A New Screening Method for Probiotic Lactic Acid Bacteria in the *Lactobacillus acidophilus* Group by Using Carbohydrate Probes

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Abstract To select probiotic strains of the *Lactobacillus* (*L.*) *acidophilus* group with high adhesion to human intestinal tracts for developing the functional yogurt, a new screening method using biotinylated carbohydrate probe (BCP) was introduced. After incubation of living cells of 30 strains of the *L. acidophilus* group with 5 kinds of BCPs which possess representative partial structures of the sugar chains constituting of human colonic mucin, the interaction between cell-surface lectins and BCP were evaluated. The highest reactivity was detected in 19 strains (about 63%) with a trisaccharide probe (GalNAc α (1 $\rightarrow$ 3)[Fuc α (1 $\rightarrow$ 2)]Gal β 1 $\rightarrow$). The cell-surface lectin activity was first clearly confirmed in the B group of the *L. acidophilus* group. The adhesion evaluation score (AE score, total sum of each absorbance of 5 BCPs samples) is considered to be useful as an indicator for selection of the probiotic strains which have high adhesiveness in human gastrointestinal tract.

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Key words : *Lactobacillus acidophilus*, Cell-surface lectin, Biotinylated carbohydrate probe, Sugar chain, Surface-layer protein

Lactobacillus (*L.*) *acidophilus* group lactic acid bacteria (LAB) isolated from human feces are considered to survive in the host's gastrointestinal tracts, and to possess beneficial effects in their host's health when they are used in the functional yogurt⁸⁾. The *L. acidophilus* group was classified into two big category of the group A [*L. acidophilus* (A₁ subgroup), *L. crispatus* (A₂), *L. amylovorus* (A₃), *L. gallinarum* (A₄)] and the group B [*L. gasseri* (B₁), *L. johnsonii* (B₂)] by DNA-DNA homology and cell-wall composition analysis^{4,5)}. Recently, *L. gasseri* (B₁ subgroup) is known to be a dominant strain among the *L. acidophilus* group living in human gastrointestinal tracts²⁾.

In the group A strains, the lectin-like proteins in surface layer protein (SLP) which was detected by hemagglutination (HA) test has recognized by Mukai *et al.*⁶⁾ and by us¹⁰⁾ to have an important role for the cell adhesion through binding to carbohydrate portions of the colonic mucous layer. However, the reactivity of the HA test is not so sensitive and the interpretation of adhesion mechanism between cell-surface lectins (receptors) and sugar chains (ligands) on erythrocytes was really complicated.

Recently, we have pointed out that the HA assay was not a good indicator for selecting strains having high adhesion to human intestinal tract and developed the new screening method with combination using

Carnoy's-fixed human colonic mucous layer and polystyrene beads coated with rat-colonic mucin (RCM) instead of human mucin⁹⁾. Although this method was useful to select probiotic LAB, the preparation of RCM and the slices of Carnoy's-fixed human colon is costly and time-consuming.

Biotinylated carbohydrate probe (BCP, commercial name is biotinylated polymeric (BP)-probe) was designed for detection of the carbohydrate binding proteins (lectins) directly on the cell membrane, organelle and in cytoplasm^{1,3)}. Podolsky⁷⁾ has reported the chemical structure of 21 kinds of acidic and neutral O-glycosidic sugar chains (mucin type) combining to human colonic mucin and the most representative sugar chain are as follows.

GalNAc α 1-3Gal β 1-3GalNAc β 1-3Gal β 1-4GlcNAc β 1-3GalNAc-Thr(or Ser)

We selected 5 kinds of BCP probes, which have the same partial structure of mono-, di- and trisaccharide moiety combining the most common sugar chains as shown above. Therefore, the probes were expected to be high-sensitive tool to detect faint lectin activity on cell-surface and to specify the lectin character instead of our former screening method using RCM.

In this paper, we describe a new screening method using BCP that can differentiate LAB strains among the *L. acidophilus* group with high adhesion to human gastrointestinal tracts.

Materials and Methods

Bacteria

Table 1 shows names and origins of 30 strains of the *Lactobacillus* (*L.*) *acidophilus* group of LAB used in this study according to the recent classification^{4,5)}. Fourteen strains were purchased from the Japan Collection of Microorganisms (JCM, Wako, Japan) and another strains were isolated from mainly human feces in Technical Research Institute of Snow Brand Milk Products Co., Ltd. (SBT, Kawagoe, Japan) or in our laboratory (LA). *L. acidophilus* SBT2062 (A₁ subgroup) was a selected a strain which showed high adhesion to RCM-coated polystyrene beads reported in our previous paper⁹⁾.

Materials

Table 1. Names and origins of 30 strains of *Lactobacillus acidophilus* group lactic acid bacteria used in this experiment

Subgroup	Strains (Origin)
A group	
<i>L. acidophilus</i> (A ₁)	JCM 1132 ^T (a), SBT 279 (a), SBT 2062 (c), LA 67(a)
<i>L. crispatus</i> (A ₂)	JCM 1185 ^T (f), JCM 8778 (a), SBT 265 (a), SBT 266 (a), LA 87 (a), LA 135 (a)
<i>L. amylovorus</i> (A ₃)	JCM 1126 ^T (j), JCM 1034 (b), JCM 2125 (b), SBT 264 (a), SBT 10021 (a)
<i>L. gallinarum</i> (A ₄)	JCM 2011 ^T (g), JCM 8782 (i), SBT 316 (d)
B group	
<i>L. gasseri</i> (B ₁)	JCM 1131 ^T (b), JCM 1025 (b), JCM 8787 (a), SBT 267 (a), SBT 269 (a), LA 39 (a), LA 158 (a), LA 187 (a)
<i>L. johnsonii</i> (B ₂)	JCM 2012 ^T (h), JCM 2122 (e), JCM 8793 (k), SBT 277 (a)

T : type strain

(a) : human feces, (b) : human intestine, (c) : unknown, (d) : commercial yogurt, (e) : sour milk, (f) : human eye, (g) : chicken crop, (h) : human blood, (i) : chicken feces, (j) : cattle waste-corn fermentation, (k) : pig feces.

Five kinds of BCP were purchased from Seikagaku Industries Co., Ltd., (Tokyo, Japan) as follows : GlcNAc (N-acetylglucosamine) β 1 $\rightarrow$ probe, NeuAc (N-acetylneuraminic acid) α 2 $\rightarrow$ probe, Gal (D-galactose) β (1 $\rightarrow$ 4) GlcNAc (N-acetyllactosamine, LacNAc) β 1 $\rightarrow$ probe, GalNAc (N-acetylgalactosamine) α (1 $\rightarrow$ 3) Gal β 1 $\rightarrow$ probe and GalNAc α (1 $\rightarrow$ 3) [Fuc (L-fucose) α (1 $\rightarrow$ 2)]-Gal β 1 $\rightarrow$ probe. Each probe had a partial structure of the most representative O-type sugar chain combined to human colonic mucin⁷⁾ as described above. Sugar content, biotin content and molecular weight of each probes were 20 mol.%, 5 mol.% and 30–40 kDa, respectively. Each BCP (1 mg) was dissolved in 2.0 ml of 10 mM sodium phosphate buffer containing 0.05% (w/v) NaCl (PBS,

pH 7.2), then stored at -20°C until using.

Preparation of 3 types of cells from *L. acidophilus* SBT 2062 strain

L. acidophilus SBT 2062 strain was successively propagated 3 times in Lactobacilli MRS broth (Difco Laboratories, Detroit, U.S.A.) at 37°C for 24 hrs. After centrifugation (8,000 rpm, 20 min) of the culture, precipitated cells were washed 3 times with PBS followed by water, and then lyophilized. The lyophilized cells (15 mg) were suspended in 2.0 ml of PBS ($\text{OD}_{660} \approx 2.0$) and swelled for 1 hr at room temperature (Sample A). After centrifugation of a part of the Sample A (0.5 ml), cells were treated with 4 M guanidine hydrochloride (GHCl) solution (1 ml) at 37°C for 2 hrs with gentle shaking to remove SLP. The cells were recovered by centrifugation (10,000 rpm, 10 min), washed 3 times with PBS followed by water, and then resuspended in 0.5 ml of PBS ($\text{OD}_{660} \approx 2.0$, Sample B). Another part of the sample A (0.5 ml) was heated at 65°C for 10 min (Sample C). These 3 types of cells (Sample A, B and C) were subjected to the binding assay with BCP.

Binding assay with BCP to 3 types of *L. acidophilus* SBT 2062 cells

Each BCP solution ($5\mu\text{l}$) was added to a cell-suspension of the Sample A, B and C ($20\mu\text{l}$) prepared from *L. acidophilus* SBT 2062. After incubation at 37°C for 2 hrs with mixing at every 15 min, the supernatant was removed by centrifugation (8,000 rpm, 20 min). Precipitated cells were washed 3 times with PBS followed by water, and then were resuspended in water ($20\mu\text{l}$). After mixing with $5\mu\text{l}$ of Horse Radish Peroxidase (HRP) conjugated streptoavidin (SA, 2,000 fold dilution with water, Zymed Laboratories INC., San Francisco, U.S.A.), the samples were incubated at 4°C for 1 hr with mixing at every 10 min. After rinsing of cells with PBS followed by water at 3 times, the cells were mixed with tetramethylbenzidine solution ($100\mu\text{l}$, TMBZ color-developing reagent kit, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and left for 10 min. After adding $100\mu\text{l}$ of the stopping solution, the supernatant obtained by centrifugation were transferred to a well of a microtiter plate (96-wells, Sumitomo Bakelite Co., Ltd.) and its absorbance was measured at 450 nm by a

microtiter plate reader Model 450 (Bio-Rad Laboratories Inc., California, U.S.A.). Three cell samples without addition of BCP were also reacted with HRP-SA and then TMBZ solution, and absorbance of the supernatant were set as blank.

Binding assay with BCP to 30 strains of the *L. acidophilus* group

The following operation was performed with 30 strains of the *L. acidophilus* group of LAB listed in Table 1. Each lyophilized cell (7.5 mg) prepared from 30 strains was suspended in PBS ($\text{OD}_{660} \approx 2.0$) and swelled for 1 hr at room temperature. These samples ($20\mu\text{l}$) were reacted with 5 kinds of BCP solution ($5\mu\text{l}$) and then with HRP-SA and TMBZ, followed by OD_{450} measurement under the same conditions as above mentioned. These experiments were repeated three times and the absorbance was expressed mean $\pm$ standard deviation. ($\bar{X} \pm \text{SD}$).

Results

Binding assay with 5 kinds of BCP to 3 types of cells from *L. acidophilus* SBT 2062 was intended to reveal the binding specificity of the probes to cell-surface of *L. acidophilus* strains. Figure 1 shows the change of binding ability on each BCP against the 3 type of cells (cell A (intact cells, white bar), cell B (cells after GHCl treatment, shaded bar) and cell C (cells after heating, black bar)). The binding ability of the probes was represented as "relative binding intensity" [$(\text{OD}_{\text{cell B or C}} / \text{OD}_{\text{cell A}}) \times 100\%$] that was calculated from each absorbance of cell samples at 450 nm.

In the cell B (shaded bar), the binding of BCP except a NeuAc-probe extremely decreased after GHCl treatment (36–63%). The result indicates that a part of binding receptors on the cell-surface was removed by the GHCl extraction treatment. In a sole increasing effect on the binding with a NeuAc-probe after GHCl treatment, binding sites may newly appear on the cell-surface after partial removal of SLP. In the cell C (black bars), the binding ability of all probes markedly decreased after heating (36–67%). The result indicates that the binding receptors locating on the cell surface were proteinaceous lectin(s) and also partially denatured or inactivated by the heating treatment. The specific binding between

Fig. 1. The change of binding activity of 5 kinds of BCP on the cells of *L. acidophilus* SBT 2062 strain after guanidine hydrochloride extraction and heating treatment.

White bar, no treatment (intact cells) ; Shaded bar, guanidine hydrochloride (GHCl) extraction ; Black bar, heating treatment (65°C, 10 min).

cell-surface lectins and BCP, which was observed in this strain of *L. acidophilus* SBT 2062 (Fig. 1), was considered to be able to apply to other strains in Table 1.

After the preliminary experiments (Fig. 1), the binding assay with 5 kinds of BCP to cell-surface lectins of the *L. acidophilus* group was performed. Figure 2 shows the ranking of well reacted 10 strains plus *L. acidophilus* SBT 2062 strain and the relative reactivity on the assay with the probes to the cells from 30 strains of the *L. acidophilus* group. The highest reactivity to all probes was observed in a strain of *L. gallinarum* JCM 8782 (A_4 subgroup, from chicken feces), therefore each absorbance was expressed as "relative reactivity" after the absorbance of JCM 8782 was set to 100%. All 30 strains reacted with more than at least one of the probes tested (each data not shown). The binding tendency of each probe to cell-surface lectins could not be classified among subgroups ($A_1 \sim B_2$) of the *L. acidophilus* group and apparently varied among 30 strains (strain-specific). The reactivity was increased in proportion to the

molecular weight of sugar portion of BCP, especially a GalNAc α (1→3)[Fuc α (1→2)]Gal(trisaccharide) β 1→probe was highly reacted ($OD_{450}=0.5-0.6$) with 19 strains (about 63%). The lectin activity on the cell-surface in the group B of the *L. acidophilus* group was first proposed in this report. These results suggest that not only the A group but the B group of the *L. acidophilus* group possess cell-surface lectins which can participate in binding with sugar chains of human colonic mucin.

Table 2 shows the ranking (best 5) and the "Adhesion Evaluation score (AE score)" for the selected 5 strains of *L. acidophilus* group with high adhesion ability calculated from the results (Fig. 2) by the BCP method. The final AE score in each strain was calculated from the sum of each absorbance from 5 BCP samples. The 5 strains (JCM 8782, 2011, 8787, 2012 and 2125) which has high evaluation score more than 2.000 was expected to be a probiotic LAB for the functional yogurt.

Fig. 2. The ranking of well-reacted 10 strains with 5 kinds of BCP among the cells from 30 strains of the *L. acidophilus* group LAB.

White bar, the A group strain ; Black bar, the B group strain.

The relative reactivity (%) of the strain of *L. acidophilus* SBT 2062 was shown as a reference data.

Table 2. Ranking (Best 5) and adhesion evaluation score of *L. acidophilus* group LAB selected with high adhesion ability and calculated from BCP binding assay

Ranking	Strains	OD ^a GlcNAc β 1-	OD NeuAc α 2-	OD Gal β 1-4- GlcNAc β 1-	OD GalNAc- α 1-3 Gal β 1-	OD GalNAc α 1-3- [Fuc α 1-2]-Gal β 1-	Adhesion ^b Evaluation score
1	<i>L. gallinarum</i> JCM 8782 (A ₄)	0.49 $\pm$ 0.09	0.52 $\pm$ 0.08	0.59 $\pm$ 0.02	0.58 $\pm$ 0.12	0.61 $\pm$ 0.04	2.784
2	<i>L. gallinarum</i> JCM 2011 ^T (A ₄)	0.47 $\pm$ 0.05	0.52 $\pm$ 0.08	0.39 $\pm$ 0.02	0.50 $\pm$ 0.09	0.59 $\pm$ 0.01	2.464
3	<i>L. gasseri</i> JCM 8787 (B ₁)	0.45 $\pm$ 0.07	0.36 $\pm$ 0.02	0.40 $\pm$ 0.04	0.51 $\pm$ 0.04	0.50 $\pm$ 0.08	2.225
4	<i>L. johnsonii</i> JCM 2012 ^T (B ₂)	0.49 $\pm$ 0.07	0.37 $\pm$ 0.06	0.49 $\pm$ 0.07	0.28 $\pm$ 0.04	0.53 $\pm$ 0.02	2.160
5	<i>L. amylovorus</i> JCM 2125 (A ₃)	0.40 $\pm$ 0.11	0.33 $\pm$ 0.09	0.46 $\pm$ 0.05	0.26 $\pm$ 0.13	0.56 $\pm$ 0.01	2.009

^a OD represents the absorbance ($\bar{X} \pm SD$) at 450 nm by using each biotinylated carbohydrate probe (BCP).

^b Adhesion Evaluation score (AE score) = The sum of each absorbance from 5 BCP samples.

Discussion

In our previous report on the lectin screening¹⁰⁾ in the *L. acidophilus* group using ovine erythrocytes, the most cells in the group A strains showed the hemagglutination (HA) activity, but no active strains were detected in the group B strains. The results led the conclusion that the A group strains of the *L. acidophilus* group have cell-surface lectins which expect to combine to colonic mucosa, but the adhesion mechanism in the group B strains remained unclear. In our later screening method⁹⁾ of the combination with rat colonic mucin (RCM) coated polystyrene beads and Carnoy's-fixed human colonic tissue, HA assay was evidenced to be a no good indicator for selection of the strains with high adhesion to the human intestinal tracts. Although the RCM beads method was useful for the mass-screening of strains in the group A, it could not still determine the adhesion activity of the B group *L. acidophilus* strains, because of the less existence and weak activity of lectins on their cell-surface and also high background problem of the beads method. Moreover, as it is not so easy to obtain RCM from rat's colon (a few mg from colons of 100 rats) and human normal colon tissue, BCP which is easy to obtain as commercial reagents is prominent.

In this report, the location of the cell-surface lectins in the group B of the *L. acidophilus* group was first confirmed through "the BCP method". This new method using 5 kinds of BCPs is expected to be a good technique for the selection of high adherent strains to the carbohydrate portion of human colonic mucin. The selected strains (Table 2) with high AE score were considered to serve in dairy, medical and pharmaceutical field as probiotic LAB having strong possibility for initial adhesion and the later colonization ability in human gastrointestinal tracts.

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References

- 1) Abramenko IV, Gluzman DF, Korchagina EYu, Zemlyanukhina TV, Bovin NV. Oligosaccharide-binding molecules on the surface of human hemopoietic and lymphoid cells. *FEBS Letters*, 307 : 283-286. 1992.
- 2) Benno Y, Endo K, Mizutani T, Namba Y, Komori T, Mitsuoka T. Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. *Applied and Environmental Microbiology*, 55 : 1100-1105. 1989.
- 3) Coppee I, Gabius HJ, Danguy A. Histochemical analysis of carbohydrate moieties and sugar-specific acceptors in the kidneys of the laboratory mouse and the golden spiny mouse (*Acomys russatus*). *Histology and Histopathology*, 8 : 673-683. 1993.
- 4) Johnson JL, Phelps CF, Cummins CS, London J, Gasser F. Taxonomy of the *Lactobacillus acidophilus* group. *International Journal of Systematic Bacteriology*, 30 : 53-68. 1980.
- 5) Lauer E, Helming C, Kandler O. Heterogeneity of the species *Lactobacillus acidophilus* (Moro) Hansen and Moquot as revealed by biochemical characteristics and DNA-DNA hybridisation. *Zentralblatt für Bakteriologie, Mikrobiologie, und Hygiene. 1. Abt, Originale, C, Allgemeine, angewandte, und ökologische Mikrobiologie C1* : 150-168. 1980.
- 6) Mukai T, Arihara K, Itoh H. Lectin-like activity of *Lactobacillus acidophilus* strain JCM 1026. *FEMS Microbiology Letters*, 98 : 71-74. 1992.
- 7) Podolsky DK. Oligosaccharide structure of human colonic mucin. *Journal of Biological Chemistry*, 260 : 8262-8271. 1985.
- 8) Sellars RL. *Acidophilus* products. In : *Therapeutic Properties of Fermented Milks*. (Robinson RK. ed) 81-116. Elsevier. New York. 1991.
- 9) Takahashi N, Saito T, Ohwada S, Ota H, Hashiba H, Itoh T. A new screening method for the selection of *Lactobacillus acidophilus* group lactic acid bacteria with high adhesion to human colonic mucosa. *Bioscience, Biotechnology and Biochemistry*, 60 : 1434-1438. 1996.
- 10) Yamada M, Saito T, Toba T, Kitazawa H, Uemura J, Itoh T. Hemagglutination activity of *Lactobacillus acidophilus* group lactic acid bacteria. *Bioscience, Biotechnology and Biochemistry*, 58 : 910-915. 1994.