DOI:10.1021/jf1035756

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Expression of *Lactobacillus reuteri* Pg4 Collagen-Binding Protein Gene in *Lactobacillus casei* ATCC 393 Increases Its Adhesion Ability to Caco-2 Cells

Hsiang-Yun Hsueh,[†] Pei-Ying Yueh,[‡] Bi Yu,[§] Xin Zhao,[#] and Je-Ruei Liu*[,†,⊗]

[†]Institute of Biotechnology and Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan, [‡]Council of Agriculture, Executive Yuan, Taipei, Taiwan, [§]Department of Animal Science, National Chung Hsing University, Taichung, Taiwan, [#]Department of Animal Science, McGill University, Montreal, Canada, and [⊗]Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

The collagen-binding protein gene *cnb* was cloned from the probiotic *Lactobacillus reuteri* strain Pg4. The DNA sequence of the *cnb* gene (792 bp) has an open reading frame encoding 263 amino acids with a calculated molecular weight of 28.5 kDa. The *cnb* gene was constructed so as to constitutively express under the control of the *Lactococcus lactis lac*A promoter and was transformed into *Lactobacillus casei* ATCC 393, a strain isolated from dairy products with poor ability to adhere to intestinal epithelial cells. Confocal immunofluorescence microscopic and flow cytometric analysis of the transformed strain *Lb. casei* pNZ-cnb indicated that Cnb was displayed on its cell surface. *Lb. casei* pNZ-cnb not only showed a higher ability to adhere to Caco-2 cells but also exhibited a higher competition ability against *Escherichia coli* O157:H7 and *Listeria monocytogenes* adhesion to Caco-2 cells than *Lb. casei* ATCC 393.

**KEYWORDS: Collagen-binding protein; adhesion; *Lactobacillus reuteri*; *Lactobacillus casei***

## INTRODUCTION

A probiotic is a live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance (*1*). Species currently being used in probiotic preparations for animal nutrition belong to the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Saccharomyces* (*2*). Of those microbial species, lactic acid bacteria, such as members of the genera *Lactobacillus*, are normal inhabitants of the intestine (*3*). There is sufficient evidence to show that some strains of probiotic *Lactobacillus* are effective at improving growth rate, feed efficiency, and disease resistance and at reducing gut shedding of enteropathogenic bacteria in livestock animals (*4*, *5*). In contrast, Watkins and Kratver (*6*, *7*) showed that the oral administration of high numbers of different *Lactobacillus* strains or the introduction commercial *Lactobacillus* to drinking water did not have any significant effect on broiler growth or feed intake. Jin et al. (*8*) attributed the failure of probiotic lactobacilli to enhance chicken production to the inability of microorganisms to colonize or survive in the gastrointestinal tract or to their inability to antagonize or competitively exclude the pathogenic bacteria. Therefore, the ideal microorganism for probiotic lactobacilli must be able to provide competition against the adhesion of gastrointestinal pathogens to the intestinal mucosa and establish itself and flourish in the intestine (*9*, *10*).

*Lactobacillus reuteri* frequently occurs in the intestinal microflora of various mammals and has been widely regarded as a potential probiotic. Strains of this species have been shown to possess the ability to adhere to epithelial cells and colonize the intestine (*11*). The collagen-binding protein Cnb is a surface-located protein from *Lb. reuteri* NCIB 11951 (*12*). Sequence comparisons have shown that Cnb is a member of the family 3 solute-binding proteins (*13*), a group of proteins that typically bind opines and polar amino acids. Most solute-binding proteins in Gram-positive bacteria are lipoproteins (*14*). Cnb is unusual in that it is not a lipoprotein and lacks the membrane anchor motif LXXC normally associated with solute-binding proteins of Gram-positive bacteria. In addition, its calculated isoelectric point is a highly alkaline 9.78. Closely related homologues of Cnb include BspA from *Lactobacillus fermentum* BR11 (*15*), a 29 kDa protein from *Lb. fermentum* RC-14 (*16*), and MapA from *Lb. reuteri* 104R (*17*). These proteins are hypothesized to be adhesion factors and probably interact with teichoic acids via electrostatic interactions due to their strong cationic properties, which are shared with the anchoring domain of *Lactobacillus* S-layer protein subunits (*18*).

In the present study, we describe the cloning of the collagen-binding protein gene *cnb* from *Lb. reuteri* Pg4, a strain isolated from the gastrointestinal tract of healthy broilers, and heterologous expression of the *cnb* gene in *Lactobacillus casei* ATCC 393, a strain isolated from dairy products that shows poor ability to adhere to intestinal epithelial cells. We examined the recombinant Cnb expression levels and the adherence ability to Caco-2 cells as well as the inhibition ability of the transformed strain *Lb. casei* pNZ-cnb against the adhesion of pathogenic bacteria to Caco-2 cells.

*Author to whom correspondence should be addressed (e-mail jrliu@ntu.edu.tw; phone 886-2-33666011; fax 886-2-33666001).

 Published on Web 11/11/2010 

## MATERIALS AND METHODS

**Bacterial Strains, Plasmids, Cell Line, and Culture Conditions.** *Lb. reuteri* Pg4 (*19*) has been deposited in the Taiwanese Bioresource Collection and Research Center with deposit no. BCRC 19618 and was used to prepare genomic DNA for amplification of the collagen-binding protein gene *cnb*. *Lb. casei* ATCC 393 (American Type Culture Collection, Manassas, VA) and *Lactobacillus* expression vector pNZ3004 (*20*) were used to express *cnb*. All PCR products intended for sequence analysis were cloned into pGEM-T Easy vector (Promega, Medison, WI). *Escherichia coli* DH5α was used in all subcloning experiments. *E. coli* BL21 (DE3) (Novagen, Madison, WI) and *E. coli* expression vector pGEX-4T-3 (GE Healthcare, Piscataway, NJ) were used to prepare the purified recombinant proteins for production of polyclonal antibody specific for Cnb. *E. coli* ATCC 43894 (O157:H7) and *Listeria monocytogenes* ATCC 13932 were used to study the competition between *Lactobacillus* and pathogens for adhesion to Caco-2 cells.

*Lactobacillus* strains were grown in de Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) at 37 °C without shaking. *E. coli* was cultured in Luria−Bertani (LB) broth (Difco Laboratories) at 37 °C in an orbital shaker at 250 rpm. *Ls. monocytogenes* was cultured in brain−heart infusion (BHI) broth (Difco Laboratories) at 37 °C in an orbital shaker at 250 rpm. Agar plates were prepared by adding agar (1.5% w/v) (Difco Laboratories) to broth.

The cancer-derived human colonic intestinal epithelial cell line Caco-2 was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Caco-2 cells were routinely grown at 37 °C in a 95% air−5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (Biological Industries, Bet-Haemek, Israel) supplemented with 4 mM L-glutamine (Biological Industries), 1.5 g/L sodium bicarbonate (Biological Industries), 4.5 g/L glucose (Sigma-Aldrich Co., St. Louis, MO), 10 mg/L human transferrin (Sigma-Aldrich Co.), and 10% fetal calf serum (Moregate Biotech, Queensland, Australia).

**DNA Isolation and Manipulation.** Genomic DNA was prepared from *Lb. reuteri* Pg4 using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). Plasmid DNA was isolated from *E. coli* using the QIAprep Miniprep Kit (Qiagen Inc.). Restriction enzymes and T4 DNA ligase (New England BioLabs Inc., Beverly, MA) were used according to the manufacturer's instructions. All other DNA manipulations were performed using established procedures (*21*). All DNA sequences were determined by an automatic sequencing service provided by Mission Biotech Inc. (Taipei, Taiwan).

**Cloning of the Collagen-Binding Protein Gene *cnb* from *Lb. reuteri* Pg4.** The *cnb* gene of *Lb. reuteri* Pg4 was amplified by PCR using the oligonucleotide forward primer cnbF, 5′ ACGCGTCGACGAAATTTTGGA 3′, and the oligonucleotide reverse primer cnbR, 5′ CCAATGCATTTATTCAGTAATA 3′ (the underlined sequences in the primers are additional sequences that represent the restriction sites for *Sal*I and *Nsi*I, respectively), designed on the basis of the Cnb gene of *Lb. reuteri* NCIB 11951 (GenBank accession no. X99716) as described by Roos et al. (*12*). These two primers were designed to place a *Sal*I site at the 5′ end and a *Nsi*I site at the 3′ end of the PCR product, respectively. PCR amplification was carried out with an initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 1 min, and a final elongation at 72 °C for 7 min. The PCR fragments encoding Cnb were digested with *Sal*I and *Nsi*I and ligated with *Sal*I−*Pst*I-digested pNZ3004 to generate pNZ-cnb (Supporting Information Figure S1), which was sequenced to ensure that there were no errors introduced by PCR.

**Preparation of Polyclonal Antibody Specific for Cnb.** The DNA sequence encoding *Lb. reuteri* CnB was amplified by PCR from pNZ-cnb using the oligonucleotide forward primer, 5′ CGCGGATCCAAATTTTGGAAGAAAGC 3′, and reverse primer, 5′ ATAAGAATGCGGCCGCTTATTCAGTAATA 3′ (the underlined sequences in the primers are additional sequences that represent the restriction sites for *Bam*HI and *Not*I, respectively). The PCR fragments encoding Cnb were digested with *Bam*HI and *Not*I and ligated with *Bam*HI−*Not*I-digested pGEX-4T-3. The *E. coli* expression vector pGEX-4T-3 is designed for the expression of the recombinant protein fused to the 224 amino acid glutathione-*S*-transferase (GST; 26.2 kDa) sequence upstream of the cloning site. The fusion tag can be removed from the recombinant target protein by protease cleavage using thrombin. The resultant plasmid, designated pGEX-cnb, was sequenced to ensure that no errors had been introduced by PCR and was used to transform *E. coli* BL21 (DE3) by standard techniques (Sambrook and Russell 2001). *E. coli* transformants were selected on LB agar plates containing ampicillin (10 $\mu$g mL$^{-1}$) (Sigma-Aldrich Co.). To produce the recombinant protein, *E. coli* BL21 transformant cells were cultured in LB broth, and cell growth was then measured turbidimetrically at 600 nm (OD600). The overnight culture was prepared and subsequently seeded at a 1:100 dilution into 5 mL of fresh LB broth. The cell cultures were maintained at 37 °C and induced with 100 $\mu$M isopropyl-$\beta$-D-thiogalactopyranoside (IPTG; Sigma-Aldrich Co.) for protein production upon reaching an OD600 of 0.5. After 4 h of induction, the cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C.

The cell pellet was resuspended in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4), sonicated for 10 min with an ultrasonicator (model XL, Misonix, Farmingdale, NY), and fractioned into supernatant and pellet fractions by subsequent centrifugation at 13000*g* for 20 min at 4 °C. The GST-tagged recombinant protein, mainly present in the supernatant, was captured on a GSTrap FF column (GE Healthcare). For thrombin on-column cleavage, 10 U of protease/mg of bound GST fusion protein was loaded onto the column and incubated at 25 °C for 16 h. Finally, the purified recombinant Cnb proteins were obtained by removal of thrombin using a HiTrap Benzamidine FF column (GE Healthcare) in series below the GSTrap FF column (Supporting Information Figure S2). The purified recombinant Cnb was used to prepare polyclonal antibodies in mice (LTK Biolab. Inc., Hsinchu, Taiwan).

**Heterologous Expression of *cnb* Gene by *Lb. casei* ATCC 393.** The pNZ-cnb plasmid was electroporated into *Lb. casei* ATCC 393 as described by Serror et al. (*22*). Subsequent to electroporation, the *Lb. casei* ATCC 393 transformants were incubated in MRS broth containing $MgCl_2$ (10 mM) at 37 °C for 3 h. The transformants were subsequently spread onto MRS agar plates containing erythromycin (10 $\mu$g/mL) and incubated at 37 °C until the appearance of transformants. The transformants, designated *Lb. casei* pNZ-cnb, were confirmed not only by direct colony PCR using cnbF and cnbR primer set but also by reverse transcription PCR (RT-PCR), Western blot, confocal immunofluorescence microscopy, and flow cytometric analysis.

**RT-PCR Analysis of RNA Isolated from *Lactobacillus* Strains.** *Lb. reuteri* Pg4, *Lb. casei* ATCC 393, and *Lb. casei* pNZ-cnb were transferred to MRS broth and incubated at 37 °C for 24 h. The cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C. Total RNA was extracted from the lactobacilli cells with TRizol reagent (Invitrogen, Carlsbad, CA). Residual DNA was removed with RNase-free DNase I (Invitrogen) treatment. RNA concentrations were measured in triplicate using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Exactly 2.0 $\mu$g of RNA was transcribed into first-strand cDNA using M-MLV reverse transcriptase (Promega) and cnbF and cnbR primer set. PCR reaction was then performed using the cDNA as template for amplification with the above primer set. The transcript levels of the housekeeping 16S rRNA gene were determined in parallel for each sample using 16S-27f (5′ AGAGTTTGATCMTGGCTCAG 3′) and 16S-1492r (5′ CGGTTACCTTGTTACGACTT 3′) primer sets.

**Gel Electrophoresis and Western Blot Analysis.** *Lb. reuteri* Pg4, *Lb. casei* ATCC 393, and *Lb. casei* pNZ-cnb cells were cultivated and harvested as described above. The cell pellet was resuspended in 1 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4), sonicated for 10 min with an ultrasonicator (model XL, Misonix), and fractioned into intracellular extract and cell-wall pellet fractions by subsequent centrifugation at 13000*g* for 20 min at 4 °C. The intracellular extract and cell-wall pellet of each *Lactobacillus* cell culture were analyzed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (*23*). Samples were placed in Laemmli buffer, boiled, and separated on 12.5% SDS-PAGE gels (Bio-Rad, Hercules, CA). Either gels were stained with Coomassie brilliant blue (Sigma-Aldrich Co.), or the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, GE Healthcare) in transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, and 20% methanol).

For Western blot analysis, the membranes were blocked in blocking buffer (25 mM Tris, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk; pH 7.5) at room temperature for 1 h and then probed with mouse polyclonal anti-Cnb antibody (1:10 dilution in PBS, LTK Biolab. Inc.) for 1 h at room temperature. Following this, the membranes were incubated with a secondary horseradish peroxidase-linked anti-mouse IgG antibody

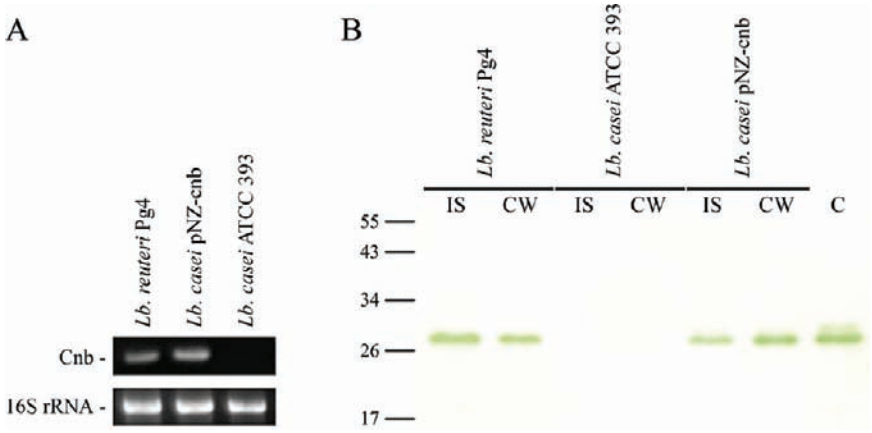


**Figure 1.** Expression of Cnb in *Lactobacillus* cells. (**A**) RT-PCR for Cnb in *Lb. rueteri* Pg4, *Lb. casei* pNZ-cnb, and *Lb. casei* ATCC 393, along with the housekeeping gene (16S rRNA). (**B**) Western blot for Cnb in the intracellular supernatant (IS) and cell-wall pellet (CW) of *Lactobacillus* cells. The purified recombinant Cnb was used as a positive control (C). The samples (2 $\mu$g of protein in each lane) were separated by SDS-PAGE with a 12.5% gel and probed with mouse polyclonal anti-Cnb antibody, followed by secondary horseradish peroxidase-linked anti-mouse IgG antibody.

(1:10000 dilution in PBS, GE Healthcare) for 1 h at room temperature. Antibody−antigen complexes were detected using ECL Western blot signal detection reagent (GE Healthcare).

**Confocal Immunofluorescence Microscopic Analysis.** *Lactobacillus* cells were cultivated and harvested as described above. The cell pellet was resuspended in 1 mL of 0.1 M PBS (pH 7.4) containing 10 g/L bovine serum albumin and incubated at room temperature for 30 min. The cells were then incubated with mouse polyclonal anti-Cnb antibody (1:10 dilution in PBS, LTK Biolab. Inc.) at room temperature for 1.5 h. Following this, the cells were incubated with the secondary goat anti-mouse IgG heavy and light chain antibody conjugated with Alexa Fluor 488 (1:2500 dilution in PBS, Invitrogen) at room temperature for 1 h. Finally, the immunostained bacterial cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C and then laid on a glass slide, air-dried, and heat-fixed. Confocal immunofluorescence microscopic analysis was performed using a Leica TCS SP5 II spectral confocal microscope mounted on a Leica DMI 6000B inverted microscope (Leica Microsystems, Heidelberg, Germany) with an HCX PLAPO CS 63×/1.4-0.6 oil immersion objective and an argon laser (458, 476, 488, 514 nm) source.

**Flow Cytometric Analysis.** *Lactobacillus* cells were immunostained as described above. Flow cytometric analysis was performed on a Cytomics FC500 Flow Cytometry System (Beckman Coulter, Inc., Miami, FL) with a 488 nm argon laser and a 525 nm emission filter. Immunostained bacterial cells were diluted to approximately $10^6$ cells/mL and delivered at a low flow rate, corresponding to 150−300 bacterial cells per second. The data were analyzed using CXP software (Beckman Coulter, Inc.) with 18000 counts analyzed in each experiment. Counts were made in triplicate for each procedure.

***Lactobacillus* Adhesion Assays.** Before adherence assays, Caco-2 cells were incubated with antibiotic-free medium at 37 °C for 24 h. Then, the Caco-2 cells were removed from tissue culture flasks with an EDTA−trypsin solution, washed three times in PBS, and resuspended in the same buffer. The cells were then incubated with mouse anti-human CD29 (integrin $\beta$ 1) monoclonal antibody conjugated with PerCP-eFluor 710 (1:10 dilution in PBS; eBioscience, Inc., San Diego, CA) at room temperature for 1.5 h. Following this, the immunostained Caco-2 cells were washed three times in PBS and then resuspended to $1 \times 10^6$ cells/mL in the same buffer.

*Lb. reuteri* Pg4, *Lb. casei* ATCC 393, and *Lb. casei* pNZ-cnb cells were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma-Aldrich Co.) as described by Logan et al. (*24*). Fluorescently labeled bacterial cells were added into the Caco-2 suspension to yield a final concentration of $10^8$ CFU/mL of bacteria and $10^6$ cells/mL of Caco-2 cells and incubated at 37 °C for 2 h. After incubation, the Caco-2 cells were washed three times in PBS to remove nonadherent bacteria. The fluorescent intensity of the Caco-2 cells with adherent lactobacilli was measured with the Cytomics FC500 Flow Cytometry System (Beckman Coulter, Inc.) using a 488 nm argon laser and a 525 nm emission filter for CFSE and a 755 nm emission filter for PerCP-eFluor 710. Flow cytometry was gated using a forward scatter (FSC) versus side scatter (SSC) dot plot to include Caco-2 cells and to exclude cellular debris and nonadherent bacteria. Cells inside the gated area were confirmed by CD29 antibody conjugated with PerCP-eFluor 710. A total of 10000 events were acquired, and the data obtained from the flow cytometry were analyzed using the CXP software (Beckman Coulter, Inc.). This program produces histograms of each individual particle sample and calculates the mean green fluorescence intensity for each cell population. Counts were made in triplicate for each procedure.

**Inhibition of Pathogenic Bacteria Adhesion.** Before adhesion competition studies, Caco-2 cells were incubated, harvested, and immunostained as described above.

In the study of competition between *Lactobacillus* and *Ls. monocytogenes* for adhesion to the Caco-2 cells, *Lactobacillus* cells were labeled with CFSE as described above, whereas *Ls. monocytogenes* cells were labeled with hexidium iodide (HI) using the Live BacLight Bacterial Gram Stain Kit (Molecular Probes, Eugene, OR) according to the protocols provided by the manufacturer.

In the study of competition between *Lactobacillus* and *E. coli* O157:H7 for adhesion to the Caco-2 cells, *Lactobacillus* cells were labeled with HI using the Live BacLight Bacterial Gram Stain Kit (Molecular Probes) according to the protocols provided by the manufacturer, whereas *E. coli* O157:H7 cells were labeled with SYTO 9 using the same kit.

Fluorescently labeled bacterial cells (*Lactobacillus* alone, pathogens alone, or *Lactobacillus* and pathogens together) were added to the Caco-2 suspension to yield a final concentration of $(1-2) \times 10^8$ CFU/mL of bacteria and $10^6$ cells/mL of Caco-2 cells and incubated at 37 °C for 2 h. After incubation, the Caco-2 cells were washed three times in PBS to remove nonadherent bacteria. The fluorescent intensity of the Caco-2 cells with adherent bacterial cells was measured with the Cytomics FC500 Flow Cytometry System (Beckman Coulter, Inc.) using a 488 nm argon laser, a 525 nm emission filter for green fluorescence (CFSE and SYTO 9), a 620 nm emission filter for red fluorescence (HI), and a 755 nm emission filter for PerCP-eFluor 710, and analyzed using CXP software (Beckman Coulter, Inc.) as described above. Counts were made in triplicate for each procedure, and the mean green fluorescence intensity and mean red fluorescence intensity for each cell population were calculated.

**Statistical Analysis.** All results were analyzed using the general linear model procedure available from the Statistical Analysis System software package version 8.1 (SAS Institute Inc., Cary, NC). Duncan's multiple-range test (*25*) was used to detect differences between treatment means. Each assay was conducted in triplicate.

**Nucleotide Sequence Accession Number.** The nucleotide sequence of *Lb. reuteri* Pg4 *cnb* has been submitted to the GenBank database under accession no. HM589858.

## RESULTS

**Cloning of *cnb* from *Lb. reuteri* Pg4.** The collagen-binding protein genes of several *Lactobacillus* strains including *Lb. reuteri* NCIB 11951 (*12*), *Lb. reuteri* DSMZ 20016 (*26*), and *Lb. fermentum* BR11 (*15*) have been characterized and cloned in

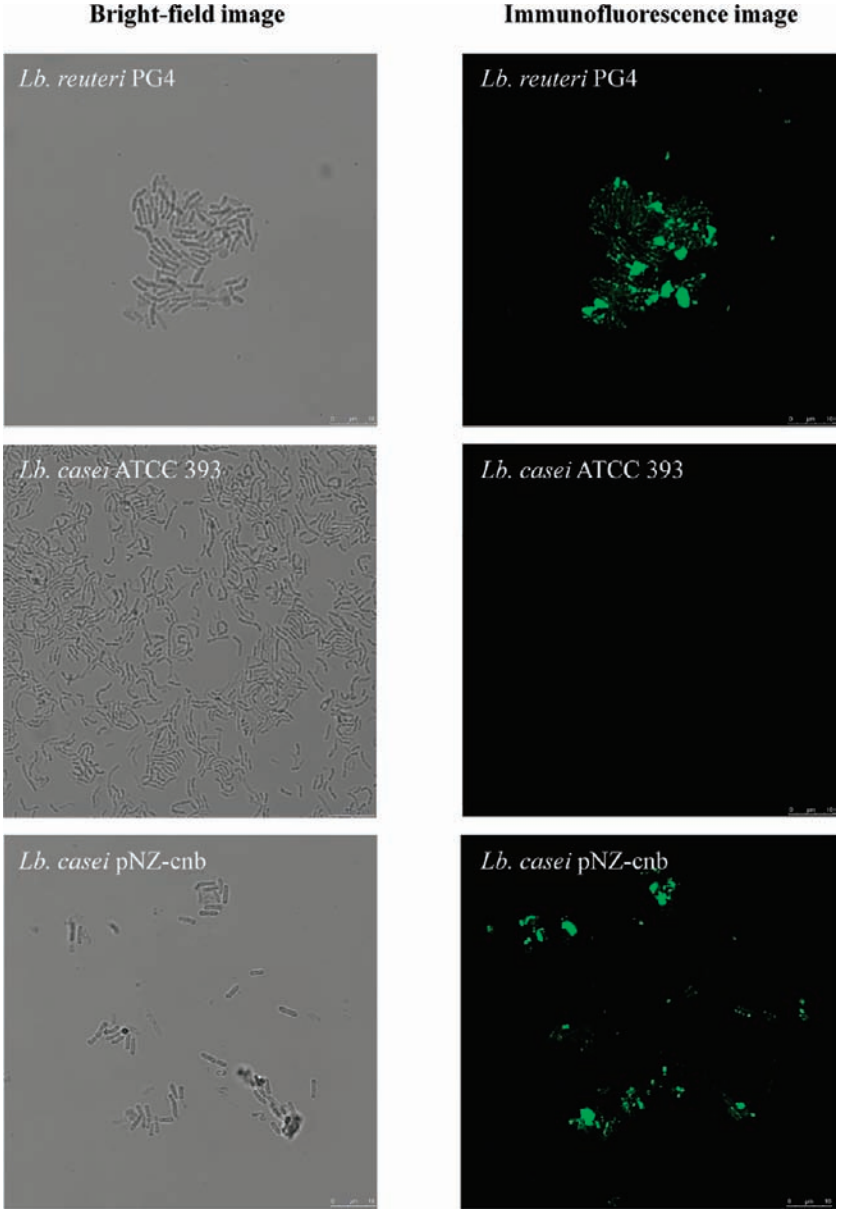


**Figure 2.** Confocal immunofluorescence microscopic examination of *Lb. reuteri* PG4, *Lb. casei* ATCC 393, and *Lb. casei* pNZ-cnb cells. Cells were labeled with mouse polyclonal anti-Cnb antibodies, followed by goat anti-mouse IgG heavy and light chain antibodies conjugated with Alexa Fluor 488.

previous studies. The oligonucleotides cnbF and cnbR were designed on the basis of the Cnb gene of *Lb. reuteri* NCIB 11951 (GenBank accession no. X99716). PCR conducted with the primers cnbF and cnbR resulted in the appearance of several amplified products, probably due to the ability of the primers to anneal with various genome fragments encoding proteins with similar properties. However, on the basis of the *cnb* sequences of *Lb. reuteri*, a 792 bp fragment was expected for the *cnb* gene of *Lb. reuteri* Pg4. A 792- bp fragment was therefore isolated, and subsequent sequence analysis revealed 96.5% nucleotide sequence identity with the *cnb* of *Lb. reuteri* NCIB 11951. Translation of the open reading frame of *cnb* revealed a protein of 263 amino acids with a predicted molecular weight of 28.5 kDa. The amino acid sequence alignment of the *Lb. reuteri* Pg4 Cnb and other proteins available from GenBank showed that the highest identity is with lr0793 protein from *Lb. reuteri* ATCC 55730 (100%, accession no. AAY86822), followed by ABC transporter substrate binding component from *Lb. reuteri* JCM 1112 (99%, accession no. YP_001841280), extracellular solute-binding protein from *Lb. reuteri* 100-23 (98%, accession no. ZP_03074063), mucus adhesion promoting protein from *Lb. reuteri* 104R (97%, accession no. CAC05301), and BspA from *Lb. fermentum* BR11 (88%, accession no. ACA45332).

**Construction and Transformation of *Lactobacillus* Expression Plasmid.** For heterologous expression of Cnb in *Lb. casei* ATCC 393, the DNA fragments of *Lb. reuteri* Pg4 encoding Cnb were cloned into the *Lactobacillus* expression vector pNZ3004, generating pNZ-cnb. The expression plasmid pNZ-cnb was used to electroporate into *Lb. casei* ATCC 393 with efficiency similar to that of pNZ3004 [$(5-10) \times 10^2$ transformants $\mu g^{-1}$ of DNA]. The presence of the *cnb* gene in the *Lb. casei* transformants (designated *Lb. casei* pNZ-cnb) was demonstrated by direct colony PCR (results not shown).

**Heterologous Expression of Cnb in *Lb. casei* ATCC 393.** RT-PCR analysis was used to confirm the transcription of Cnb in *Lb. casei* pNZ-cnb. A 0.8 kb fragment representing Cnb was RT-PCR amplified not only from the total RNA extracted from the *Lb. reuteri* Pg4 cells as expected but also from the *Lb. casei* pNZ-cnb cells, indicating that Cnb was successfully expressed by *Lb. casei* pNZ-cnb (**Figure 1A**).

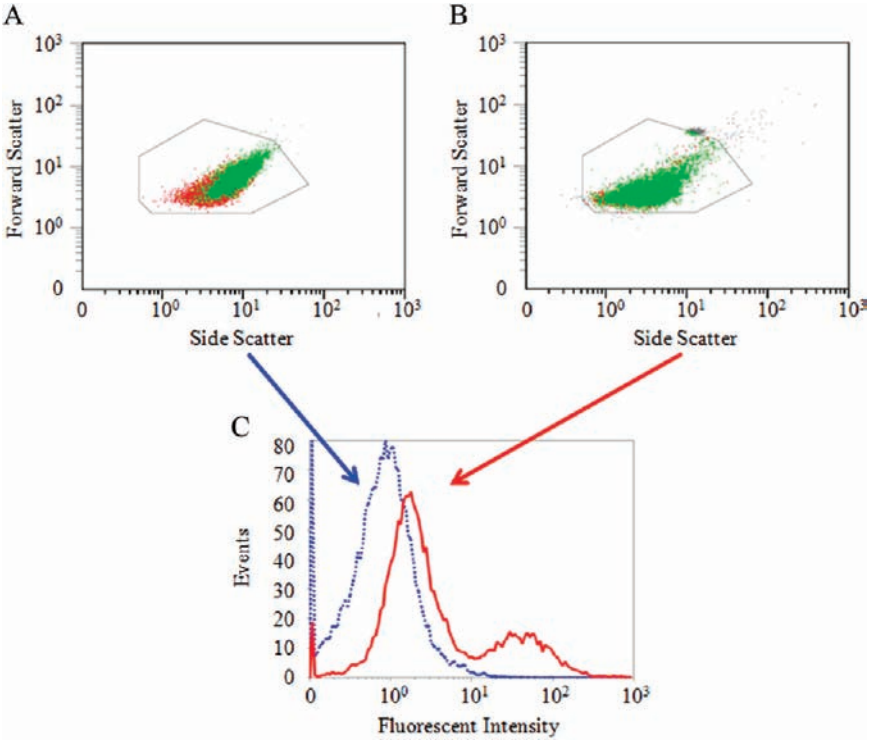


**Figure 3.** Flow cytometric analysis of *Lb. casei* ATCC 393 and *Lb. casei* pNZ-cnb: (**A**) dot plot profile of size versus granularity (forward scatter versus side scatter) analysis of the parental *Lb. casei* ATCC 393; (**B**) dot plot profile of size versus granularity (forward scatter versus side scatter) analysis of *Lb. casei* pNZ-cnb; (**C**) histogram plot of the shift in fluorescence seen with the labeled *Lb. casei* ATCC 393 (dot line) versus *Lb. casei* pNZ-cnb (solid line). Bacterial cells were labeled with mouse polyclonal anti-Cnb antibody, followed by goat anti-mouse IgG heavy and light chain antibody conjugated with Alexa Fluor 488. For each experiment, 10000 cells were analyzed. The experiment was repeated three times with highly similar results.

The expression of Cnb in *Lb. casei* pNZ-cnb cells was further confirmed by Western blot analysis using Cnb-specific polyclonal antibody. As shown in **Figure 1B**, clear bands at the expected size for Cnb (about 28.5 kDa) signal were detected in either intracellular supernatant or cell-wall fractions of *Lb. reuteri* Pg4 cells as expected (**Figure 1B**, lanes 1 and 2), and also from those of *Lb. casei* pNZ-cnb cells (**Figure 1B**, lanes 5 and 6), indicating that Cnb was successfully and constitutively expressed under control of the *Lactococcus lactis lac*A promoter.

**Confocal Immunofluorescence Microscopic and Flow Cytometric Analysis of *Lb. casei* pNZ-cnb.** Immunofluorescence labeling of *Lactobacillus* cells was performed using mouse anti-Cnb antibody as the primary antibody and Alexa Fluor 488 conjugated goat anti-mouse IgG as the secondary antibody. As shown in **Figure 2**, confocal immunofluorescence microscopy demonstrated that immunostained Cnb was observed on both *Lb. reuteri* Pg4 and *Lb. casei* pNZ-cnb cells, whereas *Lb. casei* ATCC 393 cells were not stained, indicating that Cnb was displayed on the cell surface of the former sets of *Lactobacillus* cells.

To substantiate the findings of the confocal immunofluorescence microscopic observation, flow cytometry was further used to quantitatively analyze the cell surface display of Cnb. The cell surface-displayed Cnb was stained with the primary and secondary antibodies, and *Lb. casei* ATCC 393 cells were used as a control for flow cytometry. The results obtained clearly matched the findings obtained by microscopic analysis. As anticipated, *Lb. casei* pNZ-cnb showed a greater intensity of fluorescence signals than *Lb. casei* ATCC 393 (**Figure 3**). The mean green fluorescence intensity of *Lb. casei* pNZ-cnb ($17.85 \pm 0.64$) was significantly higher than that of *Lb. casei* ATCC 393 ($1.23 \pm 0.02$) ($p < 0.05$).

**Adhesion of *Lactobacillus* to Caco-2 cells.** *Lb. reuteri* Pg4, *Lb. casei* ATCC 393, and *Lb. casei* pNZ-cnb cells were labeled with CFSE, a nonfluorescent membrane permeable ester, which is converted to a fluorescent molecule by nonspecific intracellular esterases and then covalently linked to intracellular proteins through its succinimidyl group (*27*). Fluorescently labeled bacterial cells were assessed for their ability to adhere to the Caco-2 cells. Caco-2 cells with fluorescently labeled bacteria attached showed a shift in fluorescence as a result of bacterial adhesion when compared with the autofluorescence expressed by Caco-2 cells alone (**Figure 4**). Caco-2 cells were weakly autofluorescent as seen in the scatter plot (**Figure 4A**), whereas Caco-2 cells incubated with each fluorescent-labeled *Lactobacillus* strain for 2 h shifted along the fluorescence axis in the scatter plot, indicating adhesion of bacterial cells to the Caco-2 cells (**Figure 4B**−**D**). However, the adhesion ability of *Lb. casei* ATCC 393 to Caco-2 cells was lower than that of *Lb. reuteri* Pg4. These results are consistent with our previous study (*19*). On the other hand, *Lb. casei* pNZ-cnb adhered efficiently to Caco-2 cells. There was an 8-fold increase in the mean green fluorescence intensity of Caco-2 cells incubated with *Lb. casei* pNZ-cnb ($35.33 \pm 3.14$) compared to those incubated with *Lb. casei* ATCC 393 ($4.37 \pm 0.50$). Interestingly, the mean green fluorescence intensity of Caco-2 cells incubated with *Lb. casei* pNZ-cnb was also significantly higher than that of Caco-2 cells incubated with *Lb. reuteri* Pg4 ($14.20 \pm 1.74$) ($p < 0.05$). This result demonstrated that introduction of the *cnb* gene into the *Lb. casei* ATCC 393 resulted in an increase in its adhesion ability to Caco-2 cells.

**Competition between *Lactobacillus* and *Ls. monocytogenes* for Adhesion to Caco-2 Cells.** To study the competition between *Lactobacillus* and *Ls. monocytogenes* for adhesion to intestinal epithelial cells, we performed an adhesion assay in which different *Lactobacillus* strains were added to the Caco-2 cell suspension at the same time as *Ls. monocytogenes*. *Lactobacillus* and *Ls. monocytogenes* were allowed to compete for cell adhesion at a ratio of 1:1. Representative dot plots of the HI red fluorescence

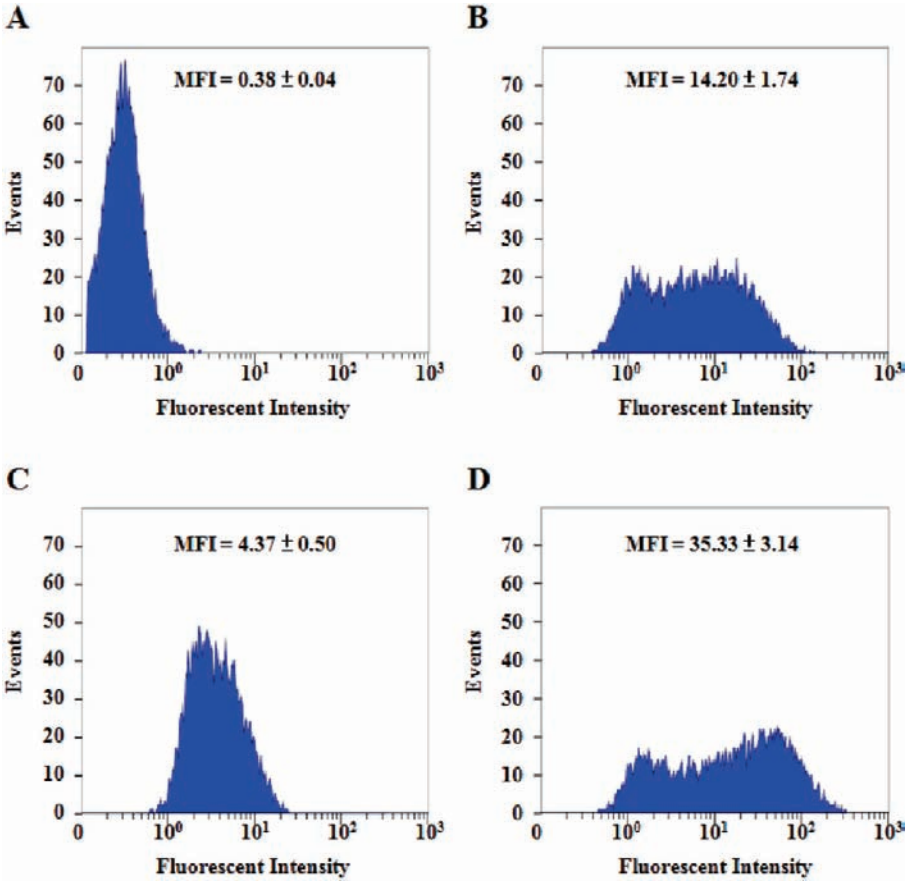


**Figure 4.** Flow cytometric analysis of *Lactobacillus* adherence to Caco-2 cells: (**A**) autofluorescence of Caco-2 cells; (**B**) Caco-2 cells exposed to fluorescently labeled *Lb. reuteri* Pg4; (**C**) Caco-2 cells exposed to fluorescently labeled *Lb. casei* ATCC 393; (**D**) Caco-2 cells exposed to fluorescently labeled *Lb. casei* pNZ-cnb. Bacterial cells were labeled with CFSE and incubated with Caco-2 cells for 2 h. For each experiment, 10000 Caco-2 cells were analyzed. Mean green fluorescence intensity (MFI) was calculated from two independent experiments performed in triplicate.

versus the CFSE green fluorescence profile of Caco-2 cells adhered with CFSE-labeled *Lactobacillus* and/or HI-labeled *Ls. monocytogenes* are shown in **Figure 5A**. The pathogenic strain of *Ls. monocytogenes* was found to adhere to Caco-2 cells efficiently. In the presence of *Lb. casei* pNZ-cnb, however, the adherence of *Ls. monocytogenes* to Caco-2 cells was significantly lower than that seen in the other groups. To substantiate the findings of the dot-plot profile analysis, the mean green fluorescence intensity and the mean red fluorescence intensity of the Caco-2 cells adhered with *Lactobacillus* and/or *Ls. monocytogenes* were calculated and are shown in **Figure 5**, panels **B** and **C**.

Caco-2 cells incubated with HI-stained *Ls. monocytogenes* exhibited a mean red fluorescence intensity of 9.61 ± 1.50, which was significantly higher than the mean red fluorescence intensity of Caco-2 cells alone (0.29 ± 0.08) ($p < 0.05$) (**Figure 5C**). In addition, there was no significant difference in mean red fluorescence intensity between Caco-2 cells with adherent *Ls. monocytogenes* in the presence or absence of *Lb. casei* ATCC 393 (9.50 ± 0.70 versus 9.61 ± 1.50) ($p > 0.05$). In the presence of *Lb. casei* pNZ-cnb, the mean red fluorescence intensity of Caco-2 cells adhered with *Ls. monocytogenes* was only 1.58 ± 0.44, which was significantly lower than the intensity of Caco-2 cells adhered with *Ls. monocytogenes* in the presence of *Lb. casei* ATCC 393 or *Lb. reuteri* Pg4 ($p < 0.05$). These results indicated that introduction of the *cnb* gene into *Lb. casei* ATCC 393 could improve its inhibition ability against the adhesion of *Ls. monocytogenes* to Caco-2 cells.

**Competition between *Lactobacillus* and *E. coli* O157:H7 for Adhesion to the Caco-2 Cells.** Representative dot plots of HI red fluorescence versus SYTO 9 green fluorescence profile of Caco-2 cells adhered with HI-labeled *Lactobacillus* and/or SYTO 9-labeled *E. coli* O157:H7 are shown in **Figure 6A**. *E. coli* O157:H7 was found to adhere to Caco-2 cells efficiently. In the presence of *Lb. casei* pNZ-cnb, the adherence of *E. coli* O157:H7 to Caco-2 cells was significantly lower than that seen in the other groups (**Figure 6A**). The mean green fluorescence intensity of Caco-2 cells incubated with SYTO 9-stained *E. coli* O157:H7 was significantly higher than that of Caco-2 cells alone (197.33 ± 19.86 versus 0.45 ± 0.07) ($p < 0.05$), indicating that *E. coli* O157:H7 could efficiently adhere to Caco-2 cells (**Figure 6B**). In the competition assays, the mean green fluorescence intensity of Caco-2 cells adhered by *E. coli* O157:H7 in the presence of *Lb. casei* pNZ-cnb was only 2.14 ± 0.45, which was significantly lower than the intensity of Caco-2 cells adhered by *E. coli* O157:H7 in the presence of *Lb. casei* ATCC 393 or *Lb. reuteri* Pg4 ($p < 0.05$). These results indicated that *Lb. casei* pNZ-cnb could decrease the adhesion of *E. coli* O157:H7 to Caco-2 cells.

## DISCUSSION

Successful establishment of infection by pathogenic bacteria requires adhesion to eukaryotic host cells, colonization of tissues, and, in certain cases, cellular invasion (*28*). Most pathogenic bacteria interacting with eukaryotic hosts express adhesive molecules on their surfaces that promote interaction with host cell receptors or with soluble macromolecules (*29*). Although it is believed that the maximum probiotic effect is achieved when probiotic lactobacilli adhere to intestinal mucosal cells (*30*), less is known about the surface molecules mediating *Lactobacillus* adhesion to the intestinal mucosa (i.e., epithelial cells, mucus

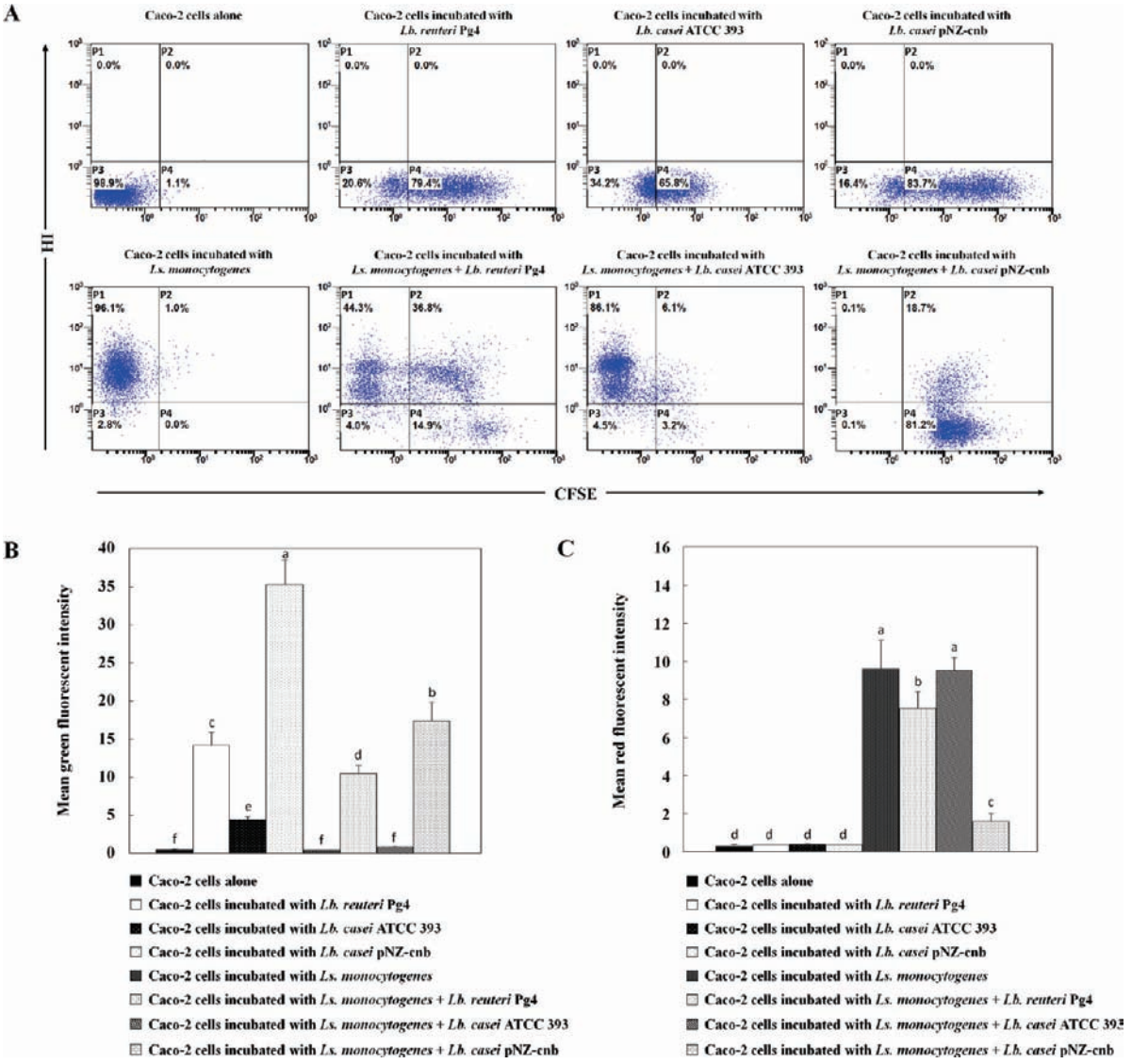


**Figure 5.** Flow cytometric analysis of adherence of CFSE-labeled *Lactobacillus* and/or HI-labeled *Ls. monocytogenes* to Caco-2 cells: (**A**) representative dot plots of HI red fluorescence versus CFSE green fluorescence of Caco-2 cells adhered with fluorescence-labeled bacteria (the percentage of cells in each quadrant is indicated); (**B**) mean green fluorescence intensity and (**C**) mean red fluorescence intensity of Caco-2 cells adhered with fluorescence-labeled bacteria [bars with different letters indicate significant difference ($p < 0.05$)].

layer, or extracellular matrices) and their corresponding receptors than about the surface molecules mediating adhesion of pathogenic bacteria (*31*). Several exported proteins produced by *Lactobacillus* have been described and characterized during recent years. It has been shown that some are able to bind to epithelial components such as mucin or extracellular matrix proteins, including fibronectin, collagen, and laminin (*32*). *Lactobacillus* strains that possess the ability to adhere to intestinal epithelial cells and/or the extracellular matrices can compete with pathogenic bacteria for the same receptors and occupy the potential binding sites in the gut (*31*). The collagen-binding protein, Cnb, is one of the reported extracellular matrix adhesion proteins produced by *Lb. reuteri* (*12*). Previous studies have demonstrated that the homologues of Cnb mediate *Lb. reuteri* adhesion to the intestinal epithelial cells and mucin (*17*, *26*). To the best of our knowledge, however, our finding that heterologous expression of the *cnb* gene in a low-adhesive *Lactobacillus* strain could increase its adhesion ability has not been reported previously. In the present study, the *cnb* gene was cloned from an adhesive *Lb. reuteri* strain Pg4 and heterologously expressed in the low-adhesive *Lb. casei* strain ATCC 393. The transformed strain *Lb. casei* pNZ-cnb not only was demonstrated to be able to display Cnb on its cell surface (**Figures 2** and **3**) but also showed greater adhesion ability to Caco-2 cells than the parental *Lb. casei* ATCC 393 (**Figure 4**). It has been reported that adherence of *Lb. casei* cells on the surface of Caco-2 cells could stimulate the up-regulation of mucin expression in Caco-2 cells, resulting in inhibition of pathogenic bacteria translocation (*33*). Further investigation is required to verify the colonization of *Lb. casei* pNZ-cnb in vivo and its interaction with the host and the microorganisms present in the host system.

*Lb. casei* ATCC 393 showed weak ability to compete with *Ls. monocytogenes* in adhering to Caco-2 cells. Introduction of the *cnb* gene into the *Lb. casei* ATCC 393 cells resulted in an increase in its adhesion ability to Caco-2 cells and improvement of its ability to compete with *Ls. monocytogenes* in adhering to Caco-2 cells (**Figure 5**). *Ls. monocytogenes* is a facultative intracellular Gram-positive bacterium that is responsible for foodborne illnesses and may lead to encephalitis, meningitis, stillbirths, and infection of the central nervous system in newborn or immunocompromised patients (*34*). The virulence of *Ls. monocytogenes* stems from its capacity to adhere, invade, and multiply within

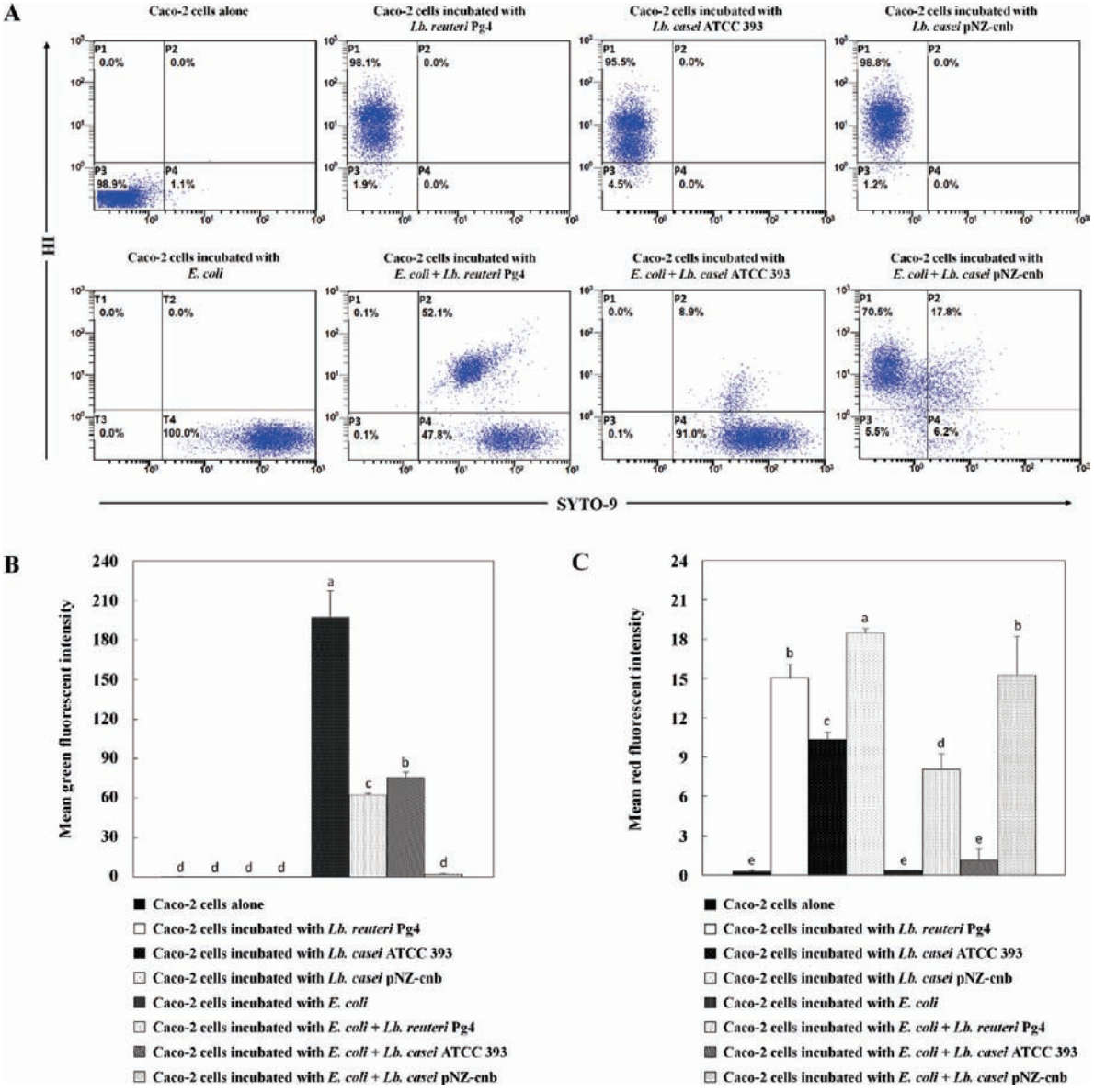


**Figure 6.** Flow cytometric analysis of adherence of HI-labeled *Lactobacillus* and/or SYTO 9-labeled *E. coli* to Caco-2 cells: (**A**) representative dot plots of HI red fluorescence versus SYTO 9 green fluorescence of Caco-2 cells adhered with fluorescence-labeled bacteria (the percentage of cells in each quadrant is indicated); (**B**) mean green fluorescence intensity and (**C**) mean red fluorescence intensity of Caco-2 cells adhered with fluorescence-labeled bacteria [bars with different letters indicate significant difference ($p < 0.05$)].

professional and nonprofessional phagocytes. Several listerial surface proteins are involved in these processes. Sequence comparisons with previously characterized proteins suggest that 12 listerial surface proteins are predicted to have a mucin-binding domain (*35*). It is known that mucin was constitutively expressed in Caco-2 cells (*36*). Therefore, the strong adhesion of *Ls. monocytogenes* to Caco-2 cells observed in this study may be attributable, in part, to listerial mucin-binding proteins. As Cnb can bind to mucin efficiently (*17*) and competes with listerial mucin-binding proteins for the same mucin receptor proteins, it is reasonable to assume that the introduction of the *cnb* gene into *Lb. casei* ATCC 393 cells could improve its competition with *Ls. monocytogenes* in adhering to Caco-2 cells.

*Lb. casei* pNZ-cnb also showed great ability to compete with *E. coli* O157:H7 in adhering to Caco-2 cells (**Figure 6**). *E. coli* O157:H7 is an enterohemorrhagic *E. coli* (EHEC) strain and causes bloody diarrhea that can progress to a fatal illness due to acute kidney failure (hemolytic uremic syndrome), particularly in children (*37*). EHEC produces a distinct histopathological lesion on intestinal epithelial cells known as the attaching and effacing (A/E) lesion, the formation of which is mediated by the EHEC outer membrane protein intimin with its translocated receptor Tir (*38*). Although EHEC also has other adhesin molecules such as pili, fimbria, Efa-1, Iha, Cah, and OmpA that allow it to adhere to host-cell surfaces (*38*), no gene encoding a putative collagen-binding protein was found in the genome of *E. coli* O157:H7 (*39*, *40*). Because the receptors for Cnb and *E. coli* adhesins were different, the inhibitory effects of *Lb. casei* pNZ-cnb on *E. coli* O157:H7 adhesion to Caco-2 cells may be attributable, in part, to the nonspecific steric hindrance of pathogen binding receptors on the Caco-2 cell surface by *Lb. casei* pNZ-cnb.

Probiotic lactobacilli are normally selected empirically from native isolates. In recent years, there has been interest in the development of methods to induce the expression of heterologous proteins in *Lactobacillus* to improve its efficiency. Examples of such potential applications of genetically modified *Lactobacillus* include strains that produce antibodies for use as recombinant live vaccines, enzymes for detoxification and fiber degradation,

and cytokines for immune intervention (*41*). *Lb. casei* ATCC 393 has been suggested as a carrier for oral vaccines, and its expression vectors have previously been constructed for surface expression or secretion of various antigens (*42*−*44*). Although Xu and Li (*44*) indicated that *Lb. casei* ATCC 393 was able to tolerate bile, survive transit in the upper gastrointestinal tract, and adhere to and colonize intestinal tracts, Edlman et al. (*45*) and Liu et al. (*19*) found that *Lb. casei* ATCC 393 showed poor ability to adhere to intestinal epithelial cells; however, introduction of the *cnb* gene into *Lb. casei* ATCC 393 cells resulted in an increase in its ability to adhere to the intestinal epithelial cells, thereby resulting in an appropriate vector for delivering heterologous antigens for oral vaccines. Future research will be directed toward coexpressing *cnb* and antigen genes in *Lb. casei* ATCC 393 and confirming the immune protection of the transformed *Lb. casei* ATCC 393 strains.

In conclusion, the collagen-binding protein gene *cnb* was cloned from *Lb. reuteri* Pg4 and heterologously expressed in *Lb. casei* ATCC 393. The transformed strain *Lb. casei* pNZ-cnb constitutively expressed Cnb on its cell surface and showed a higher ability to adhere to Caco-2 cells than its parental strain. Furthermore, *Lb. casei* pNZ-cnb also exhibited a higher competition ability against *E. coli* O157:H7 and *Ls. monocytogenes* adhesion to Caco-2 cells.

## ACKNOWLEDGMENT

Portions of this research were carried out at the Joint Center for Instruments and Researches, College of Bioresources and Agriculture, National Taiwan University. The flow cytometry and confocal microscope facilities are supported by the College of Bioresources and Agriculture, National Taiwan University.

**Supporting Information Available:** *Lactobacillus* expression plasmid and SDS-PAGE analysis of recombinant Cnb. This material is available free of charge via the Internet at http://pubs.acs.org.

## LITERATURE CITED

(1) Fuller, R. Probiotic in man and animals. *J. Appl. Bacteriol.* **1989**, *66*, 365−378.

(2) Anadon, A.; Martinez-Larranaga, M. R.; Martinez, M. A. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regul. Toxicol. Pharmacol.* **2006**, *45*, 91−95.

(3) Tannock, G. W.; Tilsala-Timisjarvi, A.; Rodtong, S.; Ng, J.; Munro, K.; Alatossava, T. Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. *Appl. Environ. Microbiol.* **1999**, *65*, 4264−4267.

(4) Patterson, J. A.; Burkholder, K. M. Application of prebiotics and probiotics in poultry production. *Poult. Sci.* **2003**, *82*, 627−631.

(5) Reid, G.; Friendship, R. Alternatives to antibiotic use; probiotics for the gut. *Anim. Biotechnol.* **2002**, *13*, 97−112.

(6) Watkins, B. A.; Kratzer, F. H. Effect of oral dosing of *Lactobacillus* strains on gut colonization and liver biotin on broiler chicks. *Poult. Sci.* **1983**, *62*, 2088−2094.

(7) Watkins, B. A.; Kratzer, F. H. Drinking water treatment with commercial preparation of a concentrated *Lactobacillus* culture for broiler chicks. *Poult. Sci.* **1984**, *63*, 1671−1673.

(8) Jin, L. Z.; Ho, Y. W.; Abdullah, N.; Jalaludin, S. Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Lett. Appl. Microbiol.* **1998**, *27*, 183−185.

(9) Kailasapathy, K.; Chin, J. Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunol. Cell Biol.* **2000**, *78*, 80−88.

(10) Amit-Romach, E.; Sklan, D.; Uni, Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poult. Sci.* **2004**, *83*, 1093−1098.

(11) Roos, S.; Jonsson, H. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiol.* **2002**, *148*, 433−442.

(12) Roos, S.; Aleljung, P.; Robert, N.; Lee, B.; Wadstrom, T.; Lindberg, M.; Jonsson, H. A collagen binding protein from *Lactobacillus reuteri* is part of an ABC transporter system? *FEMS Microbiol. Lett.* **1996**, *144*, 33−38.

(13) Tam, R.; Saier, M. H. Structural, functional, and evolutionary relationships among extracellular solute-binding receptors of bacteria. *Microbiol. Rev.* **1993**, *57*, 320−346.

(14) Hung, J.; Turner, M. S.; Walsh, T.; Giffard, P. M. BspA (CyuC) in Lactobacillus fermentum BR11 is a highly expressed high-affinity L-cystein-binding protein. *Curr. Microbiol.* **2005**, *50*, 33−37.

(15) Turner, M. S.; Timms, P.; Hafner, L. M.; Giffard, P. M. Identification and characterization of a basic cell surface-located protein from *Lactobacillus fermentum* BR11. *J. Bacteriol.* **1997**, *179*, 3310−3316.

(16) Heinemann, C.; van Hylckama Vlieg, J. E. T.; Janssen, D. B.; Busscher, H. J.; van der Mei, H. C.; Reid, G. Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiol. Lett.* **2000**, *190*, 177−180.

(17) Rojas, M.; Ascencio, F.; Conway, P. L. Purification and characterization of a surface protein from *Lactobacillus fermentum* 104R that binds to porcine small intestine mucus and gastric musin. *Appl. Environ. Microbiol.* **2002**, *68*, 2330−2336.

(18) Turner, M. S.; Hafner, L. M.; Walsh, T.; Giffard, P. M. Peptide surface display and secretion using two LPXTG-containing surface proteins from *Lactobacillus fermentum* BR11. *Appl. Environ. Microbiol.* **2003**, *69*, 5855−5863.

(19) Liu, J. R.; Yu, B.; Liu, F. H.; Cheng, K. J.; Zhao, X. Expression of rumen microbial fibrolytic enzyme genes in probiotic *Lactobacillus reuteri*. *Appl. Environ. Microbiol.* **2005**, *71*, 6769−6775.

(20) van Rooijen, J. R.; Gasson, M. J.; de Vos, W. M. Characterization of the *Lactococcus lactis* lactose operon promoter: contribution of flanking sequences and LacR repressor to promoter activity. *J. Bacteriaol.* **1992**, *174*, 2273−2280.

(21) Sambrook, J.; Russell, D. W. *Molecular Cloning: a Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.

(22) Serror, P.; Sasaki, T.; Ehrlich, S. D.; Maguin, E. Electrotransformation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* with various plasmids. *Appl. Environ. Microbiol.* **2002**, *68*, 46−52.

(23) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(24) Logan, R. P. H.; Robins, A.; Turner, G. A.; Cockayne, A.; Borriello, S. P.; Hawkey, C. J. A novel flow cytometric assay for quantitating adherence of *Helicobacter pylori* to gastric epithelial cells. *J. Immunol. Methods* **1998**, *213*, 19−30.

(25) Montgomery, D. C. *Design and Analysis of Experiments*; Wiley: New York, 1991.

(26) Miyoshi, Y.; Okada, S.; Chimura, T.; Satoh, E. A mucus adhesion promoting protein, MapA, mediates the adhesion of *Lactobacillus reuteri* to Caco-2 human intestinal epithelial cells. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 1622−1628.

(27) Weston, S. A.; Parish, C. R. New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. *J. Immunol. Methods* **1990**, *133*, 87−97.

(28) Pizarro-Cerda, J.; Cossart, P. Bacterial adhesion and entry into host cells. *Cell* **2006**, *124*, 715−727.

(29) Kline, K. A.; Flker, S.; Dahlberg, S.; Normark, S.; Henriques-Normark, B. Bacterial adhesions in host−microbe interactions. *Cell Hosts Microbe* **2009**, *5*, 580−592.

(30) Servin, A. L.; Coconnier, M. H. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. *Best Pract. Res. Clin. Gastroenterol.* **2003**, *17*, 741−754.

(31) Velez, M. P.; de Keersmaecker, S. C. J.; Vanderleyden, J. Adherence factors of *Lactobacillus* in the human gastrointestinal tract. *FEMS Microbiol. Lett.* **2007**, *276*, 140−148.

(32) Sanchez, B.; Bressollier, P.; Urdaci, M. C. Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and

molecular cross-talking with the host. *FEMS Immunol. Med. Microbiol.* **2008**, *54*, 1−17.
(33) Mattar, A. F.; Teitelbaum, D. H.; Drongowski, R. A.; Yongyi, F.; Harmon, C. M.; Coran, A. G. Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. *Pediatr. Surg. Int.* **2002**, *18*, 586−590.
(34) Jaradat, Z. W.; Bhunia, A. K. Adhesion, invasion, and translocation characteristics of *Listeria monocytogenes* serotypes in Caco-2 cell and mouse models. *Appl. Environ. Microbiol.* **2003**, *69*, 3640−3645.
(35) Bierne, H.; Cossart, P. *Listeria monocytogenes* surface proteins: from genome predictions to function. *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 377−397.
(36) Gottke, M. U.; Keller, K.; Belley, A.; Garcia, R. M.; Hollingsworth, M. A.; Mack, D. R.; Chadee, K. Functional heterogeneity of colonic adenocarcinoma mucins for inhibition of *Entamoeba histolytica* adherence to target cells. *J. Eukaryot. Microbiol.* **1998**, *45*, 17S−23S.
(37) Nataro, J. P.; Kaper, J. B. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **1998**, *11*, 142−201.
(38) Torres, A. G.; Zhou, Z.; Kaper, J. B. Adherence of diarrheagenic *Escherichia coli* strains to epithelial cells. *Infect. Immun.* **2005**, *73*, 18−29.
(39) Hayashi, T.; Makino, K.; Ohnishi, M.; Kurokawa, K.; Ishii, K.; Yokoyama, K.; Han, C. G.; Ohtsubo, E.; Nakayama, K.; Murata, T.; Tanaka, M.; Tobe, T.; Iida, T.; Takami, H.; Honda, T.; Sasakawa, C.; Ogasawara, N.; Yasunaga, T.; Kuhara, S.; Shiba, T.; Hattori, M.; Shinagawa, H. Complete genome sequence of enterohemorrhagi *Eschelichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res.* **2001**, *8*, 11−22.
(40) Kulasekara, B. R.; Jocobs, M.; Zhou, Y.; Wu, Z.; Sims, E.; Saenphimmachak, C.; Rohmer, L.; Ritchie, J. M.; Radey, M.; McKevitt, M.; Freeman, T. L.; Hayden, H.; Haugen, E.; Gillett, W.; Fong, C.; Chang, J.; Beskhlebnaya, V.; Waldor, M. K.; Samadpour, M.; Whittam, T. S.; Kaul, R.; Brittnacher, M.; Miller, S. I. Analysis of the genome of the *Escherichia coli* O157:H7 2006 spinach-associated outbreak isolate indicates candidate genes that may enhance virulence. *Infect. Immun.* **2009**, *77*, 3713−3721.
(41) Steidler, L. Genetically engineered probiotics. *Best Pract. Res. Clin. Gastroenterol.* **2003**, *17*, 861−876.
(42) Kruger, C.; Hu, Y.; Pan, Q.; Marcotte, H.; Hultberg, A.; Delwar, D.; van Dalen, P. J.; Pouwels, P. H.; Leer, R. J.; Kelly, C. G.; van Dollenweerd, C.; Ma, J. K.; Hammarstrom, L. *In situ* delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat. Biotechnol.* **2002**, *20*, 702−706.
(43) Xu, Y.; Li, Y. Construction of recombinant *Lactobacillus casei* efficiently surface displayed and secreted porcine parvovius VP2 protein and comparison of the immune responses induced by oral immunization. *Immunology* **2007**, *124*, 68−75.
(44) Xu, Y.; Li, Y. Induction of immune responses in mice after intragastric administration of *Lactobacillus casei* producing porcine parvovius VP2 protein. *Appl. Environ. Microbiol.* **2007**, *73*, 7041−7047.
(45) Edelman, S.; Westerlund-Wikstrom, B.; Leskela, S.; Kettunen, H.; Rautonen, N.; Apajalahti, N.; Korhonen, T. K. *In vitro* adhesion specificity of indigenous lactobacilli within the avian intestinal tract. *Appl. Environ. Microbiol.* **2002**, *68*, 5155−5159.

**Received for review September 15, 2010. Revised manuscript received November 1, 2010. Accepted November 2, 2010. This research was conducted using funds partially provided by grants from the National Science Council NSC 99-2918-I-002-018, Republic of China.**