J. Microbiol. Biotechnol. (2006), **16**(10), 1513–1517

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation of *Lactobacillus plantarum* from *Kimchi* and Its Inhibitory Activity on the Adherence and Growth of *Helicobacter pylori*

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Received: March 10, 2006
Accepted: April 18, 2006

Abstract One single lactic acid producing bacterium, isolated from *kimchi*, inhibited the growth and adherence of *Helicobacter pylori* to the human gastric epithelial cell line MKN-45. This isolate was identified as *Lactobacillus plantarum* and termed *L. plantarum* strain PL9011. The adherence of *H. pylori*, in the presence of live or nonviable *L. plantarum* strain PL9011 (10-fold CFU), decreased to 14–20%. The spent culture supernatant of *L. plantarum* strain PL9011 resulted in the eradication of *H. pylori*. This activity remained stable following neutralization and heat treatment, but not following pepsin treatment, thereby suggesting small peptides as the inhibitory factor. *L. plantarum* strain PL9011 did not produce any harmful metabolites or enzymes. The results obtained in this study suggest that the *L. plantarum* strain PL9011 may be a potential novel probiotic for the stomach.

Key words: *Kimchi*, lactic acid bacteria, *Lactobacillus plantarum*, *Helicobacter pylori*

Kimchi is the general term given to a group of fermented foods made from vegetables such as Chinese cabbage, radish, and garlic. The preparation of *kimchi* allows vegetables to be stored for up to several months, which is especially beneficial during the winter season when fresh vegetables are scarce. *Kimchi* fermentation is performed by various microorganisms, primarily lactic acid producing bacteria (LAB) such as *Lactobacillus brevis*, *L. plantarum*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, and *Streptococcus faecalis*, *etc.* [4, 14, 15]. LAB produce lactic acid and carbon dioxide that acidify *kimchi* and create an anaerobic state, thus suppressing the growth of aerobes [17]. In addition to these products, LAB in kimchi produce bacteriocin(s) that control other microorganisms [3, 5, 6, 12]. It has previously been reported that intestinal pathogens such as *Listeria monocytogenes* disappear from *kimchi* 2–5 days after intentional inoculation [7]. Although *kimchi* contains a large amount of various LAB, *kimchi* isolates have not as yet been developed as probiotics.

Recently, several LAB with inhibitory activity on *Helicobacter pylori* were isolated from infant feces and milk products and used in various functional foods, especially in yogurt [9, 20–22]. Herein, one lactic acid-producing bacterium isolated from *kimchi* showing the greatest inhibitory activity on *H. pylori* was selected and characterized as a probiotic candidate for gastric health.

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Materials and Methods

Isolation and Identification of Lactic Acid-Producing Bacteria with Inhibitory Activity on *H. pylori*

Kimchi was broken twice in a stomacher (Seward, Worthington, U.K.) with a 10-min cycle and 1-min rest. The supernatant was then inoculated on de Man-Rogosa and Sharpe (MRS; Difco, Sparks, U.S.A.) solid media containing 0.002% bromophenol blue. After incubation for 2–3 days at 35°C, bacteria were isolated from a single colony and incubated in MRS. Each isolate (1% of the media) was inoculated in new MRS and incubated overnight until the optical density (OD) at 600 nm reached 4. The culture supernatant of each isolate (100 μl) was added to a well, formed with a sterile Pasteur pipette, on Brucella solid media (Difco) inoculated with *H. pylori* (MacFarland, 2). One particular isolate, which developed into the largest growth inhibition zone of *H. pylori*, was identified and termed PL9001. Following confirmation of the common characteristics of LAB, PL9011 was identified according to *Bergy's Manual of Systematic Bacteriology* using an API

50CH kit (bioMerieux, Marcy-l'Etoile, France). Sequencing of PL9011 16S rDNA was performed using universal primers [8], as described previously [16], in an ABI prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, U.S.A.). The sequence was compared with the data in the GenBank (http://www.ncbi.nlm.nih.gov/).

Preparation of Bacteria

H. pylori (ATCC 43504) was grown on Brucella solid media supplemented with 10% horse serum, amphotericin B (2.5 μg/ml; Fungizone, Sigma, St. Louis, MO, U.S.A.), Skirrow's supplement (polymyxin B, 2.5 IU/ml; vancomycin, 10 μg/ml; trimethoprim, 5 μg/ml) under 10% CO_2. For the *in vitro* binding assay, bacterial cells were collected from solid media by scraping, washed twice with phosphate-buffered saline (PBS, pH 7.4), and maintained in 10 mM Tris-Cl buffer (pH 7.6) at −20°C until required. PL9011 was cultured in MRS broth. To prepare nonviable PL9011, bacterial cells were heated at 75°C for 30 min and nonviability was confirmed by inoculating on MRS solid media.

Assay of Inhibitory Activity on the Growth of *H. pylori*

The ethyl acetate extract of the spent culture supernatant (SCS) of PL9011 (1:1, v/v) was concentrated in a vacuum evaporator and dissolved in water (1/100 of the original volume). The extract was then heated at 75°C or 100°C in a water bath or at 130°C in an oil bath for various time periods to test the heat stability of the inhibitory material. The pH of the extract was adjusted from pH 2 to 9 with 1 N HCl or 1 N NaOH, to examine pH stability. For pepsin treatment, 30 unit/ml of pepsin was added to the extract (pH 2) and the reaction was stopped by neutralization after various time periods. Subsequently, 10 μl of the ethyl acetate extract was overlaid on *H. pylori* inoculated on solid media supplemented with 10% horse serum under 10% CO_2. After 3 days of incubation, growth inhibition of *H. pylori* was observed.

Adhesion of *L. plantarum* Strain PL9011 on Human Gastric Epithelial Cell Line MKN-45 Cells

Adhesion of viable PL9011 to MKN-45 cells (The Korea Cell Bank, Seoul, Korea) was observed under a light microscope following Gram-staining, and a scanning electron microscope (SEM) (model JSM-5200; JEOL, Tokyo, Japan) as previously described [20]. Briefly, PL9011 (10^8 CFU) was added to MKN-45 cells. After 1 h, unbound bacterial cells were washed three times with PBS and cells were stained with Gram-stain or treated for SEM. The number of bacterial cells attached to one MKN-45 cell was calculated by averaging the numbers of cells attached to 100 MKN-45 cells. All experiments were performed in triplicate. *Escherichia coli* ATCC 25922 was used as a control.

Observation of Inhibitory Activity of *L. plantarum* Strain PL9011 on the Adherence of *H. pylori* on MKN-45 Cells with Fluorescent Microscopy

H. pylori adherence to MKN-45 cells was observed using fluorescent microscopy as previously described [20]. MKN-45 cells (2×10^5 cells) were inoculated into a 30-mm tissue plate and cultivated for 2–3 days at 37°C under 5% CO_2. When a confluent monolayer had formed, the medium was removed and cells were washed three times with PBS. *H. pylori* (5×10^7 CFU) and PL9011 (5×10^8 CFU) were added to each plate and slowly mixed for 90 min at 37°C under 5% CO_2. MKN cells without added bacteria and MKN cells with *H. pylori* or PL9011 were used as controls. The cells were then washed three times with PBS to remove unbound bacterial cells, and subsequently fixed using 4% paraformaldehyde solution for 1 h at 4°C. After 1 h, fixed cells were washed three times with PBS-Tween 20 solution and blocked with PBS-BSA solution (1%) at 37°C for 30 min. Rabbit anti-*H. pylori* polyclonal antibody (100 μg/ml) was then added to the cells and incubated at 37°C. After 2 h, FITC-conjugated monoclonal anti-rabbit immunoglobulin (Sigma) was added to the cells. After a 2-h incubation at 37°C, cells were washed several times with PBS-Tween 20 and observed under a fluorescent microscope (excitation filter, 450 to 490 nm; diachronic mirror, 505 nm; barrier filter, 520 nm; Nikon, Tokyo, Japan).

Enzyme-Linked Immunoabsorbent Assay (ELISA)

Viable or nonviable PL9011 (1×10^8 CFU) in 100 μl RPMI 1640 medium (Gibco-BRL, New York, NY, U.S.A.) and *H. pylori* (1×10^7 CFU or 2.5×10^7 CFU) in 25 μl RPMI 1640 medium were added to confluent MKN-45 cells and incubated at 37°C. After 1 h, cells were washed three times with PBS to remove unbound bacterial cells. *H. pylori* adherence to MKN-45 cells was detected using rabbit anti-*H. pylori* polyclonal antibody, a secondary antibody (anti-rabbit IgG alkaline phosphatase conjugate, Sigma), and *p*-nitrophenol (Sigma) (1 mg/ml). Color development was stopped by the addition of 50 μl 3 M NaOH and the absorbance was measured at 405 nm. As a background, MKN-45 cells were used and treated in the same way except with no addition of *H. pylori*. A standard regression curve was obtained with increasing concentration of *H. pylori* up to 2.5×10^7 CFU.

***In Vitro* Safety Test of PL9011**

Various *in vitro* safety tests were performed as previously described [19]. These tests included production of amine, ammonia, indole, gelatin degradation, and the presence of β-glucuronidase and nitroreductase.

Statistical Analysis

Data were subjected to analysis of variance for a one-factor completely randomized design and Duncan's multiple range

tests (SAS User's Guide, 1995) procedure using a significance level of 0.05.

RESULTS

Isolation and Identification of PL9011

PL9011 identified as *L. plantarum* with the API 50CH kit and its 16S rRNA sequence matched 99.72% with several strains of *L. plantarum* in the GenBank. *L. plantarum* strain PL9011 was submitted to the Korea Culture Collection of Microorganism (KCCM) under no. KCCM-10358 and its 16S rRNA sequence was submitted to the GenBank under the accession no. AY078432.

Inhibitory Activity of *L. plantarum* Strain PL9011 on the Growth of *H. pylori*

The ethyl acetate extract of SCS of PL9011 inhibited the growth of *H. pylori*, even at 1/8 dilution (Fig. 1). Growth inhibitory activity was observed after neutralization and remained stable for 1 min at 130°C, and 30 min at 75°C or 100°C. In addition, growth inhibitory activity remained stable at a wide range of pH values (pH 2 to pH 9), but did not remain stable following pepsin treatment.

Adherence of *L. plantarum* Strain PL9011 on MKN-45 Cells

Both live and nonviable PL9011 adherence to MKN-45 cells was observed under light microscopy (Fig. 2A) and a scanning electron microscope (Fig. 2B). In the case of *E. coli* ATCC 25922, only viable bacterial cells could bind to MKN-45 cells at pH 7.0, whereas nonviable bacterial cells could not bind to MKN-45 cells at both pH values. This showed that the binding of PL9011 to MKN-45 cells was not unspecific binding.

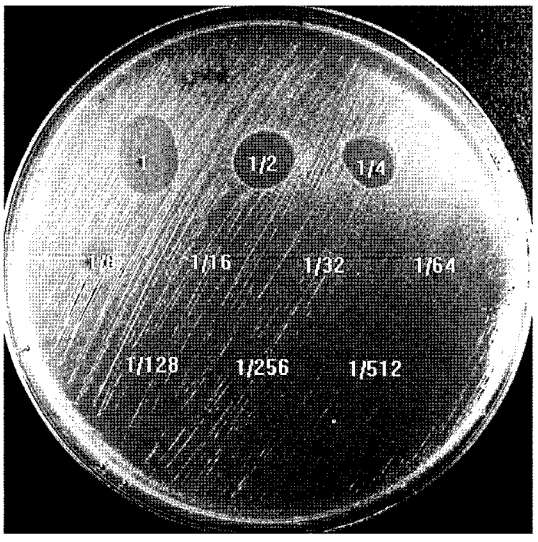

Fig. 1. Inhibitory activity of *L. plantarum* strain PL9011 on the growth of *H. pylori* using the overlay method.
An aliquot (10 μl) of the ethyl acetate extract of the spent culture supernatant of *L. plantarum* strain PL9011 was overlaid on *H. pylori* inoculated on Brucella solid medium, and the appearance of the growth inhibition zone was observed.

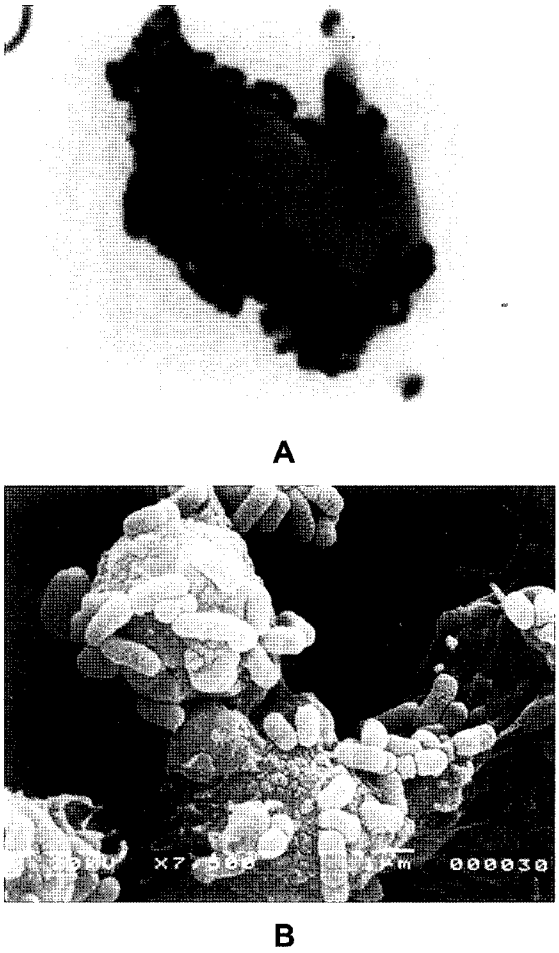

Fig. 2. *L. plantarum* strain PL9011 adhered to MKN-45 cells.
PL9011 was added to MKN-45 cells and treated as described in Materials and Methods. Adherence of PL9011 to MKN-45 cells was observed under a light microscope (×1,000) and a scanning electron microscope (×7,500).

Inhibitory Activity of *L. plantarum* Strain PL9011 on Adherence of *H. pylori* to MKN-45 Cells

Decreased binding of *H. pylori* to MKN-45 cells in the presence of live as well as nonviable PL9011 was observed using fluorescent microscopy (Fig. 3). With ELISA, the amount of *H. pylori* that adhered to MKN-45 cells decreased to 14–19% in the presence of 10-fold concentration of both viable and nonviable PL9011 (Fig. 4).

In Vitro Safety Test of *L. plantarum* Strain PL9011

PL9011 did not produce any harmful metabolites and enzymes in *in vitro* tests.

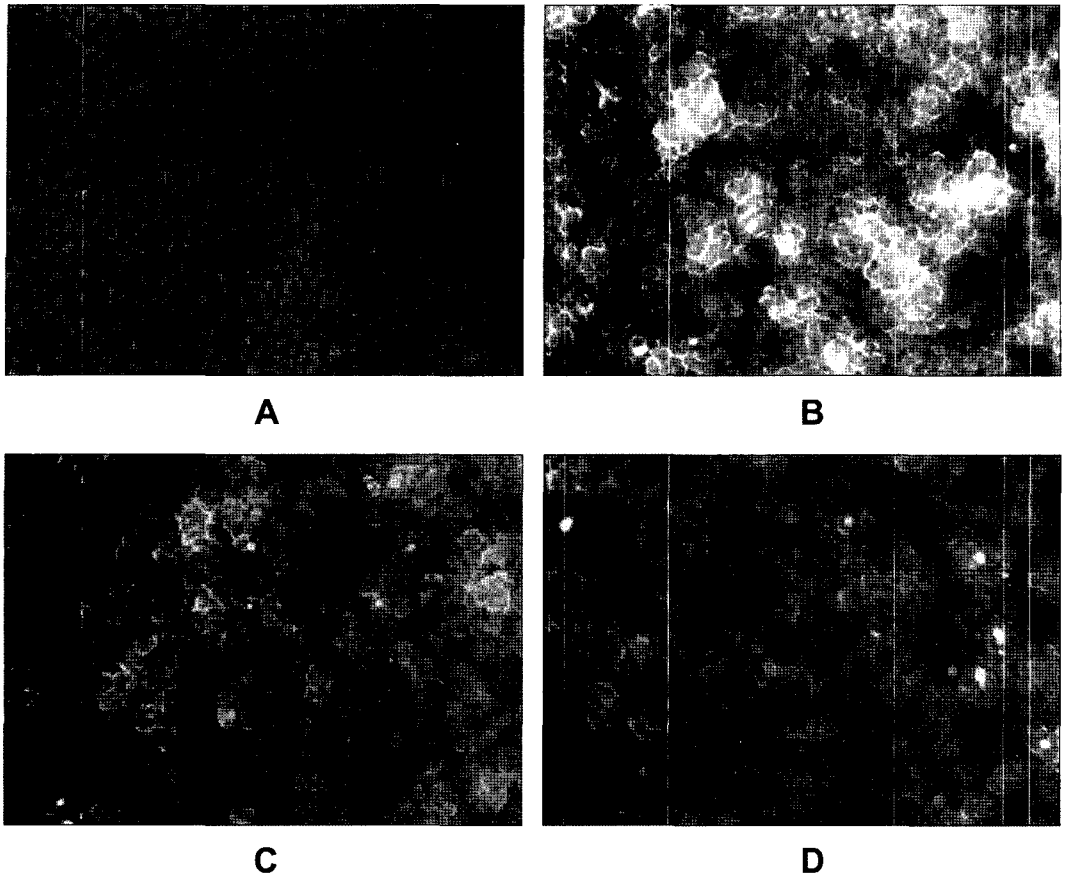

Fig. 3. *H. pylori* bound to MKN cells visualized by fluorescent microscopy.
H. pylori bound to MKN-45 cells was detected with FITC-conjugated antibody and observed under a fluorescence microscope (×40). **A**. MKN-45 cells (control); **B**. MKN-45 cells with *H. pylori*; **C**. MKN-45 cells with *H. pylori* and live *L. plantarum* strain PL9011; **D**. MKN-45 cells with *H. pylori* and non-viable *L. plantarum* strain PL9011.

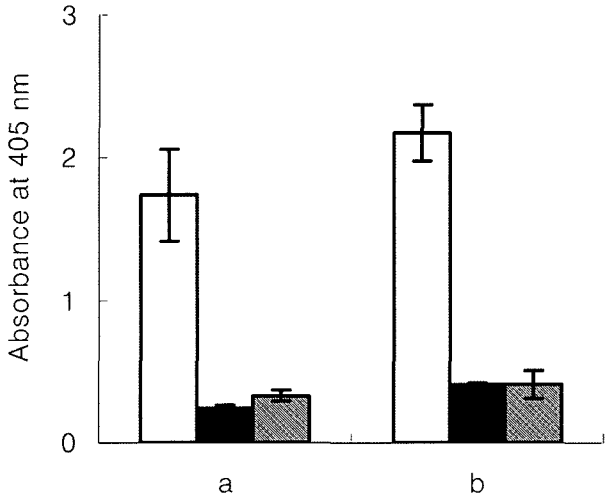

Fig. 4. ELISA of *H. pylori* bound to MKN-45 cells in the presence of *L. plantarum* strain PL9011.
A. *H. pylori* (1×10^7 CFU/well); **B**. *H. pylori* (2.5×10^7 CFU/well); □, in the absence of *L. plantarum* strain PL9011; ■, in the presence of viable *L. plantarum* strain PL9011 (1×10^8 CFU/well); ▨, in the presence of non-viable *L. plantarum* strain PL9011 (1×10^8 CFU/well).

DISCUSSION

Recently, fermented milk products have been developed with various functions in addition to promoting activity of intestinal health [1, 17]. One such product is a yogurt with inhibitory activity on *H. pylori,* providing a large market for this product in Japan and Korea. Probiotics have been shown to inhibit the infection of *H. pylori* in animal and human studies [2, 10, 13, 22]. Several LAB have been reported to inhibit the growth of *H. pylori*. These include *L. acidophilus* [9], *Bacillus subtilis*-3 [21], and *Weissella confusa* PL9001 [20], whereas only a few LAB such as *L. casei* [22] and *W. confusa* PL9001 [20] have been reported to inhibit the adherence of *H. pylori* to gastric cells.

PL9011 characterized in this study produced bacteriocin-like materials, whose activity was relatively safe at high temperatures and various pH values, but not, following pepsin treatment. This observation led to the suggestion that small-sized peptides are the growth inhibitory factors, which has also been suggested in previous reports of *L. plantarum* [11]. In addition, PL9011 adhered to MKN-45

cells and strongly inhibited the adherence of *H. pylori*. In a previous report [18], we also showed that PL9011 could inhibit the IL-8 production involved in inflammation in an animal study. Moreover, PL9011 produced no harmful metabolites and enzymes in this study.

Since PL9011 exerts anti-*H. pylori* activity via several different ways, such as inhibiting adherence of *H. pylori* and producing bacteriocin-like materials, in addition to producing lactic acid and low pH, PL9011 is not expected to show problems with resistance in antibiotic triple therapy for *H. pylori* infection. The heat stability of bacteriocin-like materials produced by PL9011 suggests a further promising application of PL9011 to various foods. Another advantage of PL9011 is that the stability of inhibitory activity on adherence of *H. pylori* remains active on nonviable heat-treated PL9011. These results suggest that PL9011 may be used as a probiotic, particularly in the case of the stomach, in addition to a food additive conferring functionality to various foods. We are currently conducting the isolation and characterization of bacteriocin-like materials of PL9011 and we anticipate that PL9011 may be another form of treatment for the prevention of gastric disease.

Acknowledgment

This work was supported by a Bahrom grant (2006) from Seoul Women's University.

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