas with an initial cell density of 6×10^5 CFU/ml, a 2.5-log reduction occurred within 1 h. On the other hand, the effect of the initial cell density of H. pylori ATCC 43504, ranging from 10^3 to 10^6 CFU/ml, was less than that of H. pylori DSM 10242 (Fig. 3B). With an initial cell density of 1×10^3 CFU/ml, effective control beyond the detection limit (<10 CFU/ml) was observed after a 12-h incubation, further supporting the strain-dependent bactericidal activity of lacticin A164. These results suggest that it would be necessary to carefully establish the quantitative relationship between the amount of the antimicrobial peptide and the cell density of clinical H. pylori strains when developing lacticin A164 as an attractive alternative to antibiotics in clinical treatments.

The morphological changes of H. pylori ATCC 43504 induced by lacticin A164 were examined by SEM. H. pylori cells without the treatment of lacticin A164 had typical spiral rod shapes, as shown in the SEM photomicrograph (Fig. 4A). When treated with lacticin A164 (4× MIC) for 2 h, the majority of cells were of the coccolid form and had extensively damaged surfaces (Fig. 4B). This result is consistent with the previous observation on the bactericidal activity of lacticin A164.

Antimicrobial activity of lacticin A164 at different growth phases. The sensitivity of H. pylori cells to lacticin A164 at each growth phase was investigated by adding 1×
MIC to culture broth at 0-, 12-, and 38-h incubations (Fig. 5). When lacticin A164 was added at zero time to the culture containing $2 \times 10^6$ CFU/ml, *H. pylori* ATCC 43504 cells did not grow during the cultivation period. The addition of lacticin A164 at the early exponential growth phase delayed growth for 8 h, after which the *H. pylori* cells began to grow. However, the maximum growth in this culture (optical density at 550 nm = 1.4 at 48 h) did not reach that of the control, whose optical density at 600 nm was 1.7 after a 36-h incubation. The growth of *H. pylori* in this culture, which was less than that of the control, indicated that $1 \times$ MIC of lacticin A164 was not sufficient to kill more than the initial cell density of $2 \times 10^6$ CFU/ml. That is, as discussed previously, the bactericidal activity of lacticin A164 was influenced by the quantity of the peptide and the cell density. In the absence of lacticin A164, *H. pylori* cells reached early stationary phase at 40 h. With the addition of lacticin A164 at the early stationary phase, however, a moderate drop in optical density values was observed with further incubation. This observation suggested that the bacteriocin might accelerate cell lysis. When quantitatively assayed, the urease activity of each growth phase of *H. pylori* culture, with or without the addition of lacticin A164, showed a similar pattern of growth inhibition (data not shown).

**Sensitivity of the bacillary or coccoid form of *H. pylori* to lacticin A164.** *H. pylori* undergoes morphological changes from a bacillary to coccoid form under unfavorable environments, including exposure to air or long incubation time, temperature shift, nutrition deficiency, and presence of antibiotics (5, 7, 25, 41, 43).

Physiologically, it is not clear how the characteristics of the coccoid form affect growth and pathogenicity. When more than 50% of the cell population exists in coccoid form, no reversion of coccoids to the infectious bacillary form was observed, even under optimum growth conditions (30). Kusters et al. (30) proposed that the coccoid form was near the death state and was irreversible. Coconnier et al. (13) postulated that ultrastructural changes in *H. pylori* ob-

**FIGURE 5.** Sensitivity of Helicobacter pylori ATCC 43504 to lacticin A164 at different growth phases. •, control; ○, addition at 0 h; ▼, addition at 12 h; ▽, addition at 38 h.
The observations that the coccoid form of cell death observed by Kusters et al. served after treatment with the supernatant of Bacillary Coccoid Morphology

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Initial cell density (log CFU/ml)</th>
<th>Without treatment</th>
<th>With treatment of lacticin A164(^a)</th>
<th>Without treatment</th>
<th>With treatment of lacticin A164(^a)</th>
</tr>
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<tbody>
<tr>
<td>Bacillary</td>
<td>4.54 ± 0.05</td>
<td>5.33 ± 0.03</td>
<td>3.08 ± 0.04</td>
<td>6.21 ± 0.03</td>
<td>2.69 ± 0.02</td>
</tr>
<tr>
<td>Coccoid</td>
<td>4.49 ± 0.03</td>
<td>5.31 ± 0.02</td>
<td>3.00 ± 0.05</td>
<td>5.97 ± 0.02</td>
<td>2.56 ± 0.03</td>
</tr>
<tr>
<td>Bacillary</td>
<td>6.43 ± 0.03</td>
<td>6.91 ± 0.02</td>
<td>6.33 ± 0.02</td>
<td>7.86 ± 0.05</td>
<td>6.24 ± 0.03</td>
</tr>
<tr>
<td>Coccoid</td>
<td>6.33 ± 0.02</td>
<td>6.81 ± 0.06</td>
<td>5.74 ± 0.04</td>
<td>7.76 ± 0.04</td>
<td>5.07 ± 0.04</td>
</tr>
</tbody>
</table>

\(^a\) Morphology of *H. pylori* was observed with a Zeiss microscope. The coccoid form was induced by exposing the bacillary cell culture to the air for 6 h.

\(^b\) Viable cells were counted by a serial 10-fold dilution using the broth medium as diluent, followed by plating on selective agar. All experiments were carried out as triplicate, and data were expressed as mean ± standard deviation.

\(^c\) Lacticin A164 was added to the cultures, after filter sterilization, to a final concentration of 1 × MIC.

In conclusion, the in vitro antimicrobial activity of lacticin A164 against *H. pylori* suggests that the peptide can be developed as an attractive substitute to one or more antibiotics in clinical treatments. Further investigations of the antimicrobial activity of lacticin A164 in vivo, by itself, or in combination with antibiotics should be carried out to develop an effective anti-*Helicobacter* regimen without any adverse effects. The treatment regimen may contribute to a reduction in *H. pylori* infections in humans.

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**REFERENCES**


ANTAGONISM OF H. PYLORI BY BACTERIOCINS


