A New Assay Using Surface Plasmon Resonance (SPR) to Determine Binding of the Lactobacillus acidophilus Group to Human Colonic Mucin

Hideaki UCHIDA,¹ Kenji FUJITANI,¹ Yasushi KAWAI,¹ Haruki KITAZAWA,¹ Akira HORI,² Kenichi SHIBA,³ Kazuya SAITO,³ and Tadao SAITO¹,¹

¹Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-Amamiyamachi 1-1, Aoba-ku, Sendai, Miyagi 981-8555, Japan
²Department of Molecular Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
³Department of Gastrointestinal and Colorectal Surgery, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

Received August 12, 2003; Accepted February 16, 2004

A new binding assay to investigate the mechanism of adhesion of lactic acid bacteria to the human intestine was established by the surface plasmon resonance technique using a biosensor BIACORE1000. Cells of 26 strains of the Lactobacillus acidophilus group as analytes were eluted onto a sensor chip on which were immobilized biotinylated A-trisaccharide polymer probes having human A-type antigen [(GalNAcα1-3(Fucα1-2)Gal)-] or human colonic mucin of blood type A (HCM-A) as ligands. In the first screening, high affinity adhesion to the A-trisaccharide BP-probe was observed in L. acidophilus OLL2769, L. crispatus JCM8778, LA205 and LA206. In the second screening, which used HCM-A, only L. acidophilus OLL2769 and L. crispatus JCM8778 were selected as adhesive strains with specific binding ability to human A-antigen. The results indicated that some strains of the L. acidophilus group could recognize and bind the sugar chain of A-antigen structure on HCM.

Key words: surface plasmon resonance; Lactobacillus acidophilus; adhesion; human A-antigen

It is generally assumed that the mucus layer overlying digestive tract tissues has a large variety of potential functions, some of which are to protect the host from infection by pathogenic intestinal bacteria or to provide a habitat for useful intestinal bacteria. It has been reported previously that the adhesive reaction of bacteria on the mucous layer is a prerequisite for initial colonization and later proliferation. Although the mechanisms of adhesion on fimbrae and flagella have been extensively investigated for many pathogenic bacteria, there have been few reports on the mechanisms of adhesion of nonpathogenic bacteria such as probiotic lactic acid bacteria (LAB). It has been suggested that lectin-like components in surface-layered proteins (SLPs) of lactobacilli play an important role in adhesion to receptors, such as sugar chains of glycolipids or glycoproteins on the surfaces of intestinal epithelial cells. But little is known about the relationship between such adhesive components on bacterial cells and receptors on the surfaces of intestinal tracts.

The Lactobacillus acidophilus group, L. salivarius, L. casei, L. plantarum, L. reuteri, and L. brevis have been the Lactobacillus species most commonly isolated from the human intestine. The L. acidophilus group has been classified on the basis of DNA sequence similarity and cell wall composition into six subgroups: A1 (L. acidophilus), A2 (L. crispatus), A3 (L. amylovorus), A4 (L. gallinarum), B1 (L. gasseri), and B1 (L. johnsonii). Since the L. acidophilus group is considered to include particularly useful probiotic bacteria like Bifidobacterium, it has been widely used in acidophilus milk and fermented milk products in the expectation that it will be functional as a probiotic through its survival and growth in the gastrointestinal tract.

The mechanism by which L. acidophilus JCM 1026 adheres to the human gastrointestinal tract has been partially clarified. Mukai et al. and Yamada et al. have confirmed, from results of hemagglutination assays, the presence of lectin-like proteins among SLPs of the L. acidophilus group. Such proteinaceous components (lectin-like proteins) in the SLP may contribute to cell adhesion by binding to carbohydrate portions of the colonic mucous layer.

It has been reported that eight kinds of acidic and...
neutral O-glycosidic (mucin-type) sugar chains combined with rat colonic mucin (RCM) and had structures very similar to those isolated from human colonic mucin (HCM). Takahashi et al. in our laboratory reported that the conventional hemagglutination assay is not a suitable method for selecting L. acidophilus strains with strong adhesion to the human intestinal tract, and hence we introduced a screening method using polystyrene beads coated with RCM. L. acidophilus SBT2062 was shown by this method to bind actively to RCM, and binding to human colonic mucosa was confirmed through histochemical staining using human colon tissues fixed with Carnoy’s fixative. But the method could not be used to screen B-group strains of the L. acidophilus group because of non-specific binding of SLP to polystyrene beads. Recently, Matsumura et al. reported a modified screening method using an RCM-coated microtiter plate for evaluating the adhesiveness of the L. acidophilus group to human intestinal tracts. This method was also used for the isolation of lectin-like proteins from the SLPs of L. acidophilus bacteria which are related to the adhesion of bacteria to human colonic mucosa. Recently, Saito et al. have proposed another screening method for the probiotic L. acidophilus group and have demonstrated the availability of carbohydrate probes.

On the other hand, there have been some reports of differences in the carbohydrate chain on HCM in each blood type. The results indicate that sugar chains expressed in HCM must differ depending on blood type. The differences in the carbohydrate chain on HCM in each blood group and the sugar chains on HCM with different blood types has been performed until now.

In this study, we developed a novel LAB binding assay by BIACORE with both carbohydrate probes and HCM of blood type A having an A-antigen structure. This method is useful in determining the interaction between bacterial cells and a human intestinal surface-like in vivo system without the requirement of any labeling process.

**Materials and Methods**

**Bacterial strains and medium.** Twenty-six strains of the L. acidophilus group listed in Table 1 were tested for the binding assay in this study. Seven strains were purchased from the Japan Collection of Microorganisms (JCM, Wako, Japan). Eighteen strains were isolated mainly from human feces of adults and infants in our laboratory and one strain was a gift from the Meiji Dairies Corporation. Our laboratory’s strains (LA) were mainly from human feces of adults and infants in our laboratory and one strain was a gift from the Meiji Dairies Corporation. Our laboratory’s strains (LA) were purchased from the Japan Collection of Microorganisms (JCM, Wako, Japan). Eighteen strains were isolated mainly from human feces of adults and infants in our laboratory.

**Preparation of bacterial cells (analyte).** Bacterial cells were harvested from the cultured MRS medium by centrifugation (3,000 × g, 4 °C, 10 min) and washed twice with HBS–EP buffer [10 mM 2-[4-(2-hydroxyethyl)-1-piperidiny] ethanol sulfonic acid (HEPES) buffer containing 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA), and 0.005% surfactant P20, pH 7.4 (BIACORE K.K., Tokyo, Japan)]. The cells were resuspended in 500 μl of HBS–EP buffer and used as analyte for the binding assay with BIACORE (Biacore K.K.).

**Preparation of HCM.** Normal sections of colon from colon-cancer patients of blood type A were obtained from the Graduate School of Medical Science, Tohoku University. The colon samples were obtained and used in accordance with the medical regulations of Tohoku University after obtaining informed consent from the patients. The mucosa was gently scraped from the colon tissue with a coverslip and collected in ice-cold TBS buffer (50 mM Tris–HCl buffer containing 0.15 M NaCl, pH 7.6) containing proteolytic inhibitors [5 mM disodium EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM N-ethyl maleimide] and then homogenized. The crude HCM was dialyzed against three sequential extractions with chloroform: methanol (2:1, v/v) and diethylether. The delipidated HCM redissolved in 4 M guanidine hydrochloride (GHCl) solution was fractionated by gel filtration chromatography with a Toyopearl HW-65F column (90 cm × 2.6 cm, Tosoh, Tokyo, Japan). Protein was monitored at 280 nm and neutral sugar was detected at 490 nm by the phenol-sulfuric acid method. The fractions containing MRS broth (Difco Laboratories, Detroit, Michigan, USA) with 1% (v/v) inoculum before the experiments.

**Materials and Methods**

**Table 1. Bacterial Strains of L. acidophilus Group Used in This Study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus (A1)</td>
<td>JCM1132&lt;sup&gt;+&lt;/sup&gt; a</td>
<td>L. amylovorus (A1)</td>
<td>JCM2125 b</td>
</tr>
<tr>
<td>L. acidophilus (A2)</td>
<td>JCM1229 b</td>
<td>OLL2769 c</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A3)</td>
<td>LA201 a</td>
<td>LA211 a</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A4)</td>
<td>LA202 a</td>
<td>L. gasseri (B1)</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A5)</td>
<td>LA203 b</td>
<td>JCM1131&lt;sup&gt;+&lt;/sup&gt; b</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A6)</td>
<td>LA204 b</td>
<td>JCM8787 a</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A7)</td>
<td>OLL206 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A8)</td>
<td>JCM1185&lt;sup&gt;+&lt;/sup&gt; a</td>
<td>LA213 c</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A9)</td>
<td>JCM8778 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A10)</td>
<td>LA205 a</td>
<td>LA215 a</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A11)</td>
<td>LA206 a</td>
<td>LA216 a</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A12)</td>
<td>LA207 a</td>
<td>L. johnsonii (B2)</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A13)</td>
<td>LA208 a</td>
<td>LA217 a</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus (A14)</td>
<td>LA209 a</td>
<td>LA219 b</td>
<td></td>
</tr>
</tbody>
</table>

a: human adult feces b: human adult intestine c: human infant feces d: unknown
the largest amounts of sugar were dialyzed against distilled water at 4 °C for 2 days and then lyophilized. The expression of human A-type antigen [(GalNAc-3(Fuc-1-2)Gal)-] in each HCM sample was then checked by the dot-blotting method using Anti-A lectin (Dominion Biologicals Limited, Canada) after periodate oxidation of HCM according to our previous method.5) These samples, labeled HCM-A, were used for the subsequent binding studies with BIACORE.

Immobilization of BP-probes and HCM. A-trisaccharide [GalNAcα1-3(Fucα1-2)Galβ1-4] and B-trisaccharide [Galβ1-3(Fucβ1-2)Galβ1] biotinyl polymer (BP) probes (Seikagaku Co., Tokyo, Japan), each consisting of a biotinylated polyacrylamide body and carbohydrate portion, were directly immobilized as a ligand onto a different flow cell of an SA sensor chip (streptavidin type, Biacore K.K.) by the biotin-avidin binding method.27) A control flow cell was made by the same procedure with an α-L-rhamnose BP-probe (Seikagaku Co.) on the same sensor chip.

Human colonic mucin of blood type A was also immobilized as a ligand on a CM5 sensor chip (carboxymethyl dextran base, Biacore K.K.) by the amine coupling method. The carboxymethylated dextran layer on the CM5 chip surface was activated by an N-ethyl-N′-(3-dimethyl-aminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) mixture to produce NHS-esters that react with radicals of primary amino groups in HCM-A molecules. After immobilization of HCM-A as a ligand, unreacted NHS-esters were deactivated by injection of a large excess of ethanolamine hydrochloride. A control flow cell was made by the same procedure except for the addition of HCM-A on the same sensor chip. The residual ligand that combined non-covalently was washed from the chip surface with a 0.75M GHCl solution. The sensor chip-bound BP-probes and the sensor chip-bound HCM-A were equilibrated in 5 mM sodium acetate buffer (pH 4.0) and HBS buffer (pH 7.4) respectively at a flow rate of 3 μl/min for 5 min at 25 °C.

BIACORE analysis. Bacterial cells (analyte) prepared from the cultured MRS broth (1 ml) were washed three times with HBS–EP buffer (pH 7.4) and then suspended in 1 ml of same buffer. The cell suspension was continuously injected onto the sensor chip, which was attached to a BIACORE 1000 instrument (Biacore K.K.), at a flow rate of 3 μl/min for 20 min at 25 °C and allowed to interact with the bound ligand of the BP-probe or HCM-A.28,29) The sensor chip was washed with HBS-EP buffer to remove unbound cells and then the resonance unit (RU) value was measured. The sensor chip surface was regenerated by elution with a 3 M GHCl solution at a flow rate of 3 μl/min for 2 min. A response of 1,000 RU represents about 1 ng/mm² by a change of concentration of bound analyte on the sensor chip surface.

Results

Isolation of HCM-A

Figure 1 shows a gel filtration chromatogram of crude HCM-A that was prepared from a sample of a normal colon from a colon-cancer patient of blood type A in a 4M GHCl solution. The fraction eluted at number 46 showed the highest concentration of neutral carbohydrate and was estimated to have a molecular weight of approximately 2,000 kDa. No. 46 fraction was dialyzed against distilled water for 2 days at 4 °C and then lyophilized. After immobilization of the HCM sample onto a PVDF membrane, anti A-lectin combined with the HCM. However, after partial destruction of the sugar portion on HCM by periodate oxidation, no adhesion of

Fig. 1. Purification of Human Colonic Mucin (HCM) by Gel Filtration Chromatography.

Human colonic mucin (HCM) from a cancer patient of blood type A was fractionated with a TOYOPEARL HW-65F column (2.6 × 95 cm) with 4 M GHCl solution at 1.0 ml/min under 20 °C. Fractions collected each 5 ml were monitored at absorbancies of 280 nm for protein (●) and 490 nm for neutral sugars (○). An arrow fraction (No. 46) was merely isolated and used for further experiment (HCM-A).
anti A-lectin was observed by the dot-blotting method (data not shown). The samples, labeled HCM-A, were used for the subsequent binding studies with BIACORE.

**Immobilization of BP-probes and HCM-A as ligands**

Figure 2 shows BIACORE sensorgrams of immobilization of four ligands (A-trisaccharide BP-probe, B-trisaccharide BP-probe, \(\alpha\)-L-rhamnose BP-probe, and HCM-A) on different flow cells of sensor chips. The binding values in the SA sensor chip (streptavidin type) combined with the biotinylated A-trisaccharide BP-probe (Fig. 2-A), B-trisaccharide BP-probe (Fig. 2-B), and \(\alpha\)-L-rhamnose BP-probe (Fig. 2-C) were 730, 530, and 250 RU respectively. The binding value in the CM5 sensor chip to which HCM-A was covalently bound by an amine coupling reaction was 3,170 RU (Fig. 2-D).

**Screening of LAB recognizing human A-antigen**

For the first screening, cells of the *L. acidophilus* group (26 strains) were analyzed using BIACORE 1000 with an SA sensor chip on which an A-trisaccharide BP-probe was immobilized as a ligand. Figure 3 shows the profiles of adhesion of 26 strains to human A-antigen of the BP-probe. Cells of four strains of *L. acidophilus* OLL2769, *L. crispatus* JCM8778, LA205, and LA206 were strongly bound to the probe with response signals ranging from 150~8,300 RU. The binding values of *L. crispatus* JCM8778 to the A-trisaccharide BP-probe on the SA sensor chip and to HCM-A on the CM5 sensor chip were 350 and 60 RU respectively (Fig. 4-A and -D). Since no binding on cells of the strain appeared to the \(\alpha\)-L-rhamnose BP-probe as a control (Fig. 4-B), the possibility of non-specific adhesion to the body of the BP-probe except for the sugar portion was excluded. Although an interaction (200 RU) with the B-trisaccharide BP-probe was also observed (Fig. 4-C), the RU value was lower than that for the A-trisaccharide BP-probe (350 RU). These results suggested that the binding of *L. crispatus* JCM8778 recognized the human A-antigen structure more strongly. Although cells of *L. crispatus* LA205 and LA206 bound well with both the A-trisaccharide BP-probe and HCM-A (data not shown), they also showed strong non-specific binding to both an SA sensor chip on which an \(\alpha\)-L-rhamnose BP-probe was immobilized and an intact CM5 sensor chip as a control.

**Discussion**

Since the BIACORE apparatus enables real-time analysis of the interaction between two components without molecular labeling by using a color coupler, biotin, or a second antibody and also enables maintenance of intact molecular structure, the assay using BIACORE is thought to be a superior method for determining actual interactions in organisms.

Several factors should be taken into consideration when using BIACORE to monitor the interactions of whole bacterial cells. The instrument generates a response with changes in the refractive index of the solution close to the gold film on the sensor surface. The effective penetration depth of the evanescent wave that arises under conditions of SPR is 0.3 \(\mu\)m. Therefore, reactive index changes occurring at depths of less than 0.3 \(\mu\)m from the surface will cause a signal change. The bacteria probably do not penetrate the dextran layer 0.1 \(\mu\)m in thickness coating the gold surface. When bacteria bind to ligands bound to the dextran surface, only a small portion of the bacterial cells close to the sensor surface contributes to the elicitation of a response signal. Furthermore, the BIACORE detection system measures the average SPR angle over an area of approximately 0.25 \(\text{mm}^2\) of the sensor surface. Since bacterial cells are large and do not cover the area measured evenly, the response signal decreases.
In this study, we first developed a novel screening method using BIACORE assays of the L. acidophilus group binding to colonic mucin samples from colon-cancer patients of blood type A, the most common blood type in Japan. Only the four strains L. acidophilus OLL2769, L. crispatus JCM8778, LA205, and LA206 (the A1 and A2 subgroups in the L. acidophilus group), were selected for the first screening, on the basis of their strong adhesion to the A-trisaccharide BP-probe, the sugar portion of which has the same structure as that of human A-antigen trisaccharide [GalNAc1-3(Fuc1-2)Gal-].

In BIACORE assays, it has been reported that RU values in studies of bacterial adhesion are usually in the range of 100 to 2,000.26–29) Since L. crispatus JCM8778 did not adhere to the L-rhamnose BP-probe as a control, unlike L. crispatus LA205 and LA206, JCM8778 was thought to bind specifically to the sugar portion of A-trisaccharide. But the strain JCM8778 also showed higher recognition (350 RU) by the A-trisaccharide BP-probe compared to recognition by the B-trisaccharide BP-probe (200 RU). These results suggest that L. crispatus JCM8778 recognized A-antigen about two times as strongly as B-antigen. Both A- and B-antigens have a consensus structure, and the only difference between them lies in their non-reducing terminus (A-antigen, GalNAc-; B-antigen, Gal-). It is not clear whether cells recognize the monosaccharide of the non-reducing terminus or the whole sequence of trisaccharide (A-, B-).

In the second screening, only L. crispatus strain JCM8778 was selected, as a strain that adheres to the A-antigen of HCM-A. The difference between the low RU value (60) on HCM-A and the high RU value (350) on A-trisaccharide perhaps reflected the existing ratio of A-antigen in the ligands of natural HCM (low) and artificial BP-probe (high). These results suggest that the commercially available A-trisaccharide BP-probes used are a good substitute in the first screening before the

**Fig. 3.** The First Screening of L. acidophilus Group Strains by BIACORE Binding Assay Using an A-Trisaccharide BP-Probe as Ligand.

Bacterial cells prepared from cultured MRS broth (1 ml) were suspended in HBS-EP buffer (pH 7.4) and then injected to an A-trisaccharide BP-probe with A-antigen structure. The analysis was done at a flow rate of 3 µl/min for 20 min at 25 °C.

**Fig. 4.** Confirmation of Specific Binding of L. crispatus Strain JCM8778 to Human A-Antigens by BIACORE Analysis.

The four ligands used in this experiment were (A) an A-trisaccharide BP-probe, (B) an α-L-rhamnose BP-probe, (C) a B-trisaccharide BP-probe and (D) an HCM-A. The solid arrow and dotted arrow indicate the injection point of cells of L. crispatus strain JCM8778 and the end injection point, respectively.
second step using HCM, which is not easy to obtain in a general laboratory.

Differences in the carbohydrate chain bound on HCM in each blood type have been reported. These results strongly indicate that sugar chains expressed in HCM differ depending on ABO blood type. We first reported that some strains of the \textit{L. acidophilus} group, especially ones isolated from human intestine, distinguished the sugar chain of \textit{A} antigen structure from that of \textit{B} antigen in this study. This result suggested that the probiotic \textit{L. acidophilus} group combining with the human intestine would be different in persons with different blood types such as \textit{A}-, \textit{B}-, \textit{H}-, and \textit{AB}-antigen on HCM. Therefore, it is important to select a probiotic strain of the \textit{L. acidophilus} group that can match the blood type in each person to the future construction of functional blood-typed yogurt.

We have established a novel bacterial binding assay using BIACORE and oligosaccharide BP-probes that enables simple and rapid detection of the interaction between the \textit{L. acidophilus} group and HCM. Another study of bacterial adhesion using other trisaccharide BP-probes (H- and AB-antigen) and HCM with B-, H- and AB-antigen structure is in progress.

\section*{Acknowledgments}

This work was partially supported by a Grant-in-Aid for Scientific Research (B) (2) (No. 10660254, 12460117) from the Japan Society for the Promotion of Science (JSPS) and the Food Science Institute Foundation (Ryoshoku-Kenkyukai). We thank to Meiji Dairies Corporation (Tokyo, Japan) for gifting \textit{L. acidophilus} OLL2769 strain.

\section*{References}


10) Fujisawa, T., Benno, Y., Yaeshima, T., and Mitsuoka, T., Taxonomic study of \textit{Lactobacillus acidophilus} group, with recognition of \textit{Lactobacillus gallinarum} sp. nov. and \textit{Lactobacillus johnsonii} sp. nov. and synonymy of \textit{Lactobacillus acidophilus} group A3 (Johnson et al., 1980) with the type strain of \textit{Lactobacillus amylovorus} (Nakamura, 1981). \textit{Int. J. Systematic Bacteriol.}, 32, 487–491 (1992).


21) Holgersson, J., Stromberg, N., and Breimer, M. E.,


