Identification of a Surface Protein from *Lactobacillus reuteri* JCM1081 That Adheres to Porcine Gastric Mucin and Human Enterocyte-Like HT-29 Cells

Bin Wang · Hong Wei · Jing Yuan · Qiurong Li · Yousheng Li · Ning Li · Jieshou Li

Abstract Adhesion of lactobacilli to the host gastrointestinal (GI) tract is considered an important factor in health-promoting effects. However, studies addressing the molecular mechanisms of the adhesion of lactobacilli to the host GI tract have not yet been performed. The aim of this work was to identify *Lactobacillus reuteri* surface molecules mediating adhesion to intestinal epithelial cells and mucins. Nine strains of lactobacilli were tested for their ability to adhere to human enterocyte-like HT-29 cells. The cell surface proteins involved in the adhesion of *Lactobacillus* to HT-29 cells and gastric mucin were extracted. The active fractions were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting with horseradish peroxidase-labeled mucin and NHS-Biotin-labeled HT-29 cells. Furthermore, tandem mass spectrometry analysis was performed to identify the surface protein that participates in adhesion. It was shown that the ability of lactobacilli to adhere to HT-29 cells *in vitro* varied considerably among different strains. The most adhesive strain was the chicken intestinal tract isolate *Lactobacillus reuteri* JCM1081 (495.07 ± 80.03 bacterial cells/100 HT-29 cells). The adhesion of *L. reuteri* JCM1081 to HT-29 cells appeared to be mediated by a cell surface protein, with an approximate molecular mass of 29 kDa. The peptides generated from the 29-kDa protein significantly matched the Lr0793 protein sequence of *L. reuteri* strain ATCC55730 (∼71.1% identity) and displayed significant sequence similarity to the putative ATP-binding cassette transporter protein CnBP.

Introduction

Lactobacilli play an important role in controlling undesirable microflora in the gastrointestinal (GI) tract of humans and animals. They have been shown to exert healthful effects in humans, such as improving the properties of the endogenous flora, and they possess inhibitory activity toward the growth of pathogenic bacteria, blocking the attachment of enteropathogens results from steric hindrance caused by the adhesion of lactobacilli [3, 4, 16]. The adhesion of lactobacilli to the intestinal mucosal surface is a critical prerequisite for exerting beneficial effects to their host organisms and is considered one of the main selection criteria for potential probiotics, as it prolongs their persistence in the intestine and thus allows the probiotic to exert its healthful effects longer [7, 19, 24]. However, difficulties involved in studying bacterial adhesion *in vivo*, especially in humans, have led to the development of *in vitro* model systems for the preliminary selection of potentially adherent strains [29, 33]. Therefore, adherence of different lactobacilli has been studied with eukaryotic cell cultures as *in vitro* models for human intestinal mucosa. One of these models is the HT-29 cell line, which originates from human colon adenocarcinoma [11, 25]. The human intestinal epithelial cell line HT-29 has been used widely in the study of human enterocytic function [5, 6]. The advantage of this cellular model is that it expresses morphological and functional differentiation *in vitro* and shows characteristics of mature enterocytes, including polarization, a functional brush border, and apical intestinal

B. Wang · Q. Li · Y. Li · N. Li · J. Li
Research Institute of General Surgery, Jin Ling Hospital,
Nanjing, Jiangsu 210002, China

H. Wei (✉) · J. Yuan
Department of Laboratory Animal Science, The Third Military
Medical University, Chongqing 400038, China
e-mail: wblee@sohu.com

hydrolases [21]. Therefore, the HT-29 cell line is actually one of the best available models to study the adhesion of lactobacilli.

To date little is known about the mechanism of adherence of lactobacilli to human enterocytes. It has already been suggested that proteinaceous compounds are involved in the attachment of bacteria to intestinal epithelial cells [6, 13, 31]. Recent studies showed that surface proteins of some lactobacilli participate in adhesion to epithelial cell lines, GI mucins, or extracellular matrix proteins [8, 12, 20, 22, 26]. To further understand the mechanisms by which lactobacilli adhere to human intestinal cells, we investigated the adhesion of nine *Lactobacillus* strains to HT-29 cells and identified a cell surface protein from *Lactobacillus reuteri* JCM1081 that is involved in its adhesion to HT-29 cells and gastric mucin.

Materials and Methods

Bacterial Strains and Growth Conditions

Lactobacillus reuteri JCM1081, *Lactobacillus johnsonii* JCM1022 and *Lactobacillus gasseri* JCM1130 were purchased from the Japan Collection of Micro-organism (JCM). *Lactobacillus acidophilus* 1.1878 and *Lactobacillus rhamnosus* 1.120 were obtained from the Chinese General Micro-organism Culture Collection (CGMCC). *L. acidophilus* L050103-12 was kindly provided by Shanghai Pharmaceutical Co., LTD. *Lactobacillus bulgaricus* was isolated from the human intestine. *Lactobacillus brevis* 6042 and *Lactobacillus plantarum* 6003 were obtained from the Chinese Industry Micro-organism Collection (CICC). All *Lactobacillus* strains were grown in De Man, Rogosa and Sharpe (MRS) (Merck) broth at 37°C for 18–24 h under aerobic or anaerobic conditions, respectively.

Cell Culture

The HT-29 cell line was kindly provided by Dr. Y. Peng (Southwest Hospital, China). The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco) supplemented with 10% (v/v) heat-inactivated (30 min, 56°C) fetal calf serum (HyClone), 2 mM L-glutamine (Sigma), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in an atmosphere of 10% CO_2/90% air. For adhesion assays, monolayers of HT-29 cells were prepared on glass cover slips that were placed in six-well tissue culture plates. Cells were seeded at a concentration of 4×10^4 cells/cm^2. All experiments and maintenance of cells were carried out at 37°C in an atmosphere of 10% CO_2/90% air. The culture medium was changed daily and replaced by fresh DMEM without antibiotics at least 1 h

before the adhesion assay. Cells were used for adhesion assays at late postconfluence after 20 days in cultures.

Adhesion Assay

The adhesion of *Lactobacillus* strains to HT-29 cells was examined as previously described [5]. Briefly, the HT-29 monolayers were washed twice with sterile phosphate-buffered saline (PBS). For each adhesion assay, 1 mL of *Lactobacillus* suspension [the final concentration of bacteria was $(1–2) \times 10^8$ colony-forming units (CFU)/mL] was mixed with 1 mL of DMEM and added to each well of the tissue culture plate, which was then incubated at 37°C for 1 h in 10% CO_2/90% air. After incubation, the monolayers were washed five times with sterile PBS, fixed with methanol, Gram-stained, and then examined microscopically under oil immersion. Each adhesion assay was conducted in duplicate with cells from three successive passages. For each glass cover slip monolayer, the number of adherent bacteria was counted in 20 random microscopic areas. Adhesion of *Lactobacillus* was expressed as the number of bacteria adhering to 100 HT-29 cells.

All *Lactobacillus* strains were metabolically radiolabeled by the addition of 5 μL methyl-1,2-[^{3}H]-thymidine (121 Ci/mmol) to the broth. The bacteria were centrifuged at 2000g and the pellet was washed twice with PBS (pH 7.2) and then resuspended in PBS. The optical density at 600 nm (OD_{600}) of a 1:5 dilution of bacterial suspensions was adjusted to 0.5 ± 0.05 to give $\sim(1–2) \times 10^8$ CFU/mL. For adhesion assays, the HT-29 monolayer cells were washed twice with sterile PBS. One milliliter of radiolabeled bacterial suspension was mixed with 1 mL of DMEM and added to each well and then incubated at 37°C for 1 h in 10% CO_2/90% air. The suspension was removed and the monolayers were washed five times with PBS. The monolayers were treated with 100 μL of 0.1 M NaOH per well at 37°C overnight to lyse both HT-29 cells and bacteria. Then scintillation liquid was added to lysed cells and the radioactivity was measured by liquid scintillation. The adhesion ratio (%) was expressed as the percentage of radioactivity recovered from the final lysed cells compared to the original bacterial suspension added.

Isolation of Bacterial Cell Surface Proteins

Cell surface proteins were prepared using a method modified from Aleljung et al. [1]. Briefly, an overnight 500-mL culture of *Lactobacillus* was harvested and washed twice with cold sterile PBS. Cell pellets were suspended in 20 mL of PBS with lysozyme (2 mg/ml) and incubated for 1 h at 37°C. After centrifugation (15 min, 10,000g), the cell pellets were resuspended in 1 M LiCl [double-distilled water (ddH$_2$O)] and incubated for 20 h at 20°C. After

centrifugation, the supernatants were collected and dialyzed overnight against demineralized water at 4°C. Finally, the dialysate was freeze-dried and stored at –20°C.

Mucin and HT-29 Cell Preparation

Partially purified mucin from the porcine stomach was obtained from Sigma. Gastric mucin was conjugated with horseradish peroxidase (HRP) by the method of Hudson and Hay [17] as previously described by Rojas and Conway [27] and Rojas et al. [26]. Briefly, the gastric mucin (4 mg/mL in carbonate buffer) was labeled with HRP (Sigma). HRP (8 mg dissolved in 2 mL of distilled water) was added to 400 μL of freshly prepared sodium periodate (0.1 M) solution. The mixture was stirred gently for 20 min at room temperature and then dialyzed overnight at 4°C against 0.001 M acetate buffer, pH 4.4. Sodium carbonate buffer (0.1 M, pH 9.5; 20 μL) was added in order to raise the pH to ∼9.0–9.5 and immediately 1 mL was mixed with 1 mL of gastric mucin. The mixtures were held at room temperature for 2 h with occasional stirring. Freshly prepared sodium borohydride solution (100 μL of a 4-mg/mL solution in distilled water) was added to reduce any free enzyme, and the mixture was dialyzed against borate buffer (0.1 M, pH 7.4). Labeled gastric mucin was mixed with equal volumes of 80% glycerol and stored at –20°C.

The HT-29 cells were washed three times with ice-cold PBS (pH 8.0) to remove amine-containing culture medium and proteins from the cells. Cells were suspended at a concentration of 2.5×10^7 cells/mL in PBS (pH 8.0). Two hundred microliters of 10 mM Sulfo-NHS-Biotin reagent solution (PIERCE) was added to 1 mL of cell suspension and incubated for 30 min at room temperature. The mixtures were washed three times with PBS + 100 mM glycine buffer to quench and remove excess Sulfo-NHS-Biotin reagent and by-products. The cell pellets were resuspended in PBS (pH 7.0) at a concentration of 1×10^7 cells/mL and were used in the next step immediately.

SDS-PAGE and Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard laboratory techniques with a 5% (w/v) stacking gel and a 12% (w/v) separating gel, in a Mini- Protean II apparatus (Bio-Rad Laboratories). The cell surface proteins of *Lactobacillus* strains were mixed with sample buffer containing SDS and mercaptoethanol, boiled for 3 min, centrifuged, and loaded on the gel. Gels were stained with Coomassie brilliant blue R-250 or transferred to Immobilon PVDF membrane (Millipore) in a semidry electroblotter (Bio-Rad) for 90 min at 15 V. The membranes were saturated by soaking the membrane in 3% (w/v) bovine serum albumin in PBS buffer

overnight at 4°C for blocking nonspecific binding, and the membranes were washed three times (20 min each time) with PBS containing 0.05% Tween-20 (PBS-T buffer). The membranes were treated with 100 μL of HRP-labeled gastric mucin in 10 mL of PBS-T buffer at 37°C for 2 h. In the cell immunoblot, the membranes were incubated with the Sulfo-NHS-Biotin-labeled HT-29 cells and diluted 1:100 in PBS-T buffer at 37°C in 10% CO_2/90% air for 2 h. The membranes were washed three times with PBS-T buffer and incubated with the HRP-labeled streptavidin and diluted 1:400 in PBS-T buffer at room temperature for 1 h. The membranes were washed three times with PBS-T buffer and then washed with 0.1 M sodium acetate (pH 5), and the reactive bands were visualized with 2.5 mg of diaminobenzidine and 2.5 μL of hydrogen peroxide (30%) dissolved in 10 mL of 0.1 M sodium acetate (pH 5).

Protein Tandem Mass Spectrometry Analysis

The cell surface proteins of *Lactobacillus* strains were separated on SDS–12% acrylamide gel. The gel was stained with Coomassie brilliant blue R-250. The band corresponding to the 29-kDa protein of *L. reuteri* JCM1081 and reacting with HRP-labeled gastric mucin and Sulfo-NHS-Biotin-labeled HT-29 cells was excised. The gel pieces were washed with 50% aqueous acetonitrile followed by rehydration with 0.1 M ammonium bicarbonate. Excised proteins were reduced with 10 mM dithioerythritol (DTE) by incubation at 56°C for 45 min and then alkylated by adding 55 mM iodoacetamide for 30 min at room temperature in the dark. After the gel pieces were dried in a speed-vacuum centrifuge, 10–15 μL of a trypsin solution (20 ng trypsin/μL in 25 mM ammonium bicarbonate containing 5 mM calcium chloride) were applied and digestion was performed overnight at 37°C. The tryptic peptides were extracted in 100 μL of 50% acetonitrile, 0.1% trifluoroacetic acid (TFA) and desalted on a Millipore ZipTip C18 (Milian S.A). After elution with methanol, the resulting tryptic peptides were lyophilized and analyzed by tandem electrospray ionization–mass spectrometry as previously described [23].

Results

Adhesion of *Lactobacillus* Strains to HT-29 Cells

Nine *Lactobacillus* strains were investigated for their ability to adhere to cultured HT-29 cells. The ability of lactobacilli to adhere to HT-29 cells varied considerably among strains and showed strain-specificity. *L. reuteri* JCM1081, originally isolated from the chicken intestine, was the most adhesive strain, followed by *L. acidophilus*

L050103-12, which was originally isolated from the human intestine. Moderate adhesion was observed with *L. johnsonii* JCM1022, *L. gasseri* JCM1130, *L. rhamnosus* 1.120, and *L. bulgaricus*, whereas *L. acidophilus* 1.1878, *L. brevis* 6042, and *L. plantarum* 6003 poorly adhered to HT-29 cells (Table 1). The strains originally isolated from the human or animal intestine all adhered at a higher level than isolates from other origins.

Electrophoresis and Western Blotting

The cell surface proteins of *Lactobacillus* strains were analyzed by denaturing discontinuous PAGE and Western blotting with HRP-labeled mucin and Sulfo-NHS-Biotin-labeled HT-29 cells. The results indicated that dominant protein bands with molecular masses of ∼29 kDa and ∼31 kDa and in the range 43–90 kDa were present in the cell extract of the most adhesive strain, *L. reuteri* JCM1081, whereas three bands of ∼43 kDa, ∼47 kDa, and ∼63 kDa were detected in the cell extracts of *L. acidophilus* L050103-12. A dominant band of ∼29 kDa and four weak bands of 41 kDa, 43 kDa, 52 kDa, and 63 kDa were detected in the cell extracts of *L. rhamnosus* 1.120 and two bands of ∼43 kDa and ∼47 kDa in *L. bulgaricus*. Only one weak protein band was detected in

L. acidophilus 1.1878 (Fig. 1), *L. brevis* 6042, and *L. plantarum* 6003 (data not shown), which showed poor adhesion to HT-29 cells.

The Western blotting profiles of cell surface proteins indicated that the strongest reacting band that bound mucin was a 29-kDa protein in the most adhesive strain, *L. reuteri* JCM1081, and moderate adhesive strain, *L. rhamnosus* 1.120 (Fig. 1). Four positive bands with approximate molecular masses of 32 kDa, 43 kDa, 47 kDa, and 63 kDa that bound mucin were found in *L. acidophilus* L050103-12 (Fig. 1). The strongest reacting band observed on Western blots using mucin was also verified by the NHS-Bbiotin-labeled HT-29 cell. A band of ∼29 kDa that bound HT-29 cells was found in *L. reuteri* JCM1081 and *L. rhamnosus* 1.120 and a 43-kDa band in *L. acidophilus* L050103-12 (Fig. 2). No reacting band that bound mucin or HT-29 cells was observed in other *Lactobacillus* strains tested. The results suggested that the 29-kDa protein, which bound mucin and HT-29 cells, could be involved in adhesion of *L. reuteri* JCM1081.

Identification of the Adhesion of *L. reuteri* JCM1081

The band with an approximate molecular mass of 29 kDa of *L. reuteri* JCM1081 was excised from the membrane and

Table 1 Adhesion of *Lactobacillus* strains to HT-29 cells in culture

Strain	Adhered bacteria/100 HT-29 cells ($\bar{x} \pm s$)	Adhesion (%)
L. reuteri JCM1081	495.07 ± 80.03	21.30 ± 1.56
L. johnsonii JCM1022	208.33 ± 32.72	9.15 ± 0.35
L. gasseri JCM1130	292.25 ± 37.26	12.05 ± 1.34
L. acidophilus 1.1878	35.43 ± 13.93	3.30 ± 0.42
L. rhamnosus 1.120	330.16 ± 66.74	13.75 ± 0.21
L. brevis 6042	59.73 ± 29.81	3.75 ± 0.50
L. plantarum 6003	95.37 ± 23.33	6.14 ± 0.85
L. acidophilus L050103-12	399.00 ± 43.89	16.25 ± 0.34
L. bulgaricus	172.25 ± 17.06	8.43 ± 0.79

Fig. 1 SDS-PAGE (**A**) and Western blotting (**B**) of cell surface proteins extracted from *Lactobacillus* strains, using HRP–mucin for blotting. Lanes: S: low-molecular-mass protein standard; 1: *L. reuteri* JCM1081; 2: *L. acidophilus* 1.1878; 3: *L. acidophilus* L050103-12; 4: *L. rhamnosus* 1.120; 5: *L. bulgaricus*; N: negative control
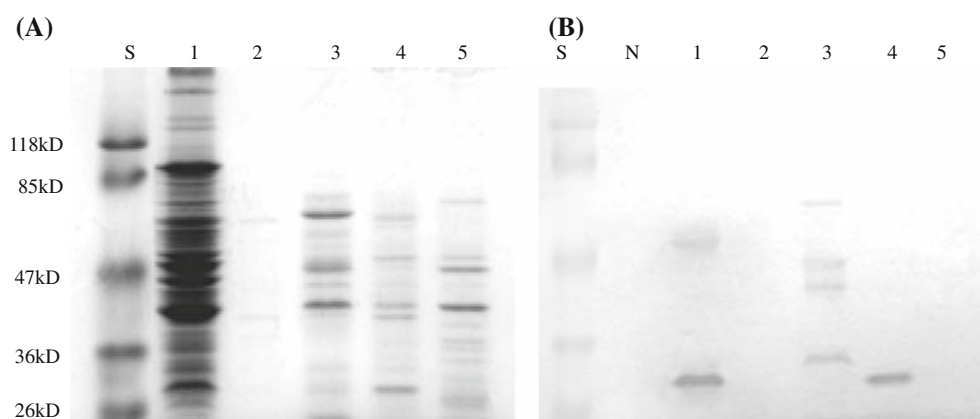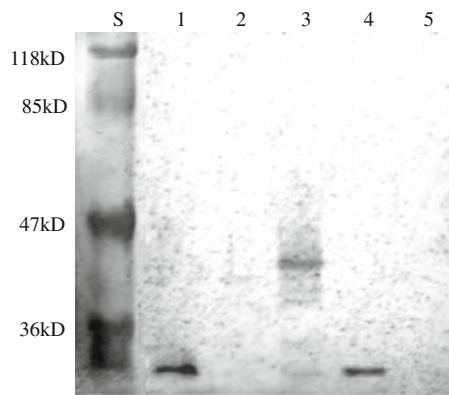
Fig. 2 Western blotting of cell surface proteins extracted from *Lactobacillus* strains, using NHS-Biotin-labeled HT-29 cells. Lanes: S: low-molecular-mass protein standard; 1: *L. reuteri* JCM1081; 2: *L. acidophilus* 1.1878; 3: *L. acidophilus* L050103-12; 4: *L. rhamnosus* 1.120; 5: *L. bulgaricus*

was analyzed by electrospray ionization–mass spectrometry. The protein was compared to the current protein databases using the program BlastP, which returned a significant identity to Lr0793 protein from *L. reuteri* ATCC55730 (~71.1% identity). The Lr0793 protein displays significant sequence similarity to a putative ATP-binding cassette transporter protein.

Discussion

The ability to adhere to intestinal epithelial cells and mucosal surfaces has been suggested to be an important property of many *Lactobacillus* strains used as probiotics [24, 32]. However, the mechanism by which lactobacilli adhere to human intestinal epithelial cells remains unclear. The nature of *Lactobacillus* adhesion determinants differs depending on the bacterial strains. Our data clearly indicate that not all *Lactobacillus* strains possess the high capacity to adhere to human intestinal epithelial cells *in vitro*. The characteristics of adhesion observed with *Lactobacillus* suggested that adhesive properties are specific and vary considerably among strains, independent of the species. This is consistent with the earlier studies [5, 30]. Also, within the same species, the number of bacteria adherent to human intestinal epithelium cells differs significantly depending on the strains [18]. The level of adherence for tested strains that were originally isolated from the human or animal intestine was found to be considerably higher than that of isolates from other origins. This appears to indicate that adhesive *Lactobacillus* strains have host-res-idential characteristics.

We further identified the surface proteins involved in the adhesion of *L. reuteri* JCM1081 to enterocytelike cells and gastric mucin by Western blot with HRP-labeled gastric

mucin and Sulfo-NHS-Biotin-labeled HT-29 cells. Our results clearly demonstrate that the strongest reacting band that bound mucin and HT-29 cells simultaneously is a 29-kDa protein in *L. reuteri* JCM1081. Mucin is a large glycosylated protein that is secreted constantly by goblet cells and is responsible for the gel-like properties of the mucus layer. It also serves as an ecological niche for both commensal and potential pathogenic microorganisms [9, 10, 15]. Thus, mucin could be regarded as a receptor for probiotic bacteria to adhere and colonize in the host GI tract. The results suggest that the 29-kDa-protein-bound mucin could be involved in adhesion of *L. reuteri* JCM1081. Furthermore, we observed that labeled HT-29 cells have specific properties to bind a 29-kDa surface protein that was extracted from *L. reuteri* JCM1081, thus suggesting that the 29-kDa surface protein of *L. reuteri* JCM1081 could recognize the receptor of HT-29 cells. Hayman et al. identified a serum-spreading factor by labeled NRK kidney cells of rat and indicated that this serum-spreading factor is associated with cell adhesion [14]. From these results, we assume that using labeled cells to hybridize with separated protein on the PVDF membrane is a feasible method for determining the protein that is involved in the adhesion of *Lactobacillus*. The results might be used as the basis to determine the interaction between these proteins and the bacterial cell, as well as the host cell surface. Aleljung et al.reported that adhesion of *L. reuteri* NCIB 11951 to collagen type I was mediated by 29-kDa and 31-kDa proteins (CnBP, EMBL accession No. X99716.1) [1]. These proteins were purified from the cell surface of *L. reuteri* NCIB 11951 and both bound radio-labeled collagen type I. The N-terminal sequence of the 29-kDa and 31-kDa proteins demonstrated the closest homologies with an internal sequence from an *Escherichia coli* trigger factor protein (EMBL accession No. P0A850) but was not similar to S-layer proteins in other *Lactobacillus* strains. A 29-kDa surface protein (MapA, EMBL accession No. AJ293860.1) from *L. fermentum* 104R that mediates binding of *Lactobacillus* to both small intestinal porcine mucus and gastric mucin has been reported [26]. From the amino acid sequence of the N-terminus, similarity with a collagen-binding protein (CnBP) of *L. reuteri* NCIB 11951 that has already been published [22, 28] became evident. Our results show that the 29-kDa surface protein of *L. reuteri* JCM1081 displays significant peptide sequence similarity to the Lr0793 protein from *L. reuteri* ATCC55730 (~71.1% identity), whereas the protein Lr0793 is homologous to the ABC transporter component CnBP, which previously has been described as a collagen-binding protein [2]. The 29-kDa surface protein of *L. reuteri* JCM1081 probably is classified as a member of the ABC transporter family, as well as CnBP from *L. reuteri* NCIB11951 and MapA from *L. fermentum* 104R.

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