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HOW TO DEVELOPE NEW PROBIOTIC WITH ANTI *Helicobacter pylori* FUNCTION

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Abstract

Lactic acid producing bacteria were isolated from baby feces and characterized to be used as a probiotic with anti *Helicobacter pylori* functions. The selected bacteria had inhibition activity on the adherance and growth of *H. pylori*. These bacteria had additional beneficial characteristics for the probiotic such as antibacterial activity, antitumor activity, immunostimulation activity, resistance to antibiotic and bile salt, ability to bind to the intestinal cells, and safe for the human use.

INTRODUCTION

Properties for lactobacilli to be effective probiotic organisms have been suggested as followings (1). The bacterial cells can: 1, adhere to cells; 2, exclude or reduce pathogenic adherence ; 3, persist and multiply; 4, produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth; 5, form normal habitat of the human intestinal tract; 6, easily proliferate in vitro (easy mass production); 7, remain viable during procession, storage and mixing in food/feed; 8, survive the upper gastro-intestinal tract; 9, be safe and therefore noninvasive, noncarcinogenic, and nonpathogenic; 10, be genetically stable, no mutations and lack of potential for making with pathogenic bacteria; 11, coaggregate and form a normal, balanced flora; 12, (pre) digest lactose (lactose intolerance); 13, have anticholesterolaemic effects; 14, have antitumor activity.

Most lactic acid producing bacteria are known to exist in the intestines and affecting the intestinal pathogens. Since the stomach has a very harsh environment with low pH and pepsin, most people assume that lactic acid bacteria cannot adhere to the stomach cells and affecting gastric pathogens because they consitute the normal intestinal microflora. Recently *Lactobacillus gasseri* (2) and *L. acidophilus* (3) were reported to adhere the gastric mucosa and *L. salivarious* (4) and *L. gasseri* (5)

were found to produce antibacterial materials against *Helicobacter pylori*.

In 1983, Warren and Marshall first isolated and characterized *Helicobacter* in the human gastric mucous membrane. *Helicobacter* is a gram-negative microorganism which was called as *Campylobacter pyloridis* and later called *H. pylori* after its helicopter morphology in the body and name of the habitat, pylorus (7).

Helicobacter pylori cause gastritis (8) and gastric carcinoma (9). Once *H. pylori* infects, it remains for several decades and is not eliminated naturally and easily reinfected being the major cause of chronic gastritis. In Korea, more than 50% of teenagers and more than 80% of adults contain *H. pylori*. *H. pylori* infects through the intake of food and attaches to the gastric mucous membrane and the duodenal mucous membrane. A disease-causing factor of *H. pylori* is urease secreted for surviving in highly acidic condition of stomach, a flagellum for maintaining mobility and the outer membrane protein having adherence to the gastric mucous membrane. For attachment to the human gastric epithelium, *H. pylori* binds to the same antigens identified by antigens of red blood cells (10). Antigens like the Lewis antigen isolated in human blood type O, are identified in the gastric mucous membrane (11).

For the removal of *Helicobacter pylori*, antibiotic drugs, restrainers on proton pumping, and gastric acid removers have been used. The method using antibiotic drugs has a side effect in that the *H. pylori* become resistant to the antibiotic and the possibility of re-infection is not prevented. The method using stomach acid remover that suppresses stomach acid secretion is a not basic solution. In addition, although the vaccine using urease has been developed, it is not effective. In the future, the development of a vaccine against *H. pylori* will be difficult, due to its complex culture conditions, which make it difficult to determine an active area for a vaccine.

In this study, lactic acid producing bacteria with anti *H. pylori* function were isolated and characterized as a new probiotic for the stomach.

MATERIALS AND METHODS

Isolation and characterization

The feces from an infant were picked by a cotton swab and inoculated on Lactobacilli MRS (Difco, MD, U.S.A.) containing 0.002% of bromophenol blue. After incubation for 3-4 days at 25℃, bacteria were isolated from a single colony and identified according to Bergy's manual of systematic bacteriology using API50CHL (bio Merieux France) and staining. Nucleotide sequencing of 16S

rRNA was performed in ABI prism 310 Genetic Analyzer (Perkin-Elmer, Cetus, U.S.A.) and compared with the database in Genbank.

Preparation of bacteria

Helicobacter pylori (ATCC 43504) inoculated on Brucella solid media containing fungizone (2.5 g/ml amphotericin B), Skirrow's supplement (0.16 mg/ml polymyxin B, 5 mg/ml vancomycin, 2.5 mg/ml trimethoprim), and 10% horse serum and incubated under 5-10% CO_2. Bacterial cells were collected by scraping, washed twice with phosphate-buffered saline (PBS, pH 7.4), and then used freshly for inoculation or kept in 10 mM Tris buffer (pH 7.0) at -20℃ for binding assay.

Binding assay to glycolipid on a TLC plate

Glycolipid was prepared and the immunochemical method to detect *H. pylori* was performed as described in the previous paper (3).

Assay of inhibition activity on *H. pylori*

H. pylori was inoculated on a solid medium and the spent culture was added to the well formed with a sterilized Pasteur pipet. After incubating in a CO_2 incubator (5%-10% CO_2), the diameter of the inhibited growth zone was measured. The inhibition activity was observed by the additional method using phenol red.

Urease test

One ml of the spent culture was mixed with 10 µl of *H. pylori* (OD_{625}=1.0), and incubated for 1 h at 37℃ under 5% CO_2. Cells were removed with centrifugation and the supernatant was diluted with the same volume of sterile water. Urease was assayed by the indophenol method.

Growth inhibition test for food-borne pathogens

The spent culture of bacterium in MRS was mixed with the same volume of BHI (2X). *Salmonella typhimurium, S. Enteritidis, E. coli O157:H7, Aeromonas hydrophila*, and *Listeria monocytogenes* grown overnight in BHI were inoculated into the mixture of the spent culture making 1% final concentration . After 5 h and 12 h, CFU was assayed with the MPN (most probable number) method.

Inhibition activity on anaerobic bacteria

Cl. perfringens (final concentration 1%) was inoculated to the medium containing the spent

culture. After 24 h incubation at 37℃, the culture was diluted, inoculated on blood agar plates, and incubated in an anaerobic jar.

Assay of immunostimulation activity

Immunostimulation activity was assayed by measuring the production of TNF-α and IL-6 by macrophage cell line Raw 264.7 with the ELISA method.

Cell adhesion assay

Intestinal cell line Caco-2 cells and gastric carcinoma cell line MKN-45 cells were cultured in two slide chamber system until confluency. Bacterial cells (1 X 10^7 CFU/ml) were added to the slide and incubated for 1 h at 37°C. The slides were washed with PBS three times to remove any unbound cells and stained with Gram stain. Cells were observed under a light microscope.

Antimutagenecity assay with SOS test

E. coli PQ37 (*F- thr leu his-4 pyrD thi galE galK or galT lac△U169 srl300::Tn10 rpoB rpsL uvrA rfa trp::Muc+ sfiA::Mud* (Ap. *lac) cts*) was cultured in LB (Luria-Bertani) containing 20 μg/ml ampicillin. The β-galactosidase assay was performed according to the method as described in a previous paper (12).

Disk test

Disk test was performed following the method of NCCLS (13).

Test for acid- and bile-resistance

The pH of MRS medium containing cysteine was adjusted with 4 N HCl and 0.1 N NaOH. OX-BILE (Difco) was added to the medium. Bacterial cells at 0.5 MacFarland unit were inoculated and the growth was assayed at 620 nm.

Hemolysis

Hemolysis activity was tested on a blood agar medium containing the whole horse blood and the presence of hemolysis was checked everyday upto 10 days.

Gelatin liquefying activity

Gelatin degrading activity was tested by stabbing bacterial cells into 12% gelatin. The tube was tilted to check the gelatin liquefaction everyday upto 10 days.

Detection of plasmid

Plasmids were extracted from cells before and after incubation overnight in the presence of 25 ㎍/ml ethidium bromide and electrophoresed in a 1% agarose gel.

Toxicity test

Total 10 rats (Sprague- Dawley), 5 females between 100~120 g and 5 males between 110~130 g were grown in cages (260 W × 420 L × 180 H mm) at 23 ± 2℃ and 50 ±10 % relative humidity. Rats were fed with 5,000 mg/kg (20 ml/kg B.W.) for 14 days. The oral toxic test was based on the 'toxic test standard for drug (1999. 12. 22)' referred as Notification No. 1999-61 of the Korean Food and Drug Administration. Referring to the established rule No. 10 of the Korean Food and Drug Administration, the material is low toxicity material in body when the value of LD_{50} is up to 5,000 mg/kg in an oral test. Therefore, in this experiment, the dosage was set to the maximum dosage 5,000 mg/kg (20 ml/kg B.W.).

RESULTS AND DISCUSSION

Among over 100 isolates, several isolates prevented *H. pylori* binding to glycolipid on a TLC plate (Fig. 1). This TLC method (3) enables mass-screening a large number of samples for its ability to bind to gastric mucus in a short time. When the selected isolates were added to the stomach cell line MKN-45 cells, they bound to the cells. These results support the previous ones published from others (2) and

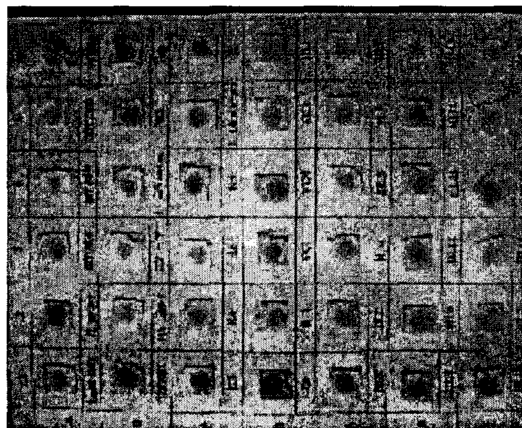

Fig. 1. Immunochemical method to screen the inhibition activity on *H. pylori* adherance to glycolipid on a TLC plate.

my lab (3) that lactic acid bacteria can bind to the gastric cells suggesting that lactic acid bacteria could be used as an inhibitor for *H. pylori* adherance. Even though these lactic acid bacteria can bind to the stomach, these are not expected to multiply and constitute the gastric microflora. Another antibacterial function of lactic acid bacteria is exerted by the production of lactic acid and bacteriocin(s) (5). Lactic acid bacteria isolated in this study inhibited the growth of *H. pylori* on solid media and this was clearly shown by the well test (Fig. 2) and urease assay (Fig. 3). These inhibitory activities were also observed with the culture supernatant neutralized with NaOH. The permeabilizing

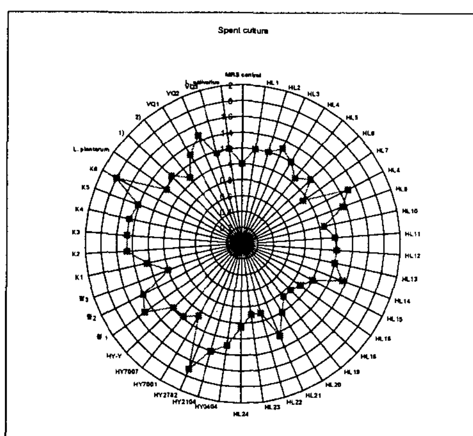

Fig. 2. Well test

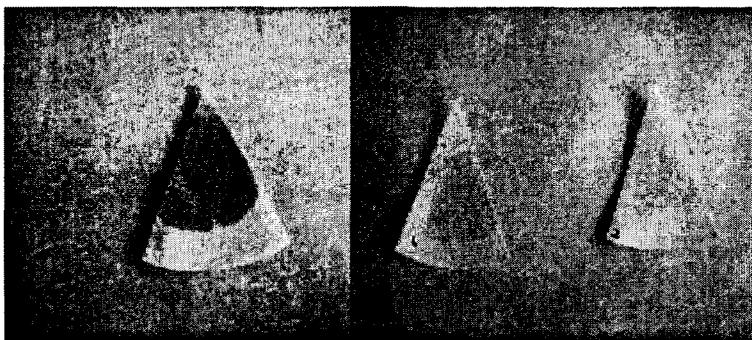

Fig. 3. Urease test

activity of bacteriocin of these bacteria was observed under a scanning electron microscope (Fig. 4). Bactericidal activity remained after the treatment with low pH and pepsin and this suggests that the bacteriocin is a non-proteinaceous factor and continues to exert its antibacterial activity in the stomach.

In addition to these characteristics for the probiotic for stomach, these bacteria possess various beneficial functions as other probiotics have. These bacteria can bind to the intestinal cell line Caco-2 and exert antibacterial effect on various food-borne pathogens including anaerobic bacteria (Fig. 5). Also these bacteria did survive at low pH and bile salt that make them to reach to the intestines. Another important characteristic is the antibacterial resistance that should not be easily transferred to the normal microflora. If the antibacterial resistance existed on plasmid, it can be easily transferred to

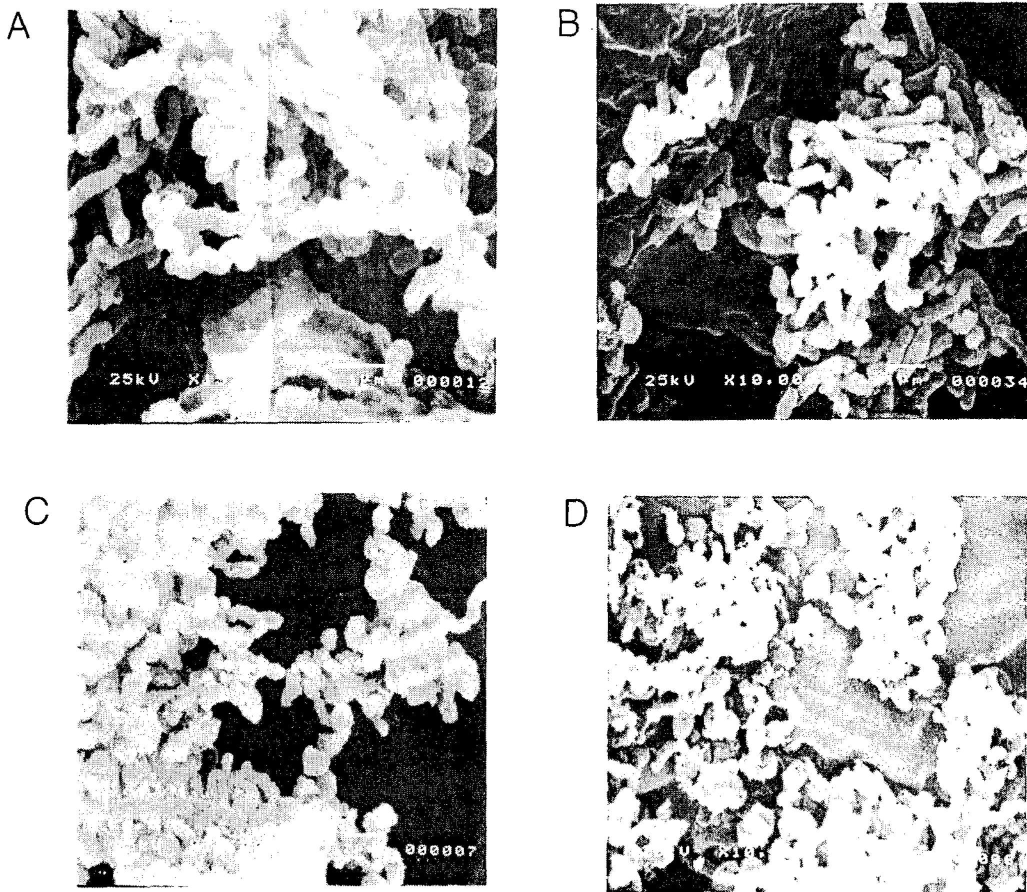

Fig. 4. Scanning electron microscope of *H. pylori* after treated with spent culture supernantant. A, no treatment; B, treated with amoxacillin; C, treated with the spent culture for 1 min ; D, treated with the spent culture for 1 h

other bacteria and causing a bigger problem. The antibiotic resistance of these lactic acid bacteria did not reside on a plasmid and was not transferred to the normal microflora.

Another checkpoint is their safety. The safety could be checked by looking for the presence of hemolysin and gelatinase that lead to the infection and toxicity to the human body. These isolates showed no hemolysis and gelatinase activity and when they were fed to rats, no signs was found in any individual rats or organs. All these results recommend these isolates as probiotics having an anti *H. pylori* function.

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