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Effect of *Enterococcus faecalis* strain PL9003 on Adherence and Growth of *Helicobacter pylori*

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Abstract The purpose of the present study was to examine the antagonistic activities of *Enterococcus faecalis* strain PL9003 (PL9003) on *Helicobacter pylori.* This strain was isolated from infant feces and found to inhibit both the growth of *H. pylori* and its *in vitro* adherence to the human gastric cell line MKN-45. The binding of PL9003 to MKN-45 was observed under a light microscope after Gram staining and under a scanning electron microscope. When detected with an FITC-conjugate antibody, both viable and nonviable PL9003 were found to decrease the number of *H. pylori* bound to MKN-45. When detected by an enzyme-linked immunoabsorbent assay, about 70% of the *H. pylori* bound on MKN-45 disappeared with the four-fold addition of viable or nonviable PL9003. The spent culture supernatant (SCS) of PL9003 also decreased the viability of *H. pylori* even after neutralization and pepsin treatment. The above results suggest that PL9003 has a potential as a new probiotic for the stomach.

Key words: Adherence, culture supernatant, *Enterococcus faecalis* PL9003, *Helicobacter pylori*, probiotic

Helicobacter is a Gram-negative microorganism that causes gastritis and gastric carcinoma [11, 12]. *H. pylori* infects through the intake of food and attaches to the gastric and duodenal mucous membranes [7, 13, 29]. After infection, it can remain in the gastric system for several decades as it is not eliminated naturally. Therefore, antibiotic drugs, restrainers on proton pumping, and gastric acid removers have all been used to remove *H. pylori*, however, the possibility of re-infection is still feasible since it becomes resistant to antibiotics. Methods using a stomach acid remover, which suppresses stomach acid secretion, cannot solve the problem. Also, due to its complex culture conditions, the development of a vaccine against *H. pylori* in the near future seems unlikely.

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It has already been well documented that lactic acid-producing bacteria (LAB) can exhibit antagonistic activity towards various intestinal human pathogens. Certain LAB synthesize antimicrobial compounds that are related to the bacteriocin family [17, 20]. Some are well-known metabolic end products of lactic acid fermentation, such as lactic and acetic acids and hydrogen peroxide [25, 30] and others remain unidentified [2, 4]. Because the stomach is a very harsh environment with a low pH and pepsin, it is usually speculated that LAB cannot inhibit gastric pathogens. However, several researchers have reported that LAB can inhibit the growth of *H. pylori in vitro* [4, 23, 24] and exhibit antagonistic activity towards *H. pylori in vivo* [1, 9, 18], and also bind to the gastric epithelium of rats [19], pigs [15], and horses [31]. Furthermore, the spent culture supernatant (SCS) of *L. acidophilus* strain LB [9], *L. acidophilus* La1 [22], and *L. gasseri* OLL 2716 (LG21) [26] all showed antagonistic activity against *H. pylori* in human volunteers.

In the current study, one LAB exhibiting antibacterial activity towards *H. pylori* was selected from various LAB isolated from infant feces and identified as *Enterococcus faecalis.* Enterococci are Gram-positive bacteria and fit in the general definition of lactic acid bacteria. They are found as a component of the natural flora of certainfoods, used as probiotics to improve the microbial balance of the intestine and to treat gastroenteritis in humans and animals [26], and as a starter in foods. In the current study, this isolate was identified, and its anti-*H. pylori* functions were characterized.

MATERIALS AND METHODS

Isolation and Identification of Lactic Acid-Producing Bacteria

Feces from infants were picked using a cotton swab and inoculated on MRS (Difco, Sparks, MD, U.S.A.) containing

0.002% bromophenol blue. After incubation for 3–4 days at 25°C, bacteria were isolated from a single colony and identified according to Bergy's Manual of Systematic Bacteriology [14] using a API20 Strep kit (bioMérieux, Marcy l'Etoile, France) and staining. This bacterium was called PL9003 and submitted to the KCCM (Korea Culture Collection of Microbes). DNA sequencing of the full 16S rRNA gene was performed in an automatic DNA sequencer (ABI Prism, Perkin-Elmer, Foster City, CA, U.S.A.) and compared with the GenBank database.

Preparation of Bacteria

The *H. pylori* (ATCC 43504) were grown on Brucella solid media [Brucella broth, fungizone (2.5 μg/ml amphotericin B) and Skirrow's supplement (2.5 IU/ml polymyxin B, 10 μg/ml vancomycin, 5 μg/ml trimethoprim)] supplemented with 10% horse serum under 5–10% CO_2. For an *in vitro* binding assay, bacterial cells were collected from the solid media by scraping, washed twice with phosphate-buffered saline (PBS, pH 7.4), and then used promptly. The PL9003 was cultured in an MRS broth or 10% skim milk. The SCS was prepared by centrifugation and filtration through a 0.45 μm syringe filter.

Assay of Inhibitory Activity of PL9003 on *H. pylori*

H. pylori was inoculated onto a medium and 100 μl of the spent culture of PL9003 was added to a well formed by a sterilized Pasteur pipette. After incubating in a CO_2 incubator (5–10% CO_2), the diameter of the inhibited growth zone was measured. The inhibitory activity was also measured using phenol red. The *H. pylori* cells were mixed with PL9003 and the mixture was inoculated by spreading on a Brucella medium containing 10% sheep serum. The colonies that appeared on the plate were blotted onto a filter paper (No. 2, Whatman International Ltd., Maidstone, England), which was then soaked with a phenol red indicator solution containing 2% urea.

Urease Assay Using the Indophenol Method

One milliliter of the SCS of PL9003 grown in skim milk was mixed with 10 μl of *H. pylori* (5×10^8 CFU/ml) and incubated for 1 h at 37°C under 5% CO_2. The bacterial cells were then removed by centrifugation and filtration through a 0.45 μm syringe filter, and the urease activity in the supernatant was then assayed using the indophenol method [16].

Cell Adhesion Assay on Gastric Cell Line MKN-45

The MKN-45 was cultured in an RPMI-1640 medium (Gibco-BRL, New York, NY, U.S.A.) containing 2 g/l sodium bicarbonate, 10% heat-inactivated fetal bovine serum, and antibiotic-antimycotic (pH 7.2). The cell monolayers were prepared in a 30-mm dish for the Gram-staining or in Lab Tak chamber slide system (Nunc Inc., Naperville, IL, U.S.A.) for the scanning electron microscopy by inoculating 3×10^5 viable cells into 2 ml of the culture medium. The medium was replaced every other day. Six-day confluent MKN-45 monolayers were washed twice with 2 ml of PBS. Then, 100 μl of PL9003 (1×10^7 CFU/ml) were added to the cells in 2 ml of the culture medium and incubated at 37°C in a 5% CO_2 incubator. After 60 min, the monolayers were washed twice with sterile PBS and fixed with methanol. After Gram staining, the cells were examined under a light microscope.

Competition for Binding Between *H. pylori* and PL9003

After the MKN-45 cells became confluent, they were washed three times with PBS and incubated in a new medium (2 ml) for another 30 min at 37°C. *H. pylori* (10^6 CFU in 10 μl) and PL9003 (10^7 CFU in 100 μl) were then added to the MKN-45 and the mixture was then incubated for 1 h. The unbound bacterial cells were removed by washing three times with PBS. The cells were then fixed in a fixing solution (100 ml 3.5% formaldehyde, 16 g Na_2HPO_4, 4 g $NaH_2PO_4 \cdot H_2O$, D.W. to 1 liter) and kept overnight at 4°C. The cells were washed three times with PBS-Tween 20 (0.05%, v/v) and blocked with 1 ml of 1% BSA in PBS at 37°C for 30 min. Thereafter, the cells were washed three times with PBS-Tween 20, and the first antibody specific to *H. pylori* (1:600 dilution) raised in rabbits was added for 1 h at 37°C. The unbound first antibody was then washed out three times with PBS-Tween 20, and the second antibody (1:100 dilution), an FITC conjugate with mouse monoclonal anti-rabbit IgG (Sigma, St. Louis, MO, U.S.A.), was added for 1 h at 37°C. The cells were then washed with PBS-Tween 20 at least three times, and were observed under a fluorescence microscope after drying (Model FDX-35, EX 450–490 nm, DM 505, BA 520 nm, Nikon, Tokyo, Japan).

Binding Assay with the Enzyme-Linked Immunosorbent Assay

The binding of *H. pylori* to MKN-45 was assayed using the enzyme-linked immunosorbent method (ELISA) [6]. Thus, the MKN-45 cells were cultured in a 96-well plate until confluency (3.0×10^5 cells/well). For competition, *H. pylori* (5×10^7) and various amounts of PL9003 were added to each well, and the *H. pylori* that bound to MKN-45 was detected by adding a rabbit anti-*Helicobacter* IgG and anti-rabbit IgG alkaline phosphatase conjugate. The amount of *H. pylori* was assayed at 430 nm in an ELISA reader (Spectra Max250, Molecular Devices, Sunnyvale, CA, U.S.A.).

Scanning Electron Microscopy

The SCS of PL9003 in the MRS broth was freeze-dried and suspended in water at 10 mg/ml concentration. One milliliter of the culture supernatant was mixed with the

same volume of *H. pylori* (A_{600}=1) in a Brucella broth. After various incubation times, the bacterial cells were washed once with PBS and twice with 0.1 M cacodylate. The bacterial cells were fixed with 1% glutaraldehyde in 0.1 M cacodylate overnight and then serially dehydrated in 25%, 50%, and 75% ethanol in 0.1 M cacodylate and finally in 100% ethanol. Thereafter, the cells were serially dehydrated in 25%, 50%, and 75% isoamyl acetate in ethanol and finally 100% isoamyl acetate. Finally, the cells were freeze-dried, coated with gold, and observed under a scanning electron microscope (Model JSM-5200, Jeol, Tokyo, Japan).

Nucleotide Sequence Accession Number

The full sequence of the 16S rRNA of *E. faecalis* PL9003 determined in the present study was assigned as GenBank accession number AF477496.

RESULTS

Isolation and Identification

Among one hundred LAB isolated from infant feces, several isolates were selected that produced a large growth inhibition zone of *H. pylori*. Then, the antibacterial activity towards *H. pylori* was confirmed by co-culturing each isolate with *H. pylori* on Brucella solid media. Because *H. pylori* forms a very tiny and translucent colony that cannot be easily detected, the colonies were blotted onto a filter paper and detected with phenol red containing urea. The colonies formed by *H. pylori* produce ammonia from urea, thereby changing the color to red upon the addition of phenol red containing urea. As shown in Fig. 1, one isolate that completely inhibited the growth of *H. pylori* was selected and it was further characterized. This isolate was identified as *E. faecalis*, based on various biochemical test results using a API20 Strep kit, and was found to have 99.1% homology with *Enterococcus faecalis*. The identification was also confirmed by sequencing the full 16S rRNA gene, and its sequence had 99.13% homology with *E. faecalis* (GenBank AB012212).

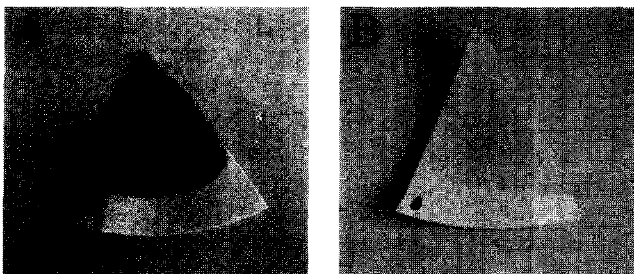

Fig. 1. *Helicobacter pylori* grown in the presence of PL9003.
PL9003 and *H. pylori* were inoculated together onto a Brucella solid medium. The growth of *H. pylori* was visualized by the addition of phenol red containing urea after the colonies had been blotted onto a filter paper. (A) Only *H. pylori*; (B) *H. pylori* and PL9003.

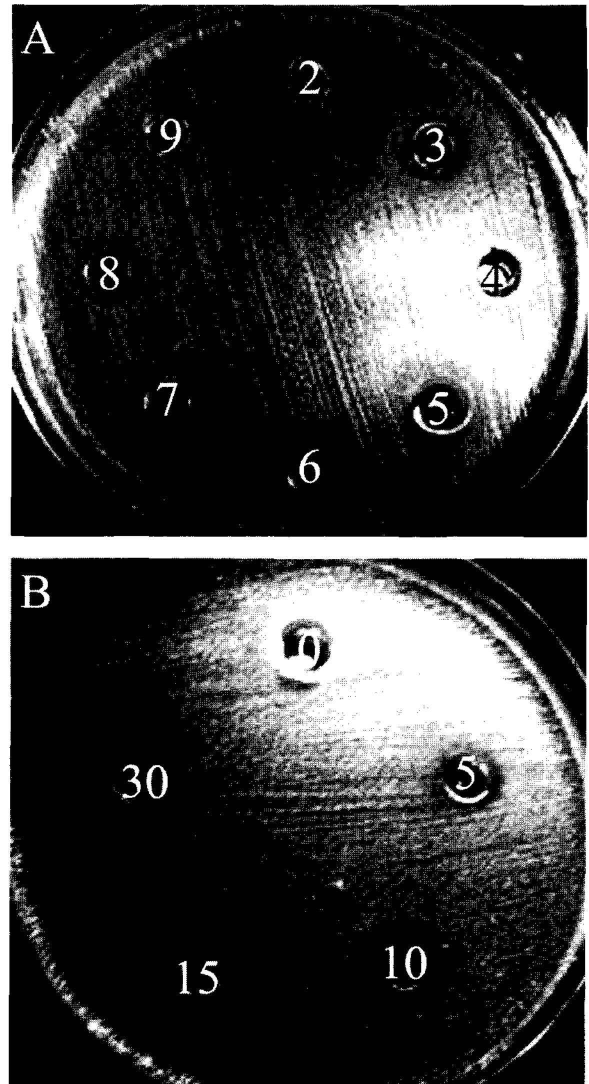

Fig. 2. Growth inhibition of *Helicobacter pylori* by ethyl acetate extract of SCS of PL9003 with low pH and after pepsin treatment.
H. pylori was inoculated onto Brucella solid media and wells were formed using a sterile Pasteur pipette. The ethyl acetate extract of the SCS was either loaded after the pH was adjusted (A) or treated with pepsin at pH 2 for various time periods and then loaded into each well after neutralization (B).

Inhibitory Activity on the Growth of *H. pylori*

The SCS of PL9003 was found to inhibit the growth of *H. pylori*. A growth inhibition zone was also observed with the SCS at various pHs as well as after pepsin treatment (Fig. 2).

To check whether the inhibitory activity of PL9003 was specifically toward the cell wall, *H. pylori* was observed with SEM after treatment with the SCS of PL9003. Before any treatment, *H. pylori* exhibited a helical form (Fig. 3A),

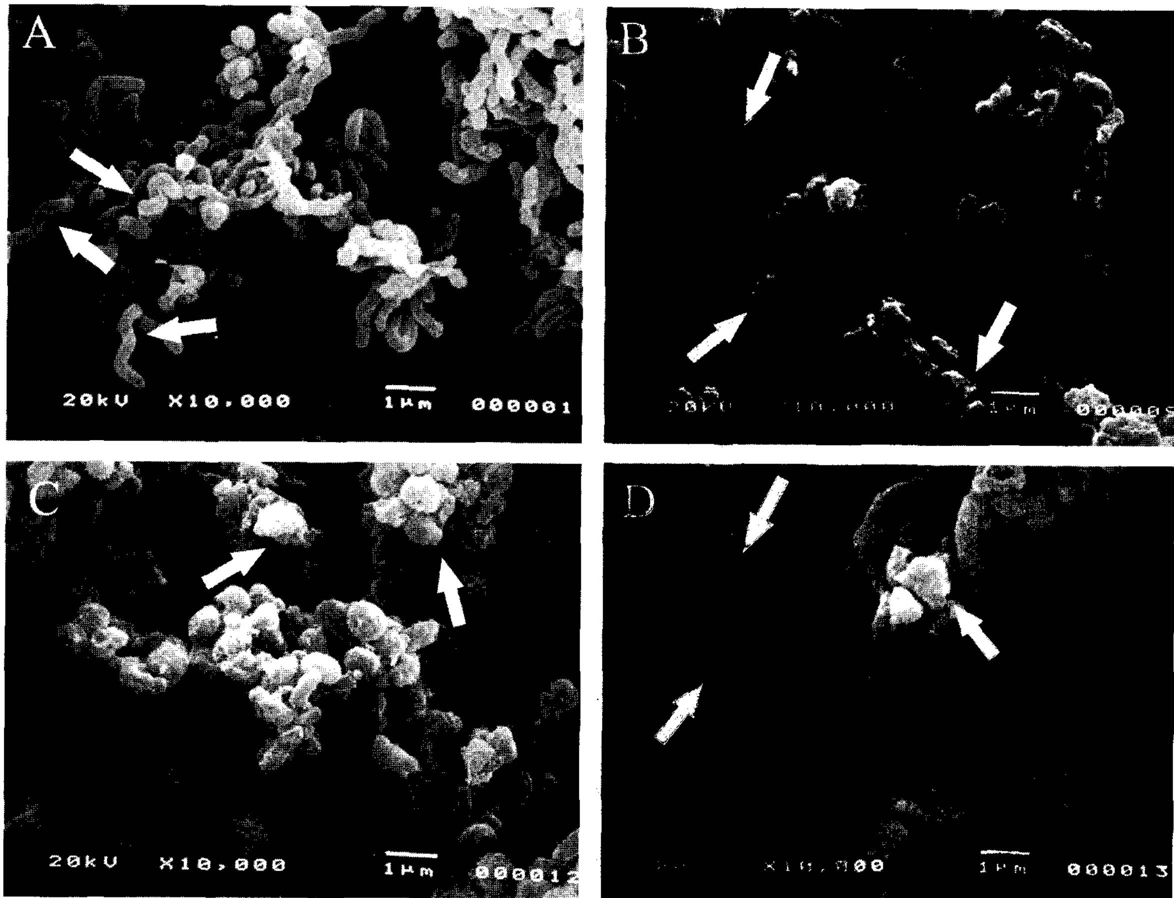

Fig. 3. Scanning electron micrograph of *Helicobacter pylori*.
H. pylori was treated with (A) control; (B) amoxicillin (0.01 μg/ml) for 5 min; (C) SCS for 5 min; (D) neutralized SCS at pH 7.0 for 5 min. (×10,000).

which was broken by the addition of amoxicillin (0.01 μg/ml). However, those cells treated with the SCS of PL9003 exhibited a coccus and necrosis morphology (Fig. 3C). Furthermore, these necrosis forms were observed even after treatment with a neutralized SCS (Fig. 3D).

Binding of PL9003 to Gastric Cell Line MKN-45

As shown in Fig. 4A, PL9003 was able to bind to the gastric cells. This binding ability was also retained in the nonviable cells which were preheated at 75°C for 15 min (Fig. 4B). The binding of PL9003 to the gastric cells was

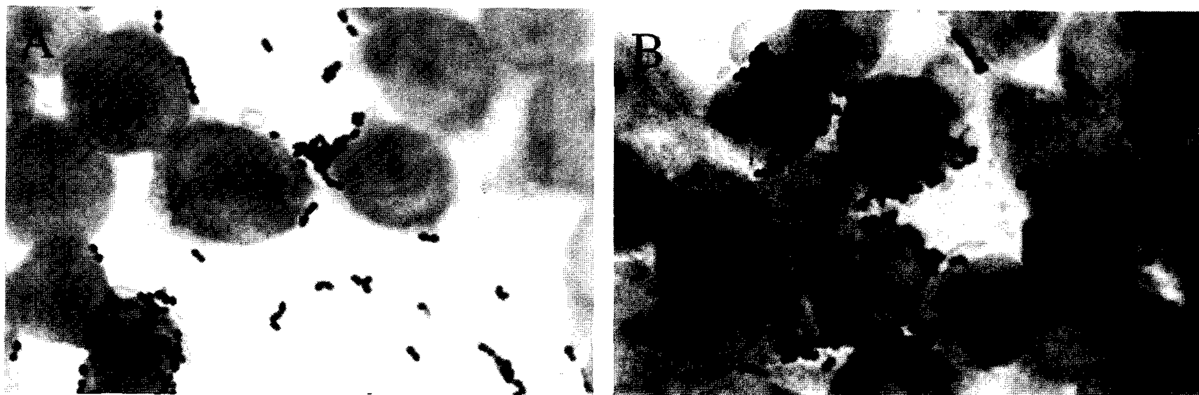

Fig. 4. Gram-stained MKN-45 after incubation with PL9003.
After 60 min of incubation with PL9003, the MKN-45 cells were washed and stained with Gram-stain (×1,000). (A) With viable PL9003; (B) with non-viable PL9003.

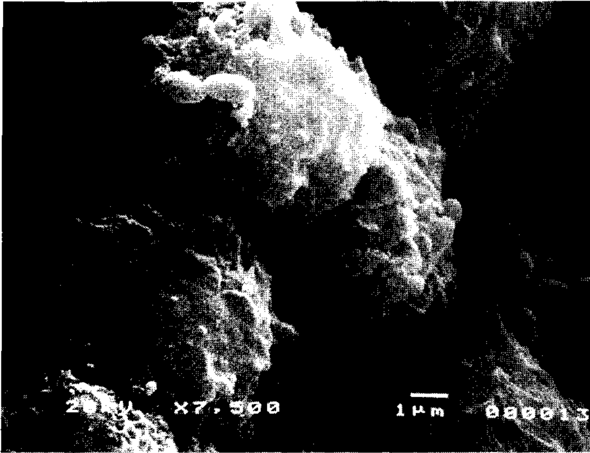

Fig. 5. Scanning electron micrograph of MKN-45 treated with PL9003.

After 60 min of incubation with PL9003, the MKN-45 was treated as described in Materials and Methods and observed under a scanning electron microscope (×7,500).

confirmed by adding PL9003 to the MKN-45 cells and observing it on the surface of the MKN-45 cells under a scanning electron microscope (Fig. 5).

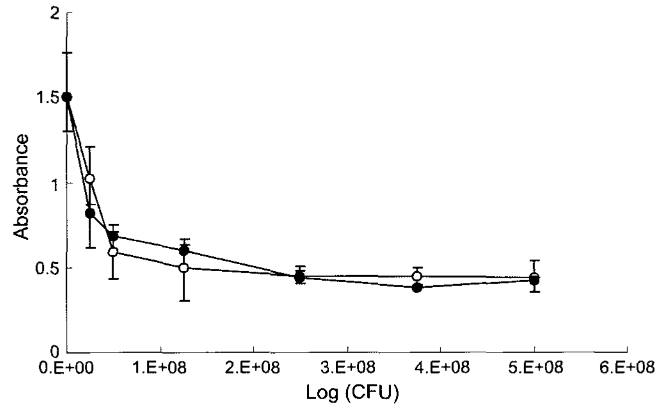

Fig. 7. Inhibitory activity of PL9003 on the adherence of *H. pylori* to MKN-45 assayed with ELISA.

H. pylori (5×10^7 CFU/well) were added to MKN-45 in the presence of various amount of viable (○) and nonviable PL9003 (●). After washing, the amount of *H. pylori* bound to MKN was assayed with ELISA. Experiments were performed five times separately.

Inhibitory Activity of PL9003 Towards *H. pylori* Binding to MKN-45

Both viable and nonviable PL9003 were found to inhibit the binding of *H. pylori* to MKN-45, observed with an

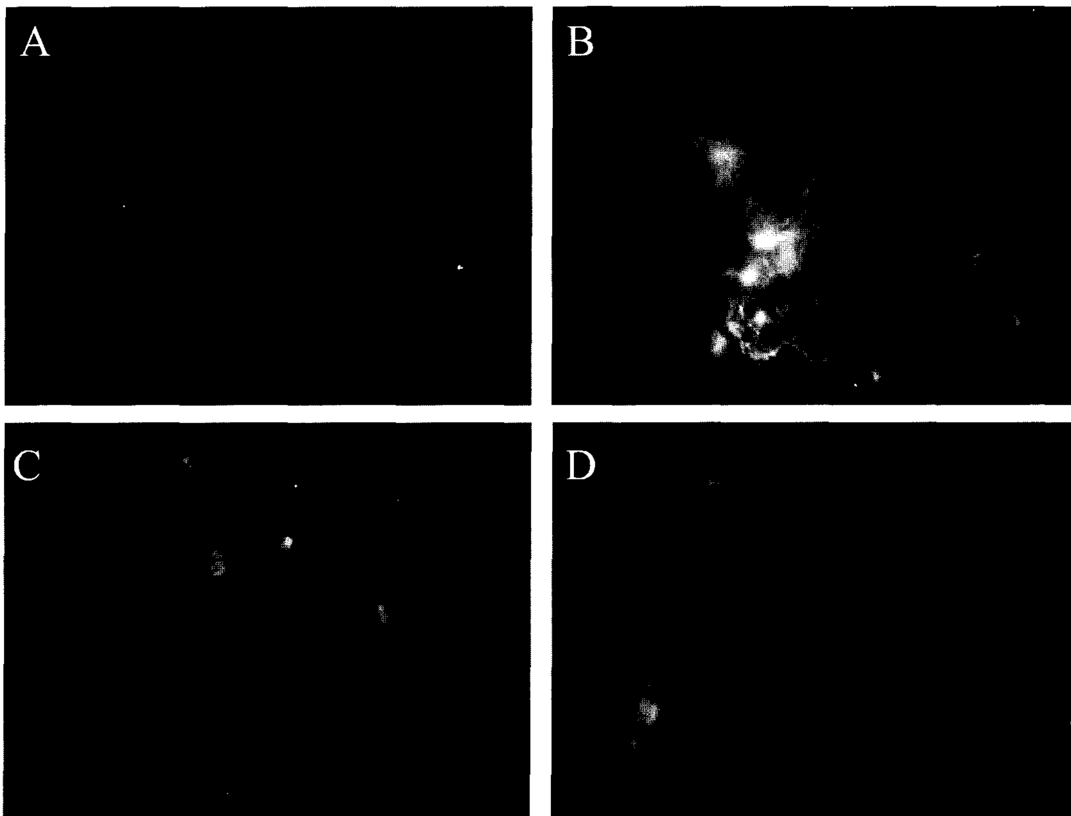

Fig. 6. Fluoresence micrograph of MKN-45 treated with *H. pylori* and PL9003.

(A) No treatment; (B) Cells treated with *H. pylori*; (C) Cells treated with *H. pylori* and viable PL9003; (D) Cells treated with *H. pylori* and non-viable PL9003 (×400).

FITC-conjugated antibody (Figs. 6C and 6D). When the *H. pylori* binding to MKN-45 was assayed using ELISA, the binding decreased to 29.25% and 28.1% in the presence of ten-fold excess amount of viable or non-viable PL9003, respectively (Fig. 7).

DISCUSSION

There have been many reports to describe that LAB can inhibit the growth of various intestinal pathogens by producing a low pH, lactic acid, and bacteriocin(s) [17, 25, 30], and also by competitive binding to the intestinal epithelial cells [3, 8]. Even though numerous research papers have been published on LAB for intestinal use, only a few reports describing the inhibitory activity of LAB on *H. pylori* have been published [21]. Only three strains, *L. salivarius*, *L. gasseri* OLL 2716 (LG21), and *Bacillus subtilis* 3, have previously been reported to inhibit the growth of *H. pylori in vitro* as well as in animals and humans [1, 9, 18, 23, 24]. This general lack of research on probiotics targeting the stomach might have been due to the assumption that LAB constitute the normal intestinal microflora and thus cannot affect gastric pathogens. However, some lactobacilli have been found to bind to the gastric cells of rats [19], pigs [15], horses [31], and humans [27], and lipoteichoic acid [28] and protein [5, 10] were suggested to be the binding moiety of LAB to gastric epithelial cells. Nevertheless, no direct evidence of LAB to compete binding with *H. pylori* has yet been presented. In particular, a nonviable LAB form has never been tested for its binding ability to stomach.

In the current study, *E. faecalis* PL9003 was shown to exhibit inhibitory activity towards *H. pylori* via two different mechanisms. One was by competitive binding to MKN-45, while the other by inhibiting the growth of *H. pylori*. When observed under a light microscope after Gram-staining and SEM, PL9003 was clearly found to bind to MKN-45, and the ELISA reader also revealed about 70% decrease in the amount of *H. pylori* bound to MKN-45 in the presence of both viable and nonviable PL9003. Another advantage of PL9003 as a potential probiotic for the stomach was that the inhibition activity remained stable in the presence of a low pH and pepsin. Since PL9003, similar to any other LAB, inhibited the growth of pathogens via several different mechanisms including the competitive binding, it would be difficult for *H. pylori* to develop resistance.

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