[Chem. Pharm. Bull. 31(5)1688—1691(1983)]

Selectivity of Utilization of Galactosyl-Oligosaccharides by Bifidobacteria[1)]

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(Received September 13, 1982)

Isogalactobiose (β-D-galactopyranosyl β-D-galactopyranoside), galsucrose (β-D-fructofuranosyl α-D-galactopyranoside) and lactosucrose (*O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-glucopyranosyl β-D-fructofuranoside) were synthesized as potential sugar sources that selectively enhance the growth of bifidobacteria in the human intestines. Isogalactobiose was synthesized by means of the Koenigs–Knorr reaction and the others by using levansucrase. The structures of these sugars were confirmed by enzymic hydrolysis. All of the sugars synthesized were utilized by bifidobacteria, but not by strains of *Lactobacillus acidophilus*, *Streptococcus faecalis* and *Escherichia coli*. However, isogalactobiose was utilized by 65%, galsucrose by 62% and lactosucrose by 41% of thirty-seven strains of *Enterobacteriaceae* isolated from nineteen adults, while raffinose, which had seemed to be the best among commercially available sugars tested, was utilized by 49% of the strains.

Keywords——isogalactobiose; galsucrose; lactosucrose; *Enterobacteriaceae*; bifidus flora; selective utilization; *Lactobacillus acidophilus*; *Streptococcus faecalis*; bifidobacterium

Bifidobacteria, main constituents of the normal intestinal flora of humans, are considered to be beneficial to health. Therefore in order to establish a bifidus flora, where bifidobacteria are predominant over other bacteria in the intestines, research has been done on vitamin-like substances for bifidobacteria, such as bifidus factors.[2)] Hirata reported that not only such vitamin-like substances but also a sufficient energy supply (sugars) were necessary to establish the bifidus flora.[3)] Therefore we have tried to find suitable sugars which will increase the number of resident bifidobacteria in the human intestines. For this purpose, *Bifidobacterium infantis* was mainly used as a representative of bifidobacteria; *Escherichia coli*, *Lactobacillus acidophilus* and *Streptococcus faecalis* were used as representatives of other bacteria competing for sugars in the jejunum. Previous research[1)] has shown that: (1) non-reducing saccharides tend to be hardly utilizable by *L. acidophilus*, *S. faecalis* and *E. coli*; (2) bifidobacteria start to consume di- or trisaccharides containing galactose without any period for adaptation; (3) bifidobacteria generally utilize mono- to tetrasaccharides; (4) as a rule, sugars consisting of galactose, glucose and fructose are fully utilized by bifidobacteria. Among commercial sugars, inulin oligomer and stachyose are consistent with items (1), (3) and (4), but bifidobacteria needed several hours to adapt to the sugars;[1b)] raffinose meets all four criteria, but raffinose was reported to be utilized by some strains of *E. coli*.[4)] Therefore, these sugars are not sufficiently selective and effective for bifidobacteria. In this study we synthesized isogalactobiose (β-D-galactopyranosyl β-D-galactopyranoside), galsucrose (β-D-fructofuranosyl α-D-galactopyranoside) and lactosucrose (*O*-β-D-galactopyranosyl-(1→4)-*O*-α-D-glucopyranosyl β-D-fructofuranoside), and these sugars were tested for selectivity, first using the same strains of bacteria as in the previous report,[1)] and then using strains of *Enterobacteriaceae* including species *E. coli*.[4)]

Experimental

Organisms——*B. infantis* S-12, *B. longum* E144b and *B. adolescentis* E288b were kindly supplied by Prof. Mitsuoka, Faculty of Agriculture, University of Tokyo, and *L. acidophilus* IAM1034, *S. faecalis* IAM-

10067 and *E. coli* K-12 were supplied by The Institute of Applied Microbiology, University of Tokyo. Thirty-seven strains of *Enterobacteriaceae* were isolated from feces of nineteen healthy men using DHL agar medium (Eiken Chemical Co., Ltd., Tokyo) and classified into genera and species using API 20E (API SYSTEM Motalieu France).

Enzymes——Levansucrase (EC 2.4.1.10) was prepared from a culture of *Bacillus subtilis*.[5] α-Galactosidase (EC 3.2.1.22) from *Mortierella vinacea* and α-glucosidase (EC 3.2.1.20) from yeast were purchased from Seikagaku Kogyo Co., Ltd., Tokyo. β-Galactosidase from *E. coli* (EC 3.2.1.23) was purchased from Sigma Chemical Co., U.S.A. β-Glucosidase (EC 3.2.1.21) from sweet almond was purchased from Miles Laboratories Inc., U.S.A. β-Fructosidase (EC 3.2.1.26) from yeast was purchased from Boehringer Mannheim-Yamanouchi Co., Ltd., Tokyo.

Synthesis of Sugars——(1) In the presence of mercuric cyanide, 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose reacted with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in nitromethane to form octa-*O*-acetyl-isogalactobiose (Koenigs–Knorr reaction). Isogalactobiose was obtained by deacetylation of the acetate with methanolic sodium methoxide. (2) According to the method of Yamamoto,[6] the reaction mixture, containing 1.0 g of sucrose, 10.0 g of galactose and levansucrase (6 units) in 8 ml of 80 mM phosphate buffer, pH 6.0, was incubated for 48 h at 30°C. Then the reaction mixture was applied to a column of charcoal, and the obtained disaccharide fraction was concentrated to a sirup. Crystalline galsucrose was obtained by leaving an aqueous solution in a refrigerator. (3) The reaction mixture, containing 1.0 g of sucrose, 20.0 g of lactose and levansucrase (6 units) in 70 ml of 80 mM phosphate buffer, pH 6.0, was incubated for 48 h at 30°C, then applied to a column of charcoal, followed by a column of Sephadex G-15 (Pharmacia Fine Chemicals, Sweden), to obtain the trisaccharide fraction. Crystalline lactosucrose powder was obtained from ethanol.

Enzymic Confirmation of Sugars——A mixture of 0.2 mg of sugar and enzyme (0.1 units) in 25 μl of 0.1 M buffer was incubated for 48 h at 37°C. The buffer was 0.1 M acetate, pH 5.5, for α-galactosidase and β-fructosidase, and 0.1 M phosphate, pH 7.5, for β-galactosidase. In the case of β-galactosidase plus α-glucosidase or β-galactosidase plus β-glucosidase, 0.1 M citrate buffer, pH 6.0, was used. After the incubation, the mixtures were spotted on pre-coated high-performance thin-layer chromatography (HPTLC) plates of Silica gel 60 (E. Merck, West Germany), and developed with a mixture of *n*-propanol–H_2O (85: 15, v/v); sugars were detected with anisaldehyde-sulfuric acid reagent.[7]

Utilization of Sugars by Microorganisms——The assay media (as double strength) for *B. infantis*, *B. longum*, *B. adolescentis*, *S. faecalis* and *Enterobacteriaceae* including *E. coli* were the same as those reported previously.[1a] The medium for *L. acidophilus* (as double strength) contained (per liter) liver extract solution prepared from 11.0 g of liver powder (Kyokuto Seiyaku Kogyo Co., Tokyo) and 950 ml of H_2O, 20.0 g of proteose peptone No. 3 (Difco Laboratories), 10.0 g trypticase peptone (BBL, U.S.A.), 6.0 g of yeast extract (Difco Laboratories), 2.0 g of Tween 80, 0.4 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of NaCl, 0.12 g of $MnSO_4$ and 0.4 g of cysteine (adjusted to pH 6.5). Sugar solution (2 mg/ml) was filtered through a membrane filter, HA type (Millipore Co., U.S.A.), and the assay medium was autoclaved. A mixture of equal volumes of the two was inoculated with 10^4 to 10^5 cells of each bacterium. *B. infantis*, *B. longum* and *B. adolescentis* were cultured for 4 d at 37°C in N_2–CO_2 (9 : 1). Other bacteria were cultured for 2 d at 37°C in air, as in the previous report.[1a] The culture medium was well mixed and the increase in absorbance at 650 nm (light path: 1 cm) was measured as an indication of growth response to the sugars.

Results

Each synthesized sugar gave only one spot on the HPTLC plate, and was non-reducing to Fehling's solution. It was found that isogalactobiose was not hydrolyzed by α-galactosidase but was by β-galactosidase (Fig. 1(a)). Galsucrose was not hydrolyzed by β-galactosidase but was by α-galactosidase or β-fructosidase (Fig. 1(b)). Lactosucrose was hydrolyzed by β-galactosidase and β-fructosidase to give mono- and disaccharide in both cases, and by β-galactosidase plus α-glucosidase to give monosaccharides only (Fig. 1 (c)). Thus, the structures of the synthesized sugars were confirmed.

Utilizations of the sugars by *B. infantis*, *B. longum*, *B. adolescentis*, *L. acidophilus*, *S. faecalis* and *E. coli* are shown in Table I. Lactose was utilized by all bacteria. In contrast, isogalactobiose, galsucrose and lactosucrose as well as raffinose were utilized only by bifidobacteria. Moreover, the three sugars were fully utilized by bifidobacteria, and faster growth of the bacteria was observed with lactosucrose than with raffinose or lactose.

Utilization of the sugars by *Enterobacteriaceae* is shown in Table II. If we assume that a sugar which gives growth equivalent to more than 25% of that obtained with glucose is utilizable by a strain, lactose was utilized by 68%, raffinose by 49%, isogalactobiose by 65%,

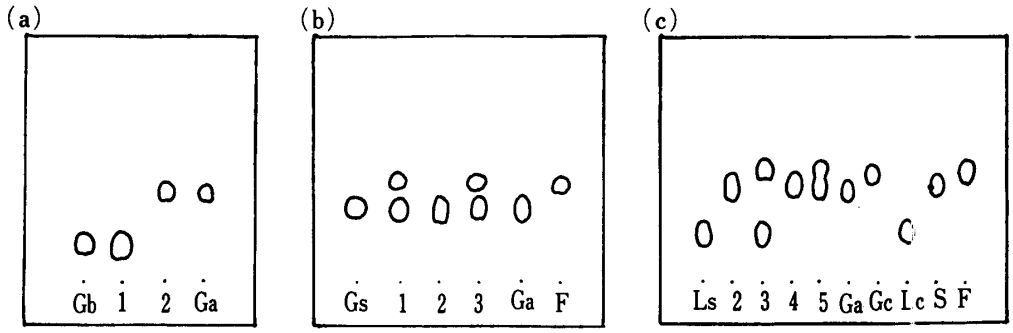

Fig. 1. TLC of Enzymic Hydrolyzates of Oligosaccharides

Isogalactobiose (a), galsucrose (b) and lactosucrose (c) were incubated with α-galactosidase 1), β-galactosidase (2), β-fructosidase (3), β-galactosidase plus β-glucosidase (4) and β-galactosidase plus α-glucosidase (5). The procedure is described in "Experimental."

Gb, isogalactobiose; Gs, galsucrose; Ls, lactosucrose; Ga, galactose; F, fructose; Gc, glucose Lc, lactose; S, sucrose.

TABLE I. Utilization of Sugars by *B. infantis, B. longum, B. adolescentis, L. acidophilus, S. faecalis* and *E. coli*

Organism	Gc	Lc	R	Gb	Gs	Ls
B. infantis	0.52[a)]	0.48	0.41	0.62	0.68	0.62
B. longum	0.57	0.46	0.46	0.58	0.36	0.58
B. adolescentis	0.56	0.53	0.51	0.24	0.37	0.39
L. acidophilus	0.49	0.47	0.00	0.00	0.00	0.01
S. faecalis	0.24	0.14	0.00	0.03	0.02	0.00
E. coli	0.46	0.39	0.00	0.07	0.04	0.01

a) Absorbance at 650 nm.
Gc, glucose ; Lc, lactose ; R, raffinose ; Gb, isogalactobiose ; Gs, galsucrose ; Ls, lactosucrose.

galsucrose by 62% and lactosucrose by 41% of the strains tested. Some strains of *Escherichia* and all strains of *Klebsiella* utilized all the sugars, including lactosucrose and raffinose. On the other hand, the two strains of *Citrobacter*, strain Nj3 and Mn2, which can produce H_2S gas, did not utilize lactosucrose, but utilized raffinose. Lactose and lactosucrose were utilized by half the strains of *Enterobacter*, while the other sugars were well utilized by almost all strains of the genus. *Hafnia* could not utilize any of the sugars.

Discussion

As expected, all three sugars which met the four criteria described in the introduction passed the screening test (Table I), indicating that non-reducing saccharides are hardly utilized by *S. faecalis, L. acidophilus* and *E. coli*. Yoshioka reported that five strains among twenty-two strains of *Escherichia* isolated from feces of infants (23%) utilized raffinose.[4)] Using *Escherichia* strains isolated from feces of adults, we found that four strains among twenty-one (19%) utilized raffinose. Thus, it appears that there is no difference in usability of raffinose between strains originating from infants and from adults.

Lactosucrose was somewhat superior to raffinose as regards selectivity. Thus, lactosucrose may enhance bifidobacteria and perhaps establish a bifidus flora in *in vivo* experiments.

The utilization of raffinose and the synthesized sugars by bacteria of *Enterobacteriaceae* other than *Escherichia* (Table II) indicates the necessity of adding several genera of *Enterobacteriaceae* to the screening system of Table I, and further, of studying *Enterobacteriaceae* in detail to find and characterize sugars which are hardly utilized by the genera.

TABLE II. Utilization of Sugars by *Enterobacteriaceae*

Genus	Species	Strains	Gc	Lc	R	Gb	Gs	Ls
Escherichia	*coli*	Nj2	0.43[a)]	0.41	0.07	0.42	0.46	0.02
		Yy1	0.41	0.45	0.01	0.35	0.28	0.13
		Tk2	0.45	0.00	0.47	0.02	0.01	0.00
		Dn1	0.47	0.00	0.00	0.38	0.43	0.00
		Ks1	0.38	0.52	0.00	0.38	0.41	0.04
		Ks2	0.34	0.00	0.01	0.08	0.00	0.01
		Hn1	0.46	0.31	0.00	0.01	0.01	0.00
		Na1	0.50	0.00	0.00	0.01	0.00	0.00
		Na2	0.47	0.48	0.00	0.39	0.26	0.00
		Ha1	0.48	0.42	0.42	0.42	0.40	0.45
		Za1	0.35	0.02	0.01	0.02	0.01	0.01
		Za2	0.43	0.06	0.00	0.01	0.01	0.00
		Mn1	0.48	0.45	0.00	0.01	0.00	0.00
		Ai1	0.48	0.00	0.00	0.01	0.00	0.00
		Ai2	0.50	0.40	0.14	0.50	0.16	0.49
		Wb1	0.45	0.48	0.39	0.27	0.35	0.64
		Mo1	0.48	0.59	0.00	0.02	0.00	0.17
		Mo2	0.54	0.35	0.01	0.29	0.01	0.01
		Ao1	0.48	0.46	0.00	0.40	0.47	0.00
		Ao2	0.22	0.30	0.00	0.00	0.00	0.00
		My1	0.38	0.41	0.00	0.04	0.00	0.00
Klebsiella	*pneumoniae*	To1	0.32	0.23	0.31	0.28	0.29	0.35
		Tk1	0.42	0.58	0.46	0.35	0.42	0.53
		Ks3	0.54	0.58	0.42	0.51	0.58	0.52
		Zt1	0.38	0.16	0.64	0.60	0.54	0.48
Klebsiella	*oxytoca*	Hn2	0.43	0.32	0.51	0.48	0.34	0.34
		Zt2	0.44	0.31	0.51	0.42	0.34	0.29
Citrobacter	*freundii*	Nj3	0.32	0.21	0.16	0.28	0.38	0.00
		To2	0.40	0.01	0.44	0.28	0.44	0.30
		Mn2	0.32	0.13	0.30	0.04	0.39	0.01
Enterobacter	*aerogenes*	Nj1	0.48	0.11	0.54	0.44	0.32	0.04
Enterobacter	*cloacae*	Ki2	0.41	0.00	0.44	0.35	0.21	0.01
		Ki3	0.37	0.00	0.35	0.33	0.15	0.29
		Se1	0.35	0.09	0.37	0.36	0.44	0.40
		Wb2	0.52	0.48	0.48	0.30	0.43	0.53
Enterobacter	*agglomerans*	Ki1	0.34	0.39	0.00	0.26	0.00	0.00
Hafnia	*alvei*	Zt3	0.35	0.00	0.00	0.00	0.00	0.00

a) Absorbance at 650nm.

Gc, glucose; Lc, lactose; R, raffinose, Gb, isogalactobiose; Gs, galsucrose; Ls, lactosucrose.

Acknowledgement The authors thank Prof. S. Tejima, Dr. M. Mori and T. Chiba of Nagoya City University for helpful advice. Thanks are also due to Dr. T. Takayanagi, Daiichi Seiyaku Co., Ltd., Tokyo, for useful advice.

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