Chem. Pharm. Bull.
33(2) 710—714 (1985)

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

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(Received May 17, 1984)

Neogalactobiose (α-D-galactopyranosyl β-D-galactopyranoside), β-D-galactopyranosyl β-D-glucopyranoside, β-D-galactopyranosyl α-D-glucopyranoside, α-D-galactopyranosyl β-D-glucopyranoside (GII), and α-D-galactopyranosyl β-D-fructofuranosyl-(2→6)-β-D-fructofuranoside (Gf) were synthesized as sugar sources which might selectively and efficiently enhance the growth of bifidobacteria in the human intestines. Gf was synthesized by using levansucrase and the others by means of the Koenigs–Knorr reaction. The structures of these sugars were confirmed by enzymic hydrolysis. All of these sugars were utilized by almost all strains of *Bifidobacterium* tested. Among them, GII, Gf and stachyose were not utilized by *Lactobacillus acidophilus* or *Streptococcus faecalis*, and were utilized by only a few strains of Enterobacteriaceae (comparable to lactosucrose). Furthermore, as regards both the growth activity of bifidobacterial cells and the generation time of the cells, the three sugars were virtually as effective as lactose or lactosucrose.

Keywords——α-D-galactopyranosyl β-D-glucopyranoside; α-D-galactopyranosyl β-D-fructofuranosyl-(2→6)-β-D-fructofuranoside; lactosucrose; raffinose; stachyose; Enterobacteriaceae; *Bifidobacterium*; selective utilization; growth activity; generation time

In order to enhance the growth of bifidobacteria in the human intestine, we are searching for sugars which are utilized selectively and efficiently by bifidobacteria. That is, the sugars should not be utilized by streptococci, lactobacilli or Enterobacteriaceae (coli-form bacteria), because these bacteria live higher in the gastro-intestinal tracts than bifidobacteria, which residue in the region from the ileum to the large intestine. We previously examined commercial sugars for selectivity. Raffinose was the most selective sugar among them.[2)] The results showed that: (1) non-reducing saccharides tend to be poorly utilizable ty *Lactobacillus, Streptococcus* and *Escherichia*; (2) bifidobacteria consume di- and trisaccharides containing galactose without riquiring 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. Taking account of these results, we synthesized galsucrose, lactosucrose and isogalactobiose. Among them, lactosucrose was the most selective but was still utilized by some Enterobacteriaceae (41% of strains), although the sugar was more selective than raffinose.[1)]

This time we added representative strains of species utilizing lactosucrose in Enterobacteriaceae to the previous screening system to find more selective sugars than lactosucrose. Several cadidate sugars which were synthesized chemically or by using the enzyme levansucrase were tested with the screening system. More selective sugars were then compared to lactosucrose in selectivity by using 37 strains of Enterobacteriaceae. Furthermore, we measured the generation time of *Bifidobacterium* on these sugars.

Experimental

The microorganisms, levansucrase and the enzymes for structural confirmation of sugars were the same as those reported in the previous paper.[1)]

Screening System for Selectivity——Strains for the new basal screening system consisted of *Bifidobacterium adolescentis* c E288b, *B. bifidum* a E419, *B. breve* b S46, *B. infantis* S-12, *B. longum* E144b, *Lactobacillus acidophilus* IAM1084, *Streptococcus faecalis* IAM10067, *Escherichia coli* K-12, *E. coli* Hal, *E. coli* Wbl, *Klebsiella pneumoniae* Tol, *K. oxytoca* Hn2, *Citrobacter freundii* Nj3, *C. freundii* To2, *Enterobacter aerogenes* Nj1 and *En. cloacae* Wb2. Among them, all strains of *Bifidobacterium* and strains Ha1, Wb1, To1, Hn2, To2 and Wb2 were able to utilize lactosucrose. The other strains were adopted as representative of the species. Assay conditions were reported in the previous paper.[1)]

Generation Time of *Bifidobacterium* on Sugars——Sugar solution (4 mg/ml) was sterilized by passing it through a membrane filter (HA type; Millipore Co., U.S.A.). The assay medium (as double strength), which was the same as for the screening system, for *bifidobacterium* was inoculated with 10^5 to 10^6 cells of *B. longum* E144b per ml and cultured at 37 °C in N_2–CO_2 (9 : 1). At intervals of 30 min, the culture was well mixed and its turbidity (absorbance at 650 nm) was measured as an indication of the cell concentration until the absorbance rose above 0.5. The generation time was defined as the period required for doubling of the absorbance of the culture.

Synthesis of Sugars——(1) In the presence of mercuric cyanide, 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose reacted with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in nitromethane to form octa-*O*-acetyl-neogalactobiose (2,3,4,6,2′,3′,4′,6′-octa-*O*-acetyl-α-D-galactopyranosyl β-D-galactopyranoside) (Koenigs–Knorr reaction). Neogalactobiose (Gb) was obtained by deacetylation of the acetate with methanolic sodium methoxide.[3)]

(2) β-D-Galactopyranosyl β-D-glucopyranoside (GI) was obtained by means of the Koenigs–Knorr reaction of 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide.

(3) α-D-Galactopyranosyl β-D-glucopyranoside (GII) was obtained by means of the Koenigs–Knorr reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide.

(4) β-D-Galactopyranosyl α-D-glucopyranoside (GIII) was obtained by means of the Koenigs–Knorr reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide.

(5) The reaction mixture, containing 1.0 g of sucrose, 3.0 g of galactose and levansucrase (1.2 units) in 3 ml of 50 mM phosphate buffer, pH 6.0, was incubated for 24 h at 37 °C. Another 1.0 g of sucrose and levansucrase (1.2 units) were added to the mixture. This process was repeated five times (finally 6.0 g of sucrose, 3.0 g of galactose, 7.2 units of levansucrase). At one day after the last addition, 100 ml of water was added to the mixture, and the solution was subjected to charcoal column chromatography. The trisaccharide fraction was concentrated *in vacuo* to 0.5 ml, and the residue was taken up in 1.0 ml of 0.05 M sodium tetraborate. The solution was applied to a QAE-Sephadex column (Pharmacia Fine Chemicals, Sweden) (borate form) and eluted with water. α-D-Galactopyranosyl β-D-fructofuranosyl-(2→6)-β-D-fructofuranoside (Gf) was eluted after sucrose and glucose. The material was run on pre-coated high-performance thin layer chromatography (HPTLC) plates of Silicagel 60 (E. Merck, West Germany) with iso-propanol–0.2 M boric acid (85 : 15, v/v), and showed an *Rf* value of 0.2. The concentrated sugar solution was then passed through a QAE-Sephadex column (OH^- form) in order to remove boric acid, and through a Sephadex G-15 column (Pharmacia Fine Chemicals). The obtained trisaccharide fraction was dried and 65 mg of Gf powder was obtained from ethanol.

Results

Each synthesized sugar was non-reducing to Fehilng's solution, and gave only one spot on the HPTLC plate. It was found that Gb was hydrolyzed by α-galactosidase or β-galactosidase. GI was not hydrolyzed by α-galactosidase or α-glucosidase but was by β-galactosidase or β-glucosidase. GII was not hydrolyzed by β-galactosidase or α-glucosidase but was by α-galactosidase or β-glucosidase. GIII was not hydrolyzed α-galactosidase or β-glucosidase but was by β-galactosidase or α-glucosidase. Gf was not hydrolyzed by β-galactosidase but was by α-galactosidase or β-fructosidase. Thus, the structures of the synthesized sugars were confirmed.

Utilization of the synthesized sugars and some other galactosyl-oligosaccharides by bifidobacteria, *L. acidophilus, S. faecalis*, and representative strains of Enterobacteriaceae are shown in Table I. All these sugars were utilized by all stains of *Bifidobacterium* except for *B. bifidum*, and they were not utilized by *L. acidophilus, S. faecalis* or *E. coli* K-12, as had been expected. GII, Gf and stachyose were utilized by fewer strains of Enterobacteriaceae than lactosucrose.

TABLE I. Utilization of Sugars by Representative Strains of Organisms

Organism		Growth (absorbance at 650 nm)								
		Gb	GI	GII	GIII	Gf	Ll	Ra	St	Ls
Bifidobacterium infantis	S-12	0.44	0.53	0.47	0.66	0.62	0.56	0.41	0.48	0.62
longum	E144b	0.54	0.68	0.56	0.59	0.71	0.42	0.46	0.49	0.58
adolescentis c	E288b	0.52	0.65	0.56	0.57	0.65	0.45	0.51	0.53	0.39
breve b	S46	0.56	0.40	0.54	0.47	0.47	0.46	0.51	0.49	0.65
bifidum a	E419	0.05	0.16	0.19	0.14	0.01	0.20	0.01	0.01	0.20
Lactobacillus acidophilus	IAM1084	0.01	0.04	0.04	0.00	0.01	0.47	0.00	0.00	0.01
Streptococcus faecalis	IAM10067	0.00	0.01	0.03	0.16	0.01	0.15	0.00	0.00	0.00
Escherichia coli	K-12	0.34	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.01
	Ha1	0.46	0.43	0.04	0.38	0.11	0.11	0.42	0.00	0.45
	Wb1	0.42	0.40	0.00	0.40	0.00	0.39	0.34	0.00	0.64
Klebsiella pneumoniae	To1	0.55	0.50	0.50	0.41	0.62	0.22	0.31	0.44	0.35
oxytoca	Hn2	0.45	0.31	0.48	0.40	0.54	0.36	0.51	0.41	0.34
Citrobacter freundii	Nj3	0.29	0.01	0.00	0.20	0.01	0.01	0.16	0.00	0.00
	To2	0.01	0.37	0.27	0.17	0.01	0.00	0.44	0.00	0.30
Enterobacter aerogenes	Nj1	0.46	0.50	0.52	0.41	0.55	0.03	0.54	0.01	0.04
cloacae	Wb2	0.31	0.46	0.26	0.42	0.55	0.16	0.48	0.00	0.53

Culture time was 4 d for *Bifidobacterium*, and 2 d for the others.
Initial sugar concentration in each medium was 1.0 mg/ml.
Conditions were as described in our previous paper.[1]
Ll, lactulose; Ra, raffinose; St, stachyose; Ls, lactosucrose.

The above two sugars were further examined with 37 strains of Enterobacteriaceae of adult origin. As shown in Table II, GII was not utilized by any strain of *Escherichia* or *Hafnia*, while Gf and stachyose were not utilized by most strains of *Escherichia* or any strain of *Citrobacter*. However, all three sugars were utilized by all strains of *Klebsiella*. On the assumption that a sugar which gave an absorbance of more than 25% of that obtained on glucose was utilized by a strain, GII was utilized by 12 strains (35%), Gf by 9 strains (24%) and stachyose by 7 strains (19%) out of 37 strains of Enterobacteriaceae of adult origin. Thus, the selectivities of the three sugars for bifidobacterial growth were found to be higher than that of lactosucrose.

The generation times of *B. longum* on the sugars are shown in Table III. The three sugars were utilized by *B. longum* almost as effectively as lactose or lactosucrose.

Discussion

Ruttloff *et al.* showed that raffinose and lactulose are not digested by mucosal enzymes in the human intestine,[4] and Dahlqvist showed that the hydrolytic activities of the mucosa toward cellobiose and gentiobiose are much lower than those toward maltose, sucrose or lactose.[5] These results suggest that our synthetic sugars (GII, Gf) and stachyose should be poorly digested. Generally, oligosaccharides indigestible by intestinal enzymes are known to be very little absorbed through the intestinal mucosa.

GII, Gf and stachyose were found to be superior to lactosucrose as regards selectivity for bifidobacterial growth. Our results indicate that di- to tetrasaccharides containing α-galactosyl, β-glucosyl and β-fructosyl residues tend to be highly selective, while α-glucosides and β-galactosides are often utilized by both bifidobacteria and other bacteria. The selectivity might be due to a difference between the sugar permeability of bifidobacteria and that of the other bacteria, as well as a difference in the hydrolytic activities of the cells. If highly selective

TABLE II. Utilization of Sugars by Enterobacteriaceae

Organism		GII	Gf	St	Ls	Gc
Escherichia coli	Nj2	0.03	0.00	0.00	0.02	0.43
	Yy1	0.05	0.01	0.00	0.12	0.41
	Tk2	0.00	0.00	0.00	0.00	0.45
	Dn1	0.00	0.01	0.00	0.00	0.47
	Ks1	0.02	0.01	0.00	0.04	0.38
	Ks2	0.01	0.00	0.01	0.01	0.34
	Hn1	0.01	0.00	0.00	0.00	0.46
	Na1	0.01	0.00	0.01	0.00	0.50
	Na2	0.02	0.00	0.01	0.00	0.47
	Ha1	0.04	0.11	0.00	0.45	0.48
	Za1	0.02	0.02	0.01	0.01	0.35
	Za2	0.00	0.01	0.01	0.00	0.43
	Mn1	0.00	0.00	0.00	0.00	0.48
	Ai1	0.02	0.01	0.01	0.00	0.48
	Ai2	0.02	0.01	0.01	0.49	0.50
	Wb1	0.00	0.00	0.00	0.64	0.45
	Mo1	0.02	0.01	0.00	0.17	0.48
	Mo2	0.02	0.01	0.01	0.01	0.54
	Ao1	0.02	0.01	0.22	0.00	0.48
	Ao2	0.00	0.01	0.00	0.00	0.22
	My1	0.02	0.00	0.01	0.00	0.38
Klebsiella pneumoniae	To1	0.50	0.62	0.44	0.35	0.32
	Tk1	0.40	0.51	0.38	0.53	0.42
	Ks3	0.47	0.61	0.55	0.52	0.54
	Zt1	0.39	0.64	0.37	0.48	0.38
oxytoca	Hn2	0.48	0.54	0.41	0.34	0.43
	Zt2	0.41	0.50	0.21	0.29	0.44
Citrobacter freundii	Nj3	0.00	0.01	0.00	0.00	0.32
	To2	0.27	0.01	0.00	0.30	0.40
	Mn2	0.00	0.00	0.00	0.01	0.32
Enterobacter aerogenes	Nj1	0.52	0.55	0.01	0.04	0.45
cloacae	Ki2	0.27	0.00	0.00	0.01	0.41
	Ki3	0.11	0.01	0.00	0.29	0.37
	Se1	0.46	0.56	0.01	0.40	0.35
	Wb2	0.26	0.55	0.00	0.53	0.52
agglomerans	Ki1	0.28	0.01	0.01	0.00	0.34
Hafnia alvei	Zt3	0.00	0.01	0.00	0.00	0.35

Culture time was 2 d.
Initial sugar concentration in the medium was 1.0 mg/ml.
Conditions were as described in our previous paper.[1)]
St, stachyose; Ls, lactosucrose; Gc, glucose.

TABLE III. Generation Time of *B. longum* on Various Sugars

Sugar	Generation time (h)
Lactosucrose	1.1
GII	1.3
Gf	0.7
Raffinose	0.6
Stachyose	1.0
Lactose	0.7

Initial sugar concentration in the medium was 2.0 mg/ml.

and efficient sugars for bifidobacteria can be found, the dosage of the sugar can be decreased and the frequency of diarrhea (*e.g.* as caused by large dosages of lactulose) should be minimized.

Some strains of Enterobacteriaceae,especially *Klebsiella*, utilized GII, Gf and stachyose well. Among the sugars without any galactose residue, amylose and soluble starch were not utilized by *Bifidobacterium* or by Enterobacteriaceae. Inulin (smaller than about 25 saccharide units) was utilized by many strains of *B. adolescentis* and *B. infantis*, but was not utilized by *B. bifidum, B. breve, B. longum* or Enterobacteriaceae. Tri- and tetrasaccharides containing fructose tended to be utilized by a few strains of Enterobacteriaceae; melezitose was not utilized at all. However, melezitose was utilized only by *B. longum* among *Bifidobacterium* of human origin, and it was reported that some streptococcal strains can utilize melezitose.[6] Therefore, neither inulin nor melezitose seems to be suitable as a general sugar source for bifidobacteria. If a melezitose analogue in which a glucose residue is converted into galactose can be synthesized, it might well be very selective and efficient.

Acknowledgment The authors thank Prof. T. Yamamoto, Osaka City University, for providing levansucrase.

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