Isolation of lactic acid bacteria bound to the porcine intestinal mucosa and an analysis of their moonlighting adhesins

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The adhesion of lactic acid bacteria (LAB) to the intestinal mucosa is one of the criteria in selecting for probiotics. Eighteen LAB were isolated from porcine intestinal mucin (PIM): ten strains of *Lactobacillus*, six strains of *Weissella*, and two strains of *Streptococcus*. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for phosphate-buffered saline (PBS) extracts from the LAB, many bands were detected in half of the samples, while a few and/or no clear bands were detected in the other half. All six of the selected LAB showed adhesion to PIM. *L. johnsonii* MYU 214 and MYU 221 showed adhesion at more than 10%. *W. viridescens* MYU 208, *L. reuteri* MYU 213, *L. mucosae* MYU 225, and *L. agilis* MYU 227 showed medium levels of adhesion at 5.9–8.3%. In a comprehensive analysis for the adhesins in the PBS extracts using a receptor overlay analysis, many moonlighting proteins were detected and identified as candidates for adhesins: GroEL, enolase, and elongation factor Tu in MYU 208; peptidase C1, enolase, formyl-CoA transferase, phosphoglyceromutase, triosephosphate isomerase, and phosphofructokinase in MYU 221; and DnaK, enolase, and phosphoglycerate kinase in MYU 227. These proteins in the PBS extracts, which included such things as molecular chaperones and glycolytic enzymes, may play important roles as adhesins.

Key words: adhesion, adhesin, moonlighting protein, lactic acid bacteria, probiotics, porcine intestinal mucin

INTRODUCTION

Lactic acid bacteria (LAB) are commonly used as probiotics. LAB have many beneficial effects; for example, they can be used to manage lactose intolerance [1], lower cholesterol [2], improve immune function [3], prevent colon cancer [4], and inhibit the adherence of various pathogens [5–7]. LAB are often isolated from the alimentary canal and feces of humans and animals [8–11] and are used in fermented foods as probiotics. One potentially important property of these organisms is their ability to interact with the mucosa or mucus in the intestinal tract, which may promote retention and host-bacteria communication. The ability of a strain to adhere to the intestinal mucosa is one of the criteria used to select probiotic microorganisms. Lactobacilli contain many types of adhesins, e.g., SlpA [12, 13], CdpA [14], Mub [15], FbpA [16], CbsA [17], Msa [18], CnBP (ABC transporter) [19, 20], MapA [21], LspA [22], bacterial cell surface carbohydrate [23], and lipoteichoic acid [24].

Recently, the idea of one gene-one protein-one function has been challenged by evidence that many proteins have multiple functions. For example, many cytosolic proteins are expressed on the bacterial cell surface and have other functions in many LAB strains. These proteins are referred to as "moonlighting proteins," which are defined as single proteins that have multiple functions. Recently,

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many moonlighting proteins have been identified; for example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase, glutamine synthetase (GS), elongation factor Tu (EF-Tu), GroEL, and DnaK exist on bacterial cell surfaces in probiotic bacteria [25–31]. Previously, we reported that GAPDH expressed on the bacterial cell surfaces of *Lactobacillus plantarum* LA 318 isolated from human intestinal tissue (transverse colon) [32] binds to human colonic mucin [28] and to the A- and B-type antigens of the ABO blood type group [33]. GAPDH is universally expressed on the cell surfaces of many lactobacilli [34]. According to Antikainen *et al.* [30], GAPDH and enolase bind to lipoteichoic acid on the bacterial cell surface by ionic bonds. We also found that GAPDH can be easily extracted using phosphate-buffered saline (PBS, pH 7.4) [28]. However, the identities and functions of moonlighting proteins, such as adhesins, are unclear. Here, we examined moonlighting proteins that can be extracted with PBS to comprehensively analyze adhesins in LAB.

MATERIALS AND METHODS

Isolation of LAB from porcine intestinal mucin and culture conditions

MRS agar was prepared by adding 1.5% agar (Wako Pure Chemical Industries, Osaka, Japan) to MRS broth (Difco Laboratories, Detroit, MI, USA). Modified LBS (mLBS) agar was prepared by adding 0.8% (w/v) Lab-Lemco Powder (Oxoid, Basingstoke, Hampshire, UK), 0.1 mol/l sodium acetate, and 3.7% (v/v) acetic acid to LBS agar (Becton Dickinson and Company, Sparks, MD, USA).

The surface mucus layer was gently scraped from porcine intestinal samples using a glass slide and incubated on MRS agar, mLBS agar, or BL agar (Nissui, Tokyo, Japan) in an anaerobic jar for 48 hr at 37°C. Colonies differing in color, form, and size were selected from each plate and propagated with MRS broth (Difco Laboratories) at 37°C; for subsequent experiments, they were stored at −80°C.

Bacterial strains were propagated twice at 37°C for 24 hr in MRS broth with 2% (v/v) inoculum before the experiments.

Identification of isolated bacteria

The isolated bacteria were identified using Gram staining, morphological observations, catalase tests, carbohydrate fermentation tests with API 50 CH (bioMérieux, St. Louis, MO, USA), and a homology search using 16S rDNA sequences. Amplification of the total 16S

rDNA was performed using the following pair of universal primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525r (5′-AGAAAGGAGGTGATCCAGCC-3′) [35]. The homology search was performed using BLAST against the DDBJ, EMBL, and GenBank databases.

Isolation and purification of PIM

Porcine intestinal mucin (PIM) in the adherent mucosa was prepared from fresh porcine intestinal tissues by scraping with slide glass. Isolation and purification of PIM were performed as described previously [32].

Lipids from crude PIM were removed using three sequential extractions with chloroform:methanol (2:1, v/v) and diethyl ether. The lipid-free PIM was dissolved in 4 mol/l guanidine hydrochloride (GHCl) solution and fractionated by gel filtration chromatography using a Toyopearl HW-65F Column (100 cm × 2.5 cm, Bio-Rad Laboratories, Hercules, CA, USA). Protein absorbance was monitored at 280 nm, and neutral sugars were detected at 490 nm using the phenol-H_2SO_4 method [36]. The fractions containing the highest sugar contents were dialyzed against distilled water at 4 °C for 2 days and then lyophilized.

Carboxyfluorescein diacetate labeling of LAB

LAB were cultured at 37°C for 18 hr in MRS broth and centrifuged (3,000 rpm, 10 min, room temperature). The pellets were washed with sterilized distilled water three times (8,000 rpm, 5 min, 4°C). The pellets were resuspended in 1 ml of sterilized distilled water, and 100 µl of 1 mmol/l carboxyfluorescein diacetate (CFDA) in PBS was added. After incubation for 30 min at 37°C in the dark, the CFDA-labeled microbial cells were washed with sterilized distilled water three times (8,000 rpm, 5 min, 4°C).

Test of adhesion of LAB to PIM

Reacti-Bind^{TM} Amine-binding Maleic Anhydride Activated Plates (Pierce, Rockford, IL, USA) that allow attachment of amine-containing proteins to microplate wells were used for microtiter plate binding assay. Purified unlabeled PIM (0.3 mg/ml) was dissolved in PBS (100 µl), added to each well, and incubated overnight at 4°C. After decantation, each well was blocked with 5% skim milk in PBS for 5 hr at 4°C; the plates were then used for the binding assay. After immobilization of PIM, each well was washed three times with 250 µl of PBS with 0.05% Tween 20 (PBS-T). Then, 100 µl of 1×10^8 cells/ml CFDA-labeled LAB cells in distilled water were added to the wells. Three parallel wells were used for each experiment. The microbial cells were allowed to

adhere for 1 hr at 37°C, and the wells were washed three times with 250 µl of PBS to remove non-adherent cells. The cells bound to PIM were released and lysed using 1% (w/v) sodium dodecyl sulfate (SDS)/0.1 mol/l NaOH solution and incubated for 1 hr at 60°C. After incubation, the fluorescence intensity (excitation, 485 nm; emission, 538 nm) of the lytic solution was measured using a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PBS was used as the control in place of PIM. The adhesion value was defined as the value of the mucin-LAB immobilized on the plate reduced by the control value. The results are shown as adhesion rates (%) and were calculated as follows:

Adhesion rate (%) = (fluorescence intensity of adherent LAB) – (fluorescence intensity of PBS control)/(initial fluorescence intensity of LAB) × 100.

Lactobacillus plantarum LA 318 was used as a positive control; it shows high adhesion to the human colonic mucin and blood type antigens (A and B antigens) [28, 32, 33].

Sample preparation for SDS-PAGE and the receptor overlay analysis

Eighteen strains isolated from PIM were cultured at 37°C for 18 hr in MRS broth (Difco Laboratories) using a 2% (v/v) inoculum. Bacterial cells after culture were washed three times with sterile distilled water. After washing, the pellets were suspended in PBS and incubated at 4°C for 30 min. After centrifugation (8,000 rpm, 5 min, 4°C), the supernatant was dialyzed, lyophilized, and used as the PBS extract sample for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the receptor overlay (RO) analysis.

SDS-PAGE

Cell surface proteins prepared from the 18 LAB were analyzed using SDS-PAGE according to the methods of Laemmli [37]. PBS extract samples were denatured in SDS buffer (60 mmol/l Tris, 25% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromophenol blue; pH 6.8) at 1 mg/ml and heated at 95 °C for 10 min. Electrophoresis was performed using polyacrylamide gels with a discontinuous buffer system, a 4.5% stacking gel, and a 12.5% separation gel (10 × 12 cm). Electrophoresis was performed using a Mini-PROTEAN III Dual Slab Cell (Bio-Rad) at a constant voltage of 125 V in running buffer containing 25 mmol/l Tris, 0.2 mol/l glycine, and 0.1% SDS. Protein bands were visualized by staining the gels with Coomassie Brilliant Blue (CBB) (Rapid CBB; Kanto Chemical Co., Inc., Tokyo, Japan). Molecular weight markers from 14 to 97 kDa (AE-1440

EzStandard, Atto Co., Ltd., Tokyo, Japan) were used.

Biotinylation of PIM and BSA

The purified PIM or bovine serum albumin (BSA; 1.0 mg) was diluted with 1 mL of 50 mmol/l borate-NaOH buffer (pH 9.0) and centrifuged (8,000 rpm, 5 min, 4°C). The supernatant (200 µl) was supplemented with 2 µl of *N,N*-dimethylformamide (DMF, Thermo Fisher Scientific) containing EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) (25 µg/ml) and was reacted for 2 hr at 4°C. After biotinylation, each sample (100 µl) was dialyzed against distilled water using Slide-A-lyzer MINI Dialysis Units (Thermo Fisher Scientific) in the dark and was used as biotinylated PIM or BSA. The biotinylated BSA was used as a control protein.

Detection of adhesins using an RO analysis

Adhesins were detected using an RO analysis according to the methods of Ilver *et al.* [38], with some modifications.

The PBS extract sample was diluted with SDS sample buffer for electrophoresis (2ME-, Wako Pure Chemical Industries) (1 mg/ml) and was applied to SDS-PAGE. After electrophoresis, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Tokyo, Japan) using a semi-dry blotting apparatus (Semi-Phor, Hoefer Scientific, San Francisco, CA, USA). The membrane was blocked with TBS-T (Tris-buffered saline with Tween-20, pH 7.5) containing 5% skim milk for 30 min. After washing with TBS-T, the membrane was soaked in TBS-T containing the biotinylated PIM (final concentration 1.0 g/ml) overnight. After washing with TBS-T, ExtrAvidin Alkaline Phosphatase Conjugate (Sigma-Aldrich) was added, followed by incubation for 30 min. After washing with TBS-T, ECF Substrate for Western Blotting (GE Healthcare, Tokyo, Japan) was added, and visualization was performed using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan) (excitation, 440 nm; emission, 560 nm).

Biotinylated molecular weight markers (Sigma-Aldrich) were used for SDS-PAGE. Biotinylated BSA was used as a control protein instead of biotinylated PIM to identify mucin-binding adhesion proteins. TBS-T was used as a negative control to exclude nonspecific binding.

N-terminal sequence analysis

After the SDS-PAGE analysis, proteins from the PBS extract were electrophoretically transferred to a PVDF membrane. Then, the membrane was visualized using Amido Black 10B (Wako Pure Chemical Industries) in 7%

Table 1.　Identification of isolated bacteria from porcine intestinal mucus

Strain no.	Culture agar	Species	Homology	Accession No.
MYU 135	mLBS	*Lactobacillus ruminis*	100%	LC163924
MYU 136	mLBS	*Lactobacillus amylovorus*	100%	LC163925
MYU 200	MRS	*Weissella viridescens*	100%	LC163926
MYU 203	MRS	*Weissella viridescens*	99%	LC163927
MYU 204	MRS	*Weissella viridescens*	100%	LC163928
MYU 205	MRS	*Weissella viridescens*	99%	LC163929
MYU 207	MRS	*Weissella viridescens*	100%	LC163930
MYU 208	MRS	*Weissella viridescens*	100%	LC163931
MYU 212	mLBS	*Streptococcus alactolyticus*	100%	LC163932
MYU 213	mLBS	*Lactobacillus reuteri*	99%	LC163933
MYU 214	mLBS	*Lactobacillus johnsonii*	100%	LC163934
MYU 215	mLBS	*Lactobacillus reuteri*	99%	LC163935
MYU 217	mLBS	*Streptococcus alactolyticus*	99%	LC163936
MYU 220	BL	*Lactobacillus reuteri*	99%	LC163937
MYU 221	BL	*Lactobacillus johnsonii*	99%	LC163938
MYU 224	BL	*Lactobacillus mucosae*	99%	LC163939
MYU 225	BL	*Lactobacillus mucosae*	99%	LC163940
MYU 227	BL	*Lactobacillus agilis*	100%	LC163941

acetic acid solution. The protein bands that corresponded to mucin-binding proteins in the RO analysis were cut from the membrane and washed three times with 10% ethanol to remove glycine from the running buffer. N-terminal amino acid sequencing was performed using Edman degradation [39]. A homology search was performed using BLAST against the DDBJ, EMBL, and GenBank databases. The molecular weight of a sample protein showing high homology to the N-terminal sequences of a sample was deduced using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/).

Statistical analyses

Adhesion tests were performed in triplicate, and results are reported as means ± SD. Statistical analyses were performed using Dunnett's test for the adhesion test after performing Kolmogorov-Smirnov's test for normality and Levene's test for equal variances.

RESULTS

Isolation and identification of LAB from PIM

Table 1 shows the isolated LAB species. Eighteen LAB were isolated from PIM, including ten strains of *Lactobacillus*, six strains of *Weissella*, and two strains of *Streptococcus*. With MRS agar and BL agar, *Caulobacter* sp., *Staphylococcus* sp., and *Enterobacteriaceae* (*Klebsiella* sp., *Escherichia* sp., or *Shigella* sp.) were detected. Only LAB (*Streptococcus* and *Lactobacillus*) were detected using mLBS agar.

SDS-PAGE of the PBS extract

Figure 1 shows the banding patterns for the SDS-PAGE analysis using PBS extracts from the LAB. Various protein banding patterns were observed in each sample. Some common bands were detected at ca. 40–60 kDa. Clear protein bands were detected in PBS extracts from MYU 200, MYU 204, MYU 207, MYU 208, MYU 213, MYU 214, MYU 221, MYU 225, and MYU 227, while a few and/or no clear bands were detected in MYU 135, MYU 136, MYU 203, MYU 205, MYU 212, MYU 215, MYU 217, MYU 220, and MYU 224. Strains showing clear bands were selected for further investigation. However, MYU 200, MYU 204, and MYU 207 were excluded from subsequent analyses because they showed similar banding patterns to that of MYU 208.

Test of adhesion of LAB to PIM

Supplemental Figure 1 shows the gel filtration chromatograph for PIM. Fractions no. 47–61, which contained high protein and sugar contents, were collected and used as a purified PIM in the adhesion test and the RO analysis.

Figure 2 shows the rate of adhesion of each selected LAB strain to purified PIM. All strains showed adhesion to purified PIM, and the adhesion rates differed significantly among strains (p<0.01). *L. plantarum* LA318, a positive control, showed high adhesion at 19.0 ± 3.7%. No significant differences were detected between the positive control and MYU 214 (16.3 ± 3.8%) and MYU 221 (11.3 ± 4.2%), whereas the adhesion rates of
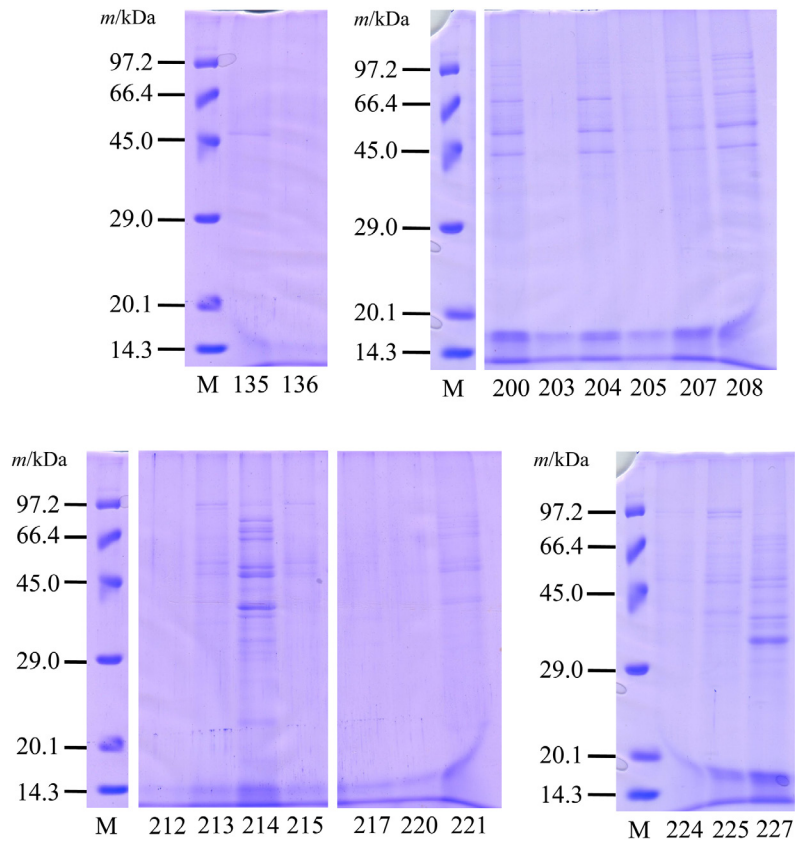
Fig. 1. SDS-PAGE of phosphate-buffered saline (PBS)-extracted proteins from lactic acid bacteria (LAB).

Bacterial cells were washed three times with sterile distilled water. The pellets were suspended in PBS and incubated at 4°C for 30 min. After centrifugation, the supernatant was dialyzed and lyophilized. SDS-PAGE was performed using a 12.5% separation gel at a constant voltage of 125 V. Protein bands were visualized using CBB staining. M: molecular weight marker. MYU strain numbers: 135–227.
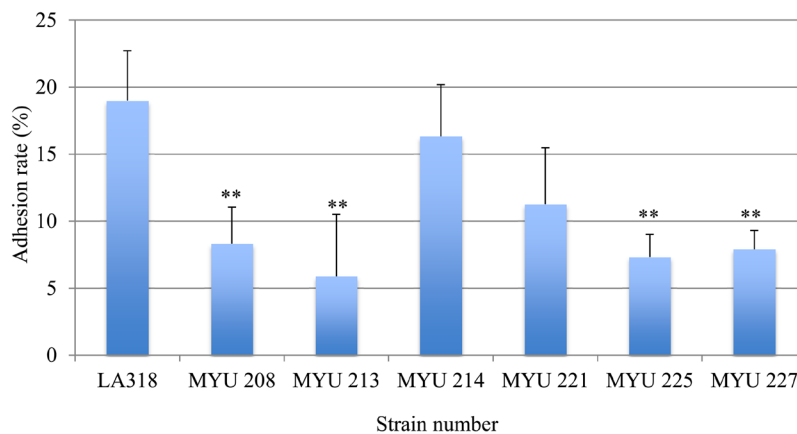

Fig. 2. Test of adhesion of selected LAB to porcine intestinal mucin (PIM).

Purified unlabeled PIM in PBS was added to each well and incubated overnight at 4°C. After blocking and washing, 100 μL of 1×10^8 cells/ml CFDA-labeled LAB cells in distilled water was added to the wells. The microbial cells were allowed to adhere for 1 hr at 37°C, and the wells were washed three times with PBS to remove non-adherent cells. The cells bound to PIM were released and lysed using 1% SDS/0.1 M NaOH solution and incubated for 1 hr at 60°C. After incubation, the fluorescence intensity (excitation, 485 nm; emission, 538 nm) of the lytic solution was measured. PBS was used as the control in place of PIM. The adhesion value was defined as the value of the mucin-LAB immobilized on the plate reduced by the control value. The results are shown as adhesion rates (%). *L. plantarum* LA 318 was used as a positive control. ** Significantly different compared with the control strain (LA 318) ($p<0.01$).
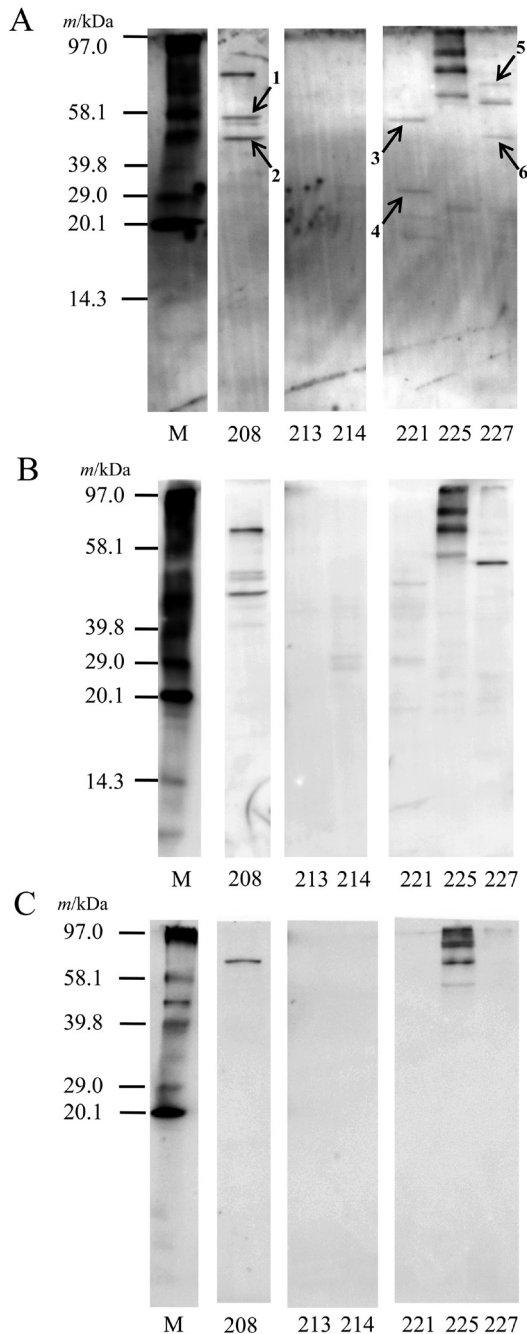
Fig. 3. Detection of adhesins in PBS extracts by a receptor overlay (RO) analysis.

After SDS-PAGE using 1 mg/ml PBS extracts, proteins were electrophoretically transferred to a PVDF membrane. The membrane was blocked with 5% skim milk. After washing, the membrane was soaked in TBS-T containing the biotinylated PIM (A), BSA (control protein) (B), or only TBS-T buffer (negative control) (C) overnight. After washing, protein bands were visualized using ExtrAvidin Alkaline Phosphatase Conjugate and ECF substrate with a luminescent image analyzer (excitation, 440 nm; emission, 560 nm). M: biotinylated molecular weight marker. The numbers (shows arrows) indicate proteins that were identified (Table 2).

MYU 208, MYU 213, MYU 225, and MYU 227 were significantly lower than that of the positive control (5.9–8.3%; $p<0.01$).

Comprehensive analysis of adhesins in PBS extracts from LAB

Figure 3 summarizes the results of the RO analysis for selected LAB strains. A band at up to 58 kDa (No. 1, Fig. 3 and Table 2) was more clear than that of BSA, a control protein. A ca. 48-kDa band (No. 2, Fig. 3 and Table 2) and a weak ca. 40-kDa band bound to PIM as well as BSA in MYU 208 but did not bind to the buffer control. No clear bands were detected in MYU 213 and MYU 214. In MYU 221, ca. 56-kDa (No. 3, Fig. 3 and Table 2) and ca. 31-kDa (No. 4, Fig. 3 and Table 2) bands were detected more clearly in PIM than in BSA and the buffer control. Four high-molecular-weight bands in MYU 225 were detected in PIM, BSA, and the buffer control, indicating nonspecific binding. In MYU 227, three bands were detected; the bands at ca. 72 kDa (No. 5, Fig. 3 and Table 2) and ca. 48 kDa (No. 6, Fig. 3 and Table 2) were mucin specific, while the ca. 64-kDa band was nonspecifc.

Identification of the adhesins of LAB

The mucin-binding proteins were identified using a homology search of N-terminal amino acid sequences, and the results are summarized in Table 2. Twenty amino acid residues were determined in all samples. A ca. 58-kDa band (No. 1, Fig. 3 and Table 2) in MYU 208 was determined to be NH$_2$-(M)AKDIKFSEDARSKMQAGVDQ. This protein was identified as GroEL based on homology comparisons of the N-terminal amino acid sequence in the databases. Two proteins at ca. 48 kDa (No. 2, Fig. 3 and Table 2) in MYU 208 were determined to be NH$_2$-(M)SAITDIYAREVLDSRGNPTV and NH$_2$-(M)AKETYERTKPHVNIGTIGHV. These proteins were identified as enolase and EF-Tu, respectively. In MYU 221, three proteins were observed at ca. 56 kDa (No. 3, Fig. 3 and Table 2) and ca. 31 kDa (No. 4, Fig. 3 and Table 2). The ca. 56-kDa band was determined to be NH$_2$-(M)VQIDNEDLKQVRSEFLDTAK, NH$_2$-MLKSVIENVHALEIFDSRGN, and NH$_2$-(M)SENEKFEQNAYAPMKGIKVI; the ca. 31-kDa band was determined to be NH$_2$-(M)AKLVLIRHGQSEANLTNQFV, NH$_2$-(M)SRTPIIAGNWKLHMNPEQTV, and NH$_2$-MIYTVTVNPALDYVMQLEKV. These proteins were identified as peptidase C1 (PepC), enolase, and formyl-CoA transferase (FRC), respectively, for the ca. 56-kDa protein and phosphoglyceromutase (PGM), triosephosphate isomerase (TPI), and phosphofructokinase (PFK),

Table 2. Identification of mucin binding proteins using a homology search of N-terminal amino acid sequences

Strain	No. identified bands[1]	Determined N-terminal sequences	Homology (%)	Homologous protein	Species	Deduced molecular weight[2]	Accession No.
MYU 208	1	AKDIKFSEDARSKMQAGVDQ	100	GroEL	*W. viridescens* and others	57,300	WP_057745829.1 etc.
	2-1	SAITDIYAREVLDSRGNPTV	100	Enolase	*W. viridescens* and others	47,900–49,100	WP_057746967.1 etc.
	2-2	AKETYERTKPHVNIGTIGHV	90–100	Elongation factor Tu (EF-Tu)	*W. viridescens* and others	43,400	WP_057747191.1 etc.
MYU 221	3-1	VQIDNEDLKQVRSEFLDTAK	100	Peptidase C1 (PepC)	*L. johnsonii* and others	51,500–51,600	WP_004896885.1 etc.
	3-2	MLKSVIENVHALEIFDSRGN	100	Enolase	*L. johnsonii* and others	46,700–46,800	WP_004897160.1 etc.
	3-3	SENEKFEQNAYAPMKGIKVI	75–90	Formyl-CoA transferase (FRC)	*L. johnsonii* and others	50,600	WP_004898834.1 etc.
	4-1	AKLVLIRHGQSEANLTNQFV	89	Phosphoglyceromutase (PGM)	*L. johnsonii* and others	26,100	WP_053106914.1 etc.
	4-2	SRTPIIAGNWKLHMNPEQTV	95–100	Triosephosphate isomerase (TPI)	*L. johnsonii* and others	27,600	WP_012846434.1 etc.
	4-3	MIYTVTVNPALDYVMQLEKV	100	Phosphofructokinase (PFK)	*L. johnsonii* and others	32,500–32,800	WP_012846697.1 etc.
MYU 227	5	SKIIGIDLGTTNSAVAVLQG	95	DnaK	*L. agilis* and others	66,000–67,400	WP_056976892.1 etc.
	6-1	SAITEIYAREVLDSRGNPTV	100	Enolase	*L. agilis* and others	47,800–48,100	WP_050611677.1 etc.
	6-2	AKLIVSDLDVKDKKVLIRVD	100	Phosphoglycerate kinase (PGK)	*L. agilis* and others	42,200–42,900	WP_050611679.1 etc.

[1]See Fig. 3.
[2]Deduced from amino acid sequences (simple protein) by the pI/Mw tool.

respectively, for the ca. 31-kDa protein. In MYU 227, a ca. 72-kDa band (No. 5, Fig. 3 and Table 2) was determined to be NH$_2$-(M)SKIIGIDLGTTNSAVAVLQG. This protein was identified as DnaK. Two proteins were at ca. 48 kDa (No. 6, Fig. 3 and Table 2) in MYU 227 and were determined to be NH$_2$-(M)AKLIVSDLDVKDKKVLIRVD and NH$_2$-(M)SAITEIYAREVLDSRGNPTV. These proteins were identified as enolase and phosphoglycerate kinase (PGK), respectively.

DISCUSSION

Adhesion to the intestinal mucosa is very important for LAB to function effectively as probiotics. There are two types of adhesins on the cell surface, membrane-associated proteins, such as the S-layer protein [12, 13], and the moonlighting proteins bound by ionic bonds. In this study, we focused on moonlighting proteins that can be easily extracted using PBS. We performed a comprehensive analysis of the adhesins in PBS extracts from LAB.

Using three different culture agars, different species were isolated. When using MRS and BL agars, species other than LAB were detected. These differences may be explained by differences in pH. In previous studies, the selectivity of culture agar for LAB was shown to be higher at lower pH values [40, 41]. However, it is necessary to select the culture agar in accordance with the intended use because an excessively low pH may obstruct even the growth of lactobacilli [42–45].

Using SDS-PAGE, various band numbers and banding patterns were observed for PBS extracts from the LAB (Fig. 1). Some strains had only a few or no proteins in PBS extracts. In our previous study, GAPDH enzymatic activity was measured in the PBS extracts of 30 lactobacilli isolated from human intestinal tissues and was detected in 21 out of 30 samples from 12-hr cultures and in all samples at 18 hr [34]. This suggests that in moonlighting proteins, such as GAPDH, accumulation is growth-time dependent. Saad et al. [46] reported that the GAPDH concentration on cell walls is growth-time dependent in L. plantarum 299v. This accumulation of GAPDH on the cell surface is related to cell injury. Similar observations were reported by Kainulainen et al. [47] for four moonlighting proteins, i.e., glutamine synthase (GS), glucose-6-phosphate isomerase (GPI), enolase, and GAPDH, from Lactobacillus crispatus ST1. However, it is not clear how moonlighting proteins are secreted onto the bacterial cell surface. Further investigations are needed to clarify their secretion mechanisms.

In the test of adhesion of selected LAB to PIM, MYU

214 showed high adhesion, but no adhesin was detected in the RO analysis for MYU 214 or MYU 213. This suggests that other membrane-associated proteins, such as S-layer proteins and/or extracellular polysaccharides (EPS), may function as adhesins.

In the comprehensive analysis of adhesins in PBS extracts using RO analysis, some moonlighting adhesins were detected (Fig. 3). In MYU 208, the molecular chaperone GroEL was identified as an adhesin (No. 1, Fig. 3 and Table 2). Bergonzelli et al. [48] reported that the GroEL of Lactobacillus johnsonii La1 (NCC 533) binds to mucin and HT29 cells in a pH-dependent manner and stimulates interleukin-8 secretion in macrophages and HT29 cells in a CD14-dependent manner. Another molecular chaperone, DnaK, was identified in MYU 227 (No. 5, Fig. 3 and Table 2). Katakura et al. [26] reported that many moonlighting proteins show affinity to invertase, a hyperglycosylated mannoprotein from Saccharomyces cerevisiae, and are found in Lactococcus lactis subsp. lactis IL1403. Of the 16 spots of affinity-purified cell wall proteins detected using two-dimensional electrophoresis, 15 were identified as cytosolic proteins, including the molecular chaperones DnaK, GroEL, and GroE, and enzymes in the central metabolic pathway, such as GAPDH, pyruvate kinase (PK), and PFK. These results suggest that DnaK has two independent binding sites, one that is specific to LAB and another that is specific to yeast agglutination.

The ca. 48-kDa band (No. 2, Fig. 3 and Table 2) in MYU 208 was identified as enolase and/or EF-Tu. Based on the molecular weight, enolase may be an adhesin, and EF-Tu may be as well. Both may be detected in a mixed state, as they have been reported as adhesins [29, 31, 48]. Similar results were observed for the ca. 48-kDa band in MYU 227 (No. 6, Fig. 3 and Table 2), which was associated with enolase and/or PGK. In this case, the molecular weight suggests that enolase may also be an adhesin. Sánchez et al. [49] reported that GAPDH and PGK are expressed on the cell surface of Lactobacillus rhamnosus GG but that none of the proteins bind to mucin or fibronectin. A weak band at ca. 40 kDa was also detected in MYU 208. This most likely corresponded to GAPDH, as many reports have shown that 40-kDa GAPDH binds to various substances in the intestinal tract, such as mucin, extracellular matrix, and intestinal epithelial cells [25, 28, 50, 51]. However, the protein could not be clearly identified because the band was very weak and showed nonspecific binding.

Three different proteins were identified in the ca. 56-kDa band (No. 3, Fig. 3 and Table 2) and the ca. 31-kDa band (No. 4, Fig. 3 and Table 2) in MYU 221. The

ca. 56-kDa protein contained PepC, enolase, and FRC. Donkor *et al.* [52] reported amino peptidase activity in both extracellular and intracellular extracts from some *Lactobacillus helveticus* strains, suggesting the presence of general amino peptidases, such as PepC and PepN. Shihata and Shah [53] found similar aminopeptidase activity in many strains of LAB and bifidobacteria at both extracellular and intracellular levels. FRC catalases transfer coenzyme A (CoA) from formate to oxalate in the first step of oxalate degradation and has a CoA binding site [54]. To our knowledge, however, no study has demonstrated that PepC and FRC bind to mucin. Additional experiments using recombinant proteins are needed to demonstrate whether these proteins are adhesins.

PGM, TPI, and/or PFK were identified as candidate adhesins in the ca. 31-kDa band from MYU 221 (No. 4, Fig. 3 and Table 2). PGM is a plasminogen-binding protein in *Bifidobacterium lactis* BI07, as are DnaK, GS, enolase, and bile salt hydrolase [55]. TPI is an adhesin that binds to Caco-2 cells, in addition to GAPDH and EF-Tu in *L. plantarum* 423 [56]. PFK is an adhesin in *L. lactis* subsp. *lactis* IL1403; it binds to mannoprotein [26]. These three proteins may not be separable by SDS-PAGE. They were detected as a single band owing to their highly similar molecular weights. At least one of the proteins is an adhesin that binds to PIM, but additional experiments are needed to clarify whether all three proteins are adhesins.

In this study, 18 LAB were isolated from PIM, and various moonlighting adhesins were detected in the PBS extracts of the LAB based on an RO analysis. These proteins in the PBS extracts, which included such things as molecular chaperones and glycolytic enzymes, may play important roles as adhesins. Recently, many studies have demonstrated that moonlighting proteins bind to various substances, such as mucin [28], A and B blood type antigens [33], sulfated carbohydrates [31], intestinal epithelial cells [56], fibronectin and collagen [50], actin [57], and plasminogen [29]. These reports suggest the importance of moonlighting proteins on the cell surfaces of LAB as adhesins. The binding mechanisms are not clear, but some moonlighting proteins may bind to PIM via mechanisms other than ionic bonds because the moonlighting proteins detected in this study remained after washing with PBS-T, which contained many salts and Tween 20. Previously, we reported that GAPDH may be an *N*-acetylgalactosamine and a galactose recognition lectin-like protein and that the trisaccharide structure is important for binding to blood group antigens [33]. Moreover, the surface GAPDH of *Streptococcus*

pyogenes ATCC 700294 binds to the N-terminal domain (D1) of uPAR (urokinase plasminogen activator receptor)/CD87 as its receptor on human pharyngeal cells, while uPAR-D1 more specifically binds to the C-terminal alpha-helix and two immediate flanking regions of the S-loop of the GAPDH molecule [58]. Streptococcal GAPDH shows multiple binding activities to plasmin(ogen) [59, 60], fibronectin, lysozyme, myosin, and actin [61]. It is difficult to explain these observations of multibinding by ionic bonds alone. Furthermore, the multiple functionalities of moonlighting proteins, other than their functions as adhesins, are enhanced with tPA- and uPA-mediated plasminogen activation [62], protecting LAB from environmental stress [55, 63–65] and stimulating the immune response of the host [48, 51, 66]. We can use these moonlighting proteins as functional markers to select useful probiotic LAB strains in the future. Additional studies are needed to clarify the binding mechanisms and the various functions of moonlighting proteins, including adhesins.

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