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Display of *Fibrobacter succinogenes* β-Glucanase on the Cell Surface of *Lactobacillus reuteri*

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S *Supporting Information*

ABSTRACT: The aim of this study was to display a rumen bacterial β-glucanase on the cell surface of a probiotic *Lactobacillus reuteri* strain. The β-glucan degrading ability and the adhesion capability of the genetically modified strain were evaluated. The β-glucanase (Glu) from *Fibrobacter succinogenes* was fused to the C-terminus of collagen-binding protein (Cnb) from *L. reuteri* and then expressed by *L. reuteri* Pg4 as a recombinant Cnb−Glu−His_6 fusion protein. Confocal immunofluorescence microscopy and flow cytometric analysis of the transformed strain *L. reuteri* pNZ-cnb/glu demonstrated that Cnb−Glu−His_6 fusion protein was displayed on its cell surface. In addition, *L. reuteri* pNZ-cnb/glu acquired the capacity to break down barley β-glucan and showed higher adhesion capability, in comparison with the parental strain *L. reuteri* Pg4. To the best of the authors' knowledge, this is the first report of successful display of fibrolytic enzymes on the cell surface of intestinal lactobacilli.

KEYWORDS: cell-surface display, *Lactobacillus reuteri*, *Fibrobacter succinogenes*, β-glucanase

INTRODUCTION

Although, in many areas of the world, the majority of monogastric animal diets are corn−soybean based, barley remains the major feed grains in certain regions of the United States and in European countries. The endosperm cell wall of barley contains a high proportion of indigestible fiber known as nonstarch polysaccharide (NSP). The NSP in barley is mainly β-glucan, which has large proportions of β-1,4 or β-1,3 glucosidic linkages. Animals, such as poultry, do not synthesize the enzyme β-glucanase to degrade this structural polysaccharide. Thus, the undigested β-glucan is often problematic for monogastric animals fed such a barley-based diet, due to its low digestibility and viscousness, which results in a reduced of digesta passage rate, decreased digestive enzyme diffusion, endogenous losses, and stimulation of bacterial proliferation.[1,2] In addition, the sticky droppings also reportedly create management problems in poultry farming.[3] Therefore, it is not surprising that addition of β-glucanase into barley-based diets for monogastric animals decreases viscosity and consequently reduces the antinutritional effect of β-glucan, leading to better production performance.[4] However, enzyme supplementation substantially increases feed cost. As lactobacilli possess the mucosal surface-colonizing property and have the potential to express proteins at specific mucosal sites, an alternative and less expensive strategy might be to develop lactobacilli with the capacity to digest plant cell-wall structural carbohydrates by introduction of heterologous genes encoding fibrolytic enzymes.[5] Previous studies demonstrated that probiotic strains of *Lactobacillus* could acquire the capacity to break down β-glucan via introduction of a heterologous β-glucanase gene into their cells,[6,7] and these transformed *Lactobacillus* strains could survive and secrete β-glucanase in the broiler gastrointestinal tract, decrease digesta viscosity, and enhance weight gain in birds fed a barley-based diet.[8]

In recent years, there has been interest in the development of methods for expression of heterologous proteins on the cell surface of lactobacilli to improve their efficiency. For the most part, surface display systems using lactobacilli have been investigated as recombinant mucosal vaccines, because being able to adhere to and colonize the intestinal tract is important and desirable for a vaccine delivery system.[9,10] Protein surface display systems using lactobacilli can be divided into two classes, covalent and noncovalent anchoring systems. The covalent anchoring system uses the C-terminal cell-wall anchoring motif LPXTG, whereas noncovalent surface attachment systems utilize either the surface (S)-layer protein from *Lactobacillus acidophilus* or *Lactobacillus brevis* [11] or BspA from *Lactobacillus fermentum*.[9,10] BspA is a surface-located protein from *L. fermentum* BR11.[12] Sequence comparisons show that BspA is a member of solute-binding protein family 3, as defined by Tam and Saier.[13] Closely related homologues of BspA include the collagen binding protein Cnb from *Lactobacillus reuteri* NCIB 11951,[14] a 29 kDa protein from *L. fermentum* RC-14,[15] and MapA from *L. reuteri* 104R.[16] These proteins probably interact with teichoic acids via electrostatic interactions due to their strong cationic properties, which

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are shared with the anchoring domain of *Lactobacillus* S-layer protein subunits.[10] In previous studies, BspA was used as carrier protein to display several heterologous proteins, such as *Streptococcus salivarius* glucosyltransferase, human immunodeficiency virus-derived antigens, and human cystic fibrosis transmembrane regulator protein on cell surfaces of *L. fermentum*.[9,10,17] To the best of our knowledge, however, functional display of a heterologous fibrolytic enzyme on the cell surface of intestinal lactobacilli has not been reported previously.

In the present study, the *Fibrobacter succinogenes* β-glucanase was fused to the C-terminus of *L. reuteri* collagen-binding protein Cnb. The fusion proteins were heterologously expressed and displayed on the cell surface of *L. reuteri* Pg4, which is a probiotic strain isolated from the gastrointestinal tract of a healthy broiler. The heterologous enzyme production and the adhesion capability of this genetically modified *L. reuteri* strain were examined.

■ MATERIALS AND METHODS

Bacterial Strains, Plasmids, Cell Line, and Culture Conditions. *L. reuteri* Pg4[6] was used to prepare genomic DNA for amplification of the collagen-binding protein gene *cnb*. *Lactobacillus* expression vector pNZ3004[18] and *L. reuteri* Pg4 were used for expressing recombinant proteins. All PCR products intended for sequence analysis were cloned into the pGEM-T Easy vector (Promega, Madison, WI). *Escherichia coli* DH5α was used in all subcloning experiments. *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. Agar plates were prepared by adding agar (1.5% w/v) (Difco Laboratories) to the 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 modified Eagle 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 *L. 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.[19] All DNA sequences were determined by an automated sequencing service provided by Mission Biotech Inc. (Taipei, Taiwan).

Construction of Plasmids for Cell-Surface Display. The *cnb* gene of *L. reuteri* Pg4 was amplified by PCR using the oligonucleotide forward primer cnbF 5′ ACGCGTCGACGAAATTTTGGAAGAAAGCAC 3′ and the oligonucleotide reverse primer cnbR 5′ CCAATGCATTCAGTGGTGGTGGTGGTGGTGCTCGAGTGCGG CCGCTTCAGTAATA 3′, designed based on the Cnb gene of *L. reuteri* NCIB 11951 (GenBank accession no. X99716) as described by Roos et al.[14] These two primers were designed to place a *Sal*I site at the 5′ end and a *Not*I−*Xho*I−His_6−*Nsi*I site at the 3′ end of the PCR product, respectively (the underlined sequences in the primers). 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, which was used to transform *E. coli* DH5α competent cells and sequenced to ensure that there were no errors introduced by PCR.

The DNA sequence encoding the catalytic domain of *F. succinogenes* β-glucanase (1,3/1,4-β-D-glucan 4-glucanohydrolase, GenBank accession no. M33676) was amplified by PCR from pNZ-glu[6] using the oligonucleotide forward primer gluF 5′ ATAAGAATGCGGCCGCAGTTAGCGCAAAG 3′ and the oligonucleotide reverse primer gluR 5′ CGCTCGAGCGATTGCGGAGCAGG 3′. These two primers were designed to place a *Not*I site at the 5′ end and a *Xho*I site at the 3′ end of the PCR product, respectively (the underlined sequences in the primers). The PCR fragments encoding β-glucanase were digested with *Not*I and *Xho*I and ligated with *Not*I−*Xho*I-digested pNZ-cnb to generate the expression plasmid pNZ-cnb/glu, which was used to transform *E. coli* DH5α competent cells and sequenced to ensure that there were no errors introduced by PCR.

Transformation of Plasmid DNA. The plasmids pNZ-glu, pNZ3004, pNZ-cnb, and pNZ-cnb/glu were electroporated into *L. reuteri* Pg4 as described by Serror et al.[20] Subsequent to electroporation, the *L. reuteri* Pg4 transformants (designated *L. reuteri* pNZ3004, *L. reuteri* pNZ-glu, *L. reuteri* pNZ-cnb, and *L. reuteri* pNZ-cnb/glu, respectively) were incubated in MRS broth containing $MgCl_2$ (10 mM) at 37 °C for 3 h with the transformants subsequently spread on MRS agar plates containing erythromycin (10 μg/mL, Sigma-Aldrich Co.) and incubated at 37 °C until the transformant colonies appeared. The transformants were confirmed by direct colony PCR using two primer sets of gluF/gluR and cnbF/gluR, confocal immunofluorescence microscopy, and flow cytometric analysis. Finally, the β-glucanase activities of the transformants were analyzed.

Confocal Immunofluorescence Microscopic Analysis. *L. reuteri* Pg4 and its transformed strains were transferred to MRS broth and incubated statically at 37 °C for 24 h. The cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C. The cell pellet was resuspended in 1 mL of 100 mM phosphate-buffered saline (PBS; pH 7.4) containing 10 g/L bovine serum albumin and incubated at room temperature for 30 min. The bacterial cells were then incubated with mouse monoclonal anti-His_6 antibody (diluted 1:1000 in PBS, GE Healthcare, Piscataway, NJ) at room temperature for 1.5 h. Following this, the bacterial cells were incubated with the secondary goat anti-mouse IgG heavy and light chain antibody conjugated with Alexa Fluor 488 (diluted 1:2500 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 resuspended in PBS. 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 (Leica Microsystems) and an argon laser (458, 476, 488, 514 nm) light source.

Flow Cytometric Analysis. *Lactobacillus* cells were immunostained as described above. Flow cytometric analysis was performed using 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/s. The data obtained from the flow cytometry were analyzed using CXP software (Beckman Coulter, Inc.), with 18000 counts analyzed in each experiment. Counts were made in triplicate for each procedure.

***β*-Glucanase Production by *L. reuteri* Strains.** For estimation of enzyme activity, 10 mL of MRS broth was inoculated with 0.1 mL (for a 1% suspension) of an overnight culture of *L. reuteri* Pg4 or its transformed strains and was incubated statically at 37 °C for 24 h. After centrifugation at 5000*g* for 20 min at 4 °C, the extracellular supernatant and cell pellet were harvested separately. The cell pellet was resuspended in 1 mL of 100 mM PBS (pH 7.4), sonicated for 10 min with an ultrasonicator (model XL, Misonix, Farmingdale, NY), and fractioned into intracellular extract and cell-wall fractions by subsequent centrifugation at 13000*g* for 20 min at 4 °C. β-Glucanase activities of the extracellular

supernatant, intracellular extract, and cell-wall fraction of each *Lactobacillus* cell culture were analyzed using the Congo red plate method described by Teather and Wood[21] and by measuring the amounts of reducing sugar released from 0.5% (w/v) barley β-glucan (Megazyme International, Wicklow, Ireland) in 50 mM sodium citrate buffer (pH 5.0) using the dinitrosalicyclic acid (DNS) reagent method as described by Liu et al.[6] One unit of β-glucanase activity was defined as that releasing 1 μmol of reducing sugar/min from β-glucan under the assay conditions. Specific activity was expressed as units per milligram of protein. Total protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) against a standard curve of bovine serum albumin (Sigma-Aldrich Co.).

Gel Electrophoresis and Zymographic Analysis. A sensitive zymography technique modified from that of Beguin[22] was used to identify β-glucanase using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). In brief, the intracellular extract and cell-wall fraction of each *L. reuteri* cell culture were electrophoretically separated using 12.5% SDS-PAGE containing 0.1% β-glucan. After electrophoresis, the gels were washed three times for 30 min at 4 °C in 100 mM acetate buffer (pH 5.0) containing 25% isopropanol to remove the SDS and then incubated in the acetate buffer for 40 min at 40 °C. Then, the gels were washed in distilled water three times to remove the acetate, stained in 0.1% Congo red solution for 15 min at room temperature, and destained with 1 M NaCl to enable visualization of regions of enzyme activity within the gel. Light yellow bands indicating degradation of β-glucan were visible on a deep red background.

Growth Characteristics of *L. reuteri* Strains. For the analysis of growth characteristics, 10 mL of MRS broth or erythromycin-containing MRS broth was, respectively, inoculated at 1% with an overnight culture of *L. reuteri* Pg4 or its transformed strains and incubated statically at 37 °C for 48 h. During the incubation period, samples were taken every 4 h for counting the cell numbers using the standard agar plate method and measuring turbidity at 600 nm (OD_{600}).

In Vitro Adhesion Assay. Before adhesion 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 PBS. The Caco-2 cells were then incubated with mouse anti-human CD29 (integrin β 1) monoclonal antibody conjugated with PerCP-eFluor 710 (diluted 1:10 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 1×10^6 cells/mL in PBS.

Lactobacilli cells were cultivated, harvested, and resuspended in 1 mL of 100 mM PBS (pH 7.4) as described above and were then labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma-Aldrich Co.) as described by Logan et al.[23] Fluorescently labeled lactobacilli were added into the Caco-2 suspension to yield a final concentration of 10^8 CFU/mL of lactobacilli 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 lactobacilli. The fluorescent intensity of the Caco-2 cells with adherent lactobacilli was measured using the Cytomics FC500 Flow Cytometry System (Beckman Coulter, Inc.) with a 488 nm argon laser and a 525 nm emission filter for CFSE detection and a 755 nm emission filter for PerCP-eFluor 710 detection. With use of a dot plot display of forward scatter (FSC) versus side scatter (SSC), flow cytometry was gated to include Caco-2 cells and to exclude cellular debris and nonadherent bacteria (Supporting Information Figure S1). Cells inside the gated area were confirmed by staining with CD29 antibody conjugated with PerCP-eFluor 710. For each analysis, 10000 events were acquired, and the flow cytometric data obtained were analyzed using 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.

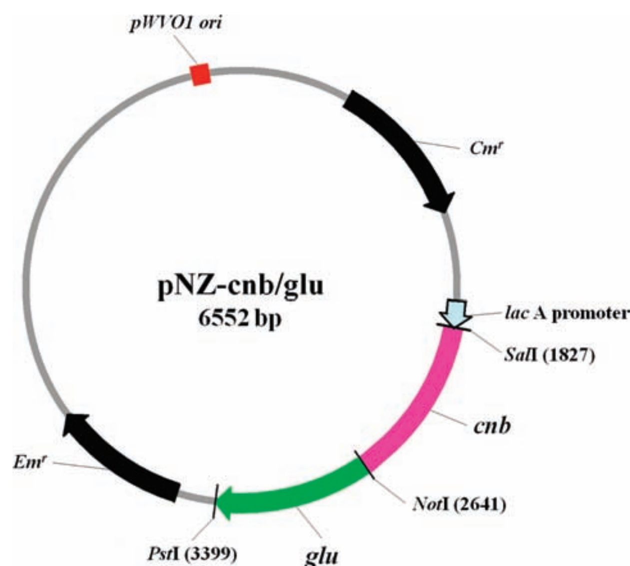

Figure 1. *Lactobacillus* expression plasmid harboring *Lactobacillus reuteri* collagen-binding protein gene, *cnb*, and *Fibrobacter succinogenes* β-glucanase gene, *glu*.

Statistical Analysis. All results were analyzed using the general linear model procedure available with Statistical Analysis System software version 8.1 (SAS Institute Inc., Cary, NC). Duncan's multiple-range test[24] was used to detect differences between treatment means. Each experiment was conducted in triplicate and repeated three times.

RESULTS

Construction of Cell-Surface Expression Plasmids. To construct a lactobacilli cell-surface display vector, the DNA fragments encoding Cnb of *L. reuteri* Pg4 were amplified by PCR and cloned into the *Lactobacillus* expression vector pNZ3004, generating pNZ-cnb. To further display β-glucanase on the cell surfaces of the lactobacilli, the DNA fragments encoding β-glucanase of *F. succinogenes* were subcloned into pNZ-cnb, generating pNZ-cnb/glu (Figure 1). The recombinant Cnb−His_6 and Cnb−Glu−His_6 fusion proteins were expected to be constitutively expressed under the control of the *Lactococcus lactis lac*A promoter and to electrostatically interact with teichoic acids[10] and be displayed on the cell surfaces of the lactobacilli.

Heterologous Expression of *β*-Glucanase in *L. reuteri* Pg4. The expression plasmids pNZ-glu, pNZ-cnb, and pNZ-cnb/glu were used to electroporate into *L. reuteri* Pg4 with efficiency similar to that of pNZ3004 ((5−10) $\times 10^2$ transformants/μg of DNA). The presence of the *glu* or *cnb-glu* genes in the *L. reuteri* transformants was demonstrated by direct colony PCR (results not shown). The expression of β-glucanase in *L. reuteri* Pg4 and its transformed strains was further confirmed using the Congo red plate assay. *L. reuteri* Pg4, *L. reuteri* pNZ3004, and *L. reuteri* pNZ-cnb were devoid of β-glucanase activity (Figure 2A). The intracellular extract of *L. reuteri* pNZ-glu and *L. reuteri* pNZ-cnb/glu produced yellow halo zones on the β-glucan-containing plates, indicating that β-glucanase was functionally expressed in the cytoplasm of these two *L. reuteri* transformed strains. In *L. reuteri* pNZ-glu, the extracellular supernatant produced a larger degradation zone on the

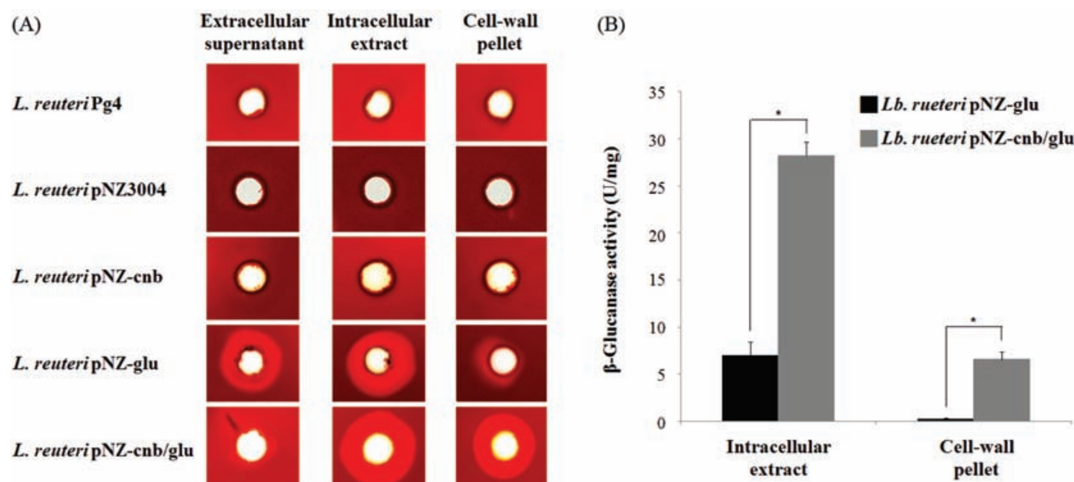

Figure 2. Plate test for β-glucanase activity of *Lactobacillus reuteri* Pg4 and its transformed strains. (A) Extracellular supernatant, intracellular extract, and cell-wall pellet fractions of each *L. reuteri* strain were inoculated onto agar plates containing barley β-glucan, incubated, and stained with Congo red. (B) Specific activities of intracellular extract and cell-wall pellet fractions of *L. reuteri* pNZ-glu and *L. reuteri* pNZ-cnb/glu were determined. Evidence of enzyme activity was defined as the release of 1 μmol of reducing sugar equivalents per minute from barley β-glucan. The bars represent standard errors of the means calculated from three independent experiments performed in triplicate. Pairs of bars marked with asterisks were significantly different ($P < 0.05$).

β-glucan-containing plate than the cell-wall fraction did, indicating that the recombinant β-glucanase was not cell-wall associated but secreted into the extracellular medium. In contrast, the cell-wall fraction of *L. reuteri* pNZ-cnb/glu produced a larger clear halo on the β-glucan-containing plate than its extracellular supernatant did, indicating the Cnb−Glu−His_6 fusion proteins were not secreted into the extracellular medium but mainly present in the intracellular extract and cell-wall fraction of *L. reuteri* pNZ-cnb/glu (Figure 2A). To substantiate the findings of the Congo red plate assay, β-glucanase activities were demonstrated by determining the specific activities of the intracellular extracts and cell-wall fractions of *L. reuteri* pNZ-glu and *L. reuteri* pNZ-cnb/glu. The results obtained clearly matched the findings obtained in the plate assays (Figure 2B). The β-glucanase activities of the intracellular extract and cell-wall fraction of *L. reuteri* pNZ-cnb/glu were 28.2 ± 1.4 and 6.6 ± 0.8 U/mg, respectively, which were significantly higher than those of *L. reuteri* pNZ-glu (7.0 ± 1.4 and 0.2 ± 0.1 U/mg, respectively, $P < 0.05$).

The β-glucanase activities of *L. reuteri* Pg4 and its transformed strains were further confirmed by zymography. The respective molecular sizes of the Cnb and Glu proteins were approximately 29 and 28 kDa, and that of the Cnb−Glu−His_6 fusion protein was, therefore, approximately 57 kDa. In the zymographic analysis of β-glucan-containing SDS-PAGE gels, the intracellular extract and cell-wall fraction from *L. reuteri* pNZ-glu revealed β-glucanase activity bands of about 29- kDa (Figure 3, lanes 5 and 6), whereas the analogue of *L. reuteri* pNZ-cnb/glu revealed β-glucanase activity bands of about 57 kDa (Figure 3, lanes 7 and 8). These results demonstrate that the Cnb−Glu−His_6 fusion proteins were successfully expressed by *L. reuteri* pNZ-cnb/glu and presented in association with its cell wall.

Confocal Immunofluorescence Microscopic and Flow Cytometric Analysis of *L. reuteri* pNZ-cnb/glu. Immunofluorescence labeling of the lactobacilli cells was performed using mouse anti-His_6 antibody as the primary antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG as the secondary antibody. As shown in Figure 4, confocal immunofluorescence microscopy demonstrated that *L. reuteri* pNZ-cnb and *L. reuteri* pNZ-cnb/glu

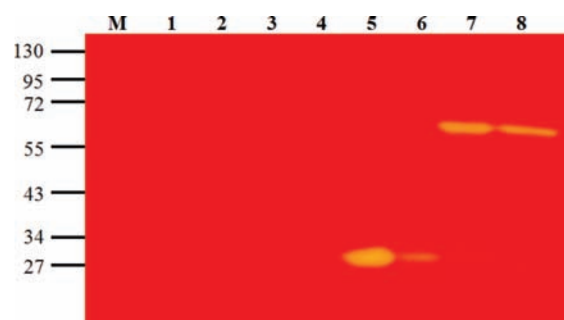

Figure 3. Zymography of β-glucanase in SDS-PAGE gels. The intracellular extracts and cell-wall pellet fractions of *Lactobacillus reuteri* transformants were analyzed using glucan-containing SDS-PAGE, renatured, and then zymographed. Lanes: M, molecular weight marker; 1, intracellular extract of *L. reuteri* Pg4; 2, cell-wall pellet of *L. reuteri* Pg4; 3, intracellular extract of *L. reuteri* pNZ-cnb; 4, cell-wall pellet of *L. reuteri* pNZ-cnb; 5, intracellular extract of *L. reuteri* pNZ-glu; 6, cell-wall pellet of *L. reuteri* pNZ-glu; 7, intracellular extract of *L. reuteri* pNZ-cnb/glu; 8, cell-wall pellet of *L. reuteri* pNZ-cnb/glu (2 μg of protein in each lane).

cells were immunostained, whereas *L. reuteri* Pg4 and *L. reuteri* pNZ3004 cells were not, indicating that Cnb−His_6 and Cnb−Glu−His_6 fusion proteins were successfully displayed on the cell surface of the *L. reuteri* pNZ-cnb and *L. reuteri* pNZ-cnb/glu.

To substantiate the findings of the immunofluorescence microscopy, quantitative flow cytometry was further used to analyze the cell surface display of Cnb−His_6 and Cnb−Glu-His_6 fusion proteins. The cell-surface-displayed Cnb−His_6 and Cnb−Glu−His_6 fusion proteins were stained with the primary and secondary antibodies, and *L. reuteri* Pg4 cells were used as a control for flow cytometry. The results obtained clearly matched the findings obtained by immunofluorescence microscopy. As anticipated, *L. reuteri* pNZ-cnb and *L. reuteri* pNZ-cnb/glu displayed greater fluorescence signals than the *L. reuteri* Pg4 control (Figure 4C,F,I).

Growth Characteristics of *L. reuteri* Pg4 and Its Transformed Strains. When cultured in MRS medium at 37 °C,

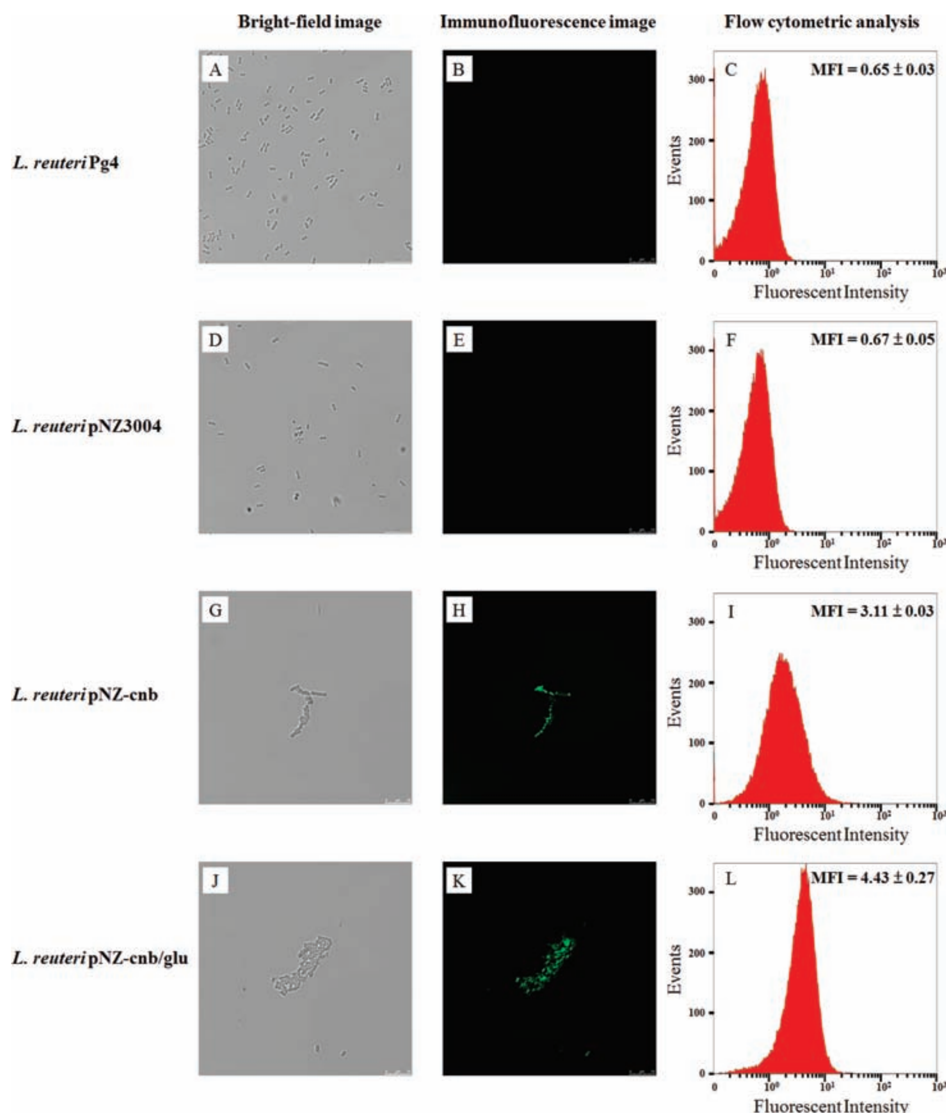

Figure 4. Confocal immunofluorescence microscopic examination and flow cytometric analysis of *Lactobacillus reuteri* cells: (A−C) *L. reuteri* PG4; (D−F) *L. reuteri* pNZ3004; (G−I) *L. reuteri* pNZ-cnb; (J−L) *L. reuteri* pNZ-cnb/glu. Cells were labeled with mouse monoclonal anti-His_6 antibodies, followed by goat anti-mouse IgG heavy and light chain antibodies conjugated with Alexa Fluor 488. For flow cytometric analysis, 10000 cells were analyzed in each experiment. The green fluorescent intensity of the lactobacilli cells was measured by flow cytometry at 525 nm emission wavelengths. Mean green fluorescence intensity (MFI) was calculated from two independent experiments performed in triplicate.

L. reuteri Pg4 reached stationary growth after 16 h of fermentation, with a concentration of 10.80 ± 0.12 log CFU/mL. The growth patterns of *L. reuteri* pNZ3004, *L. reuteri* pNZ-cnb, and *L. reuteri* pNZ-cnb/glu were similar to that observed for *L. reuteri* Pg4. The cell counts did not differ significantly among *L. reuteri* pNZ3004, *L. reuteri* pNZ-cnb, *L. reuteri* pNZ-cnb/glu, and *L. reuteri* Pg4 throughout the fermentation period (data not shown).

Adhesion of *L. reuteri* Pg4 and Its Transformed Strains to Caco-2 Cells. *L. reuteri* Pg4, *L. reuteri* pNZ3004, *L. reuteri* pNZ-cnb, and *L. reuteri* pNZ-cnb/glu cells were labeled with CFSE, a nonfluorescent membrane permeable ester, which is converted to a fluorescent molecule by nonspecific intracellular esterases, and covalently linked to intracellular proteins through its succinimidyl group.[25] 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. Caco-2 cells are weakly autofluorescent, which is seen in the scatter plot (Figure 5A), whereas Caco-2 cells incubated with each fluorescently 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 5B−E). The mean green fluorescence intensity of Caco-2 cells incubated with CFSE-stained *L. reuteri* Pg4 was significantly greater than that of Caco-2 cells alone (35.63 ± 2.80 vs 0.42 ± 0.11) ($P < 0.05$), indicating that *L. reuteri* Pg4 efficiently adhered to Caco-2 cells (Figure 5B). There was a 2.9-fold increase in the mean green fluorescence intensity of Caco-2 cells incubated with *L. reuteri* pNZ-cnb (102.70 ± 7.71) compared to that incubated with *L. reuteri* Pg4, indicating that constitutive expression of the recombinant Cnb−His_6 protein in *L. reuteri* pNZ-cnb resulted in an increase in the adhesion ability to Caco-2 cells. Interestingly, the

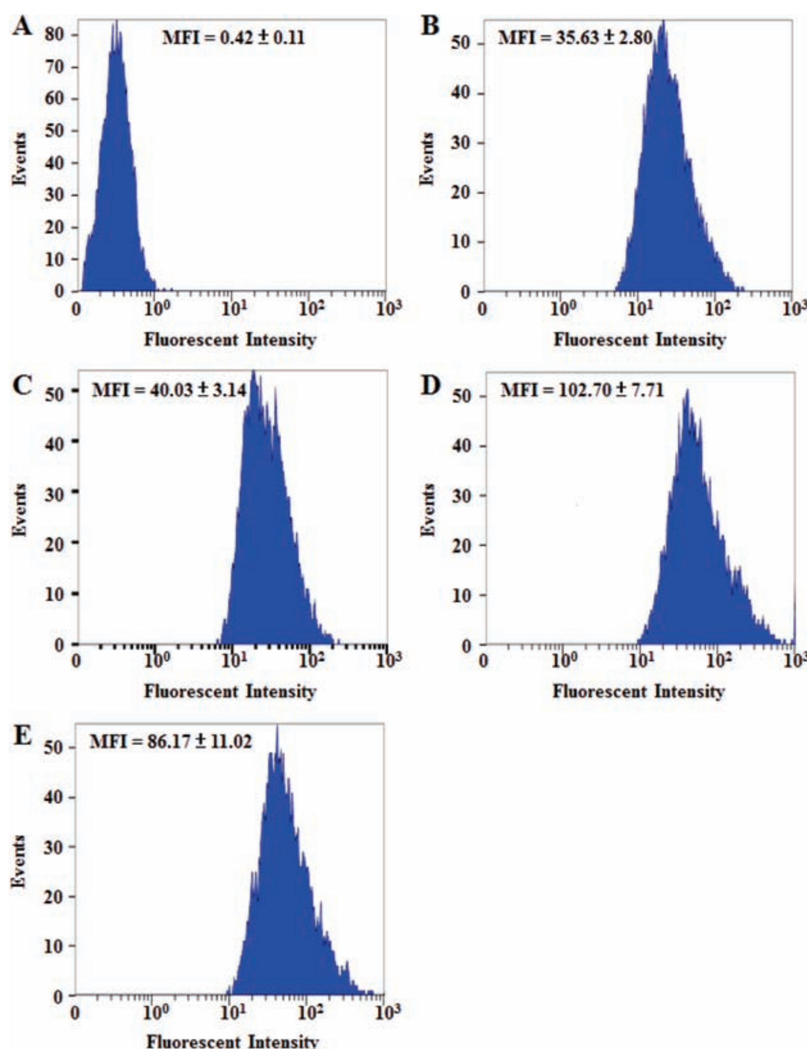

Figure 5. Flow cytometric analysis of *Lactobacillus* adherence to Caco-2 cells: (A) autofluorescence of Caco-2 cells; (B) Caco-2 cells exposed to fluorescently labeled *L. reuteri* Pg4; (C) Caco-2 cells exposed to fluorescently labeled *L. reuteri* pNZ3004; (D) Caco-2 cells exposed to fluorescently labeled *L. reuteri* pNZ-cnb; (E) Caco-2 cells exposed to fluorescently labeled *L. reuteri* pNZ-cnb/glu. Bacterial cells were labeled with CFSE and incubated with Caco-2 cells for 2 h. For each experiment, 10000 Caco-2 cells were analyzed. The green fluorescent intensity of the Caco-2 cells with adherent lactobacilli was measured by flow cytometry at 525 nm emission wavelength. Mean green fluorescence intensity (MFI) was calculated from two independent experiments performed in triplicate.

mean green fluorescence intensity of Caco-2 cells incubated with *L. reuteri* pNZ-cnb/glu (86.17 ± 11.02) was also significantly higher than that incubated with *L. reuteri* Pg4 ($P < 0.05$). These results demonstrated that display of Cnb−Glu−His_6 fusion proteins on the cell surface of *L. reuteri* Pg4 also increased its adhesion ability to Caco-2 cells.

DISCUSSION

Probiotics, which contain viable organisms and exert a beneficial effect on animal performance through modification of the gastrointestinal (GI) microflora, offer great potential both as feed additives and as replacements for antibiotics.[26] Using probiotics that secrete specific enzymes can provide additional benefits by reducing the cost of enzyme supplementation in that these enzyme genes could be replicated and expressed by the probiotics within the digestive tract. In addition, probiotics could directly deliver the enzyme to the target site, typically the intestine, where most of the feed digestion and nutrient absorption occurs. With a view to improve feed digestion and nutrient absorption, previously we induced *L. reuteri* Pg4 to express *F. succinogenes* β-glucanase and demonstrated that the transformed *L. reuteri* strain acquired the capacity to break down β-glucan, which decreased the digesta viscosity and improved body weight gain and feed conversion efficiency in broilers administered barley-based diets.[6,27] In the present study, *F. succinogenes* β-glucanase was fused to the C-terminus of the *L. reuteri* Cnb protein, which is assumed to electrostatically interact with cell-wall teichoic acids and thus be displayed on the cell surfaces of *L. reuteri* pNZ-cnb/glu. Our results demonstrated that the recombinant β-glucanase was successfully expressed and secreted by *L. reuteri*. Furthermore, the secreted Cnb−Glu−His_6 fusion proteins were associated with the cell surface of *L. reuteri* pNZ-cnb/glu, which was confirmed by confocal immunofluorescence microscopy and flow cytometric analysis (Figure 4). To the best of our knowledge, and following a thorough review of the relevant literature, this is the first report of successful display of fibrolytic enzymes on the cell surface of intestinal lactobacilli.

The cell-wall anchoring motif LPXTG and the S-layer protein are the two most commonly used anchor proteins for *Lactobacillus* surface display systems.[11,28] The LPXTG motif contains a cleavage site for sortase, which cleaves polypeptides between the threonine and the glycine of the conserved LPXTG motif and catalyzes the formation of amide bonds between the threonine at the C-terminal end of polypeptides and cell-wall cross-bridges.[29] Therefore, the difference in sortase activity between different strains could result in an insufficient display of target protein when using the LPXTG motif for cell-surface display.[28] In addition to the LPXTG motif, the S-layer proteins are also used for cell-surface display of heterologous proteins. Although S-layer proteins are expressed at very high levels, a major limitation of S-layer-based expression systems is that many fusion partners disrupt the ability of the S-layer subunit to polymerize into the S-layer lattice.[28] In contrast, another noncovalent surface attachment system utilizing BspA protein is proven to be versatile and capable of accommodating very large fusion partners.[17] However, BspA could be washed from cells even by dilute salt solutions.[10] In our study, Cnb, a closely related homologue of BspA, was used as the anchor protein for the display of β-glucanase on the cell surface of *L. reuteri*. The cell-wall fraction of *L. reuteri* pNZ-cnb/glu produced a larger clear halo on the β-glucan-containing plate than its extracellular supernatant did, indicating the Cnb−Glu−His_6 fusion proteins were present in the cell-wall fraction of *L. reuteri* pNZ-cnb/glu and were not easy to disassociate from the cell walls (Figure 2A). Thus, it is reasonable to suggest that Cnb could be used as a fusion partner to display heterologous proteins on the cell surfaces of lactobacilli.

Maximum probiotic effect is achieved when probiotic lactobacilli adhere to the intestinal mucosal cells.[30] Several exported proteins produced by lactobacilli are able to bind to epithelial component such as mucin or extracellular matrix proteins, including fibronectin, collagen, and laminin.[31] *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 these potential binding sites in the gut.[32] The collagen-binding protein Cnb is one of the reported extracellular matrix adhesion proteins produced by *L. reuteri*.[14] Previous studies demonstrated that Cnb mediated *L. reuteri* adhesion to the intestinal epithelial cells.[16,33] In our study, the *F. succinogenes* β-glucanase was fused to the C-terminus of Cnb and was displayed on the cell surface of *L. reuteri* pNZ-cnb/glu. The display of Cnb−His_6 or Cnb−Glu−His_6 fusion proteins on the cell surface of *L. reuteri* pNZ-cnb or *L. reuteri* pNZ-cnb/glu increased bacterial adhesion ability to epithelial cells (Figure 5). The adherence of lactobacilli on the surface of Caco-2 cells stimulated the up-regulation of mucin expression in Caco-2 cells, resulting in inhibition of pathogenic bacteria translocation.[34] Further investigation is required to verify the colonization of *L. reuteri* pNZ-cnb/glu in vivo and their interaction with the host and the microorganisms present in the host system. In addition, *L. reuteri* pNZ-cnb and *L. reuteri* pNZ-cnb/glu presented a higher cell autoaggregation when compared to *L. reuteri* Pg4 (Figure 4). Following our investigations of the literature, there is no research found that Cnb mediated the cell autoaggregation of *Lactobacillus*. For this reason, future research will be directed to determining whether or not Cnb plays a role in *Lactobacillus* cell autoaggregation.

In conclusion, we successfully used a surface-located protein, Cnb, as a fusion partner to direct the rumen bacterial β-glucanase to the cell surface of *L. reuteri* Pg4, and we demonstrated that display of the Cnb−Glu−His_6 fusion protein on the cell surfaces increased the adhesion capability of this genetically modified *L. reuteri* strain. Further studies to evaluate the potential of *L. reuteri* pNZ-cnb/glu for use as a probiotic in poultry diets and to verify its colonization and interaction with the host and microorganisms present in the host system are now in progress.

■ ASSOCIATED CONTENT

Supporting Information. Dot plot profiles of size versus granularity (forward scatter versus side scatter) analysis of the Caco-2 cells adhered with fluorescently labeled lactobacilli. This material is available free of charge via the Internet at http://pubs.acs.org.

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