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Difference in the xylitol sensitivity of acid production among *Streptococcus mutans* strains and the biochemical mechanism

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Xylitol inhibits the glycolysis and growth of *Streptococcus mutans*, but to different degrees among strains. Thus, we studied the biochemical mechanism through which the inhibition varies, using *S. mutans* strains ATCC 31989, NCTN 10449, and NCIB 11723, which are highly sensitive, moderately sensitive, and resistant to xylitol, respectively, under strictly anaerobic conditions such as those found in deep layers of dental plaque. Xylitol (30 mM) decreased the rate of acid production from glucose (10 mM) in ATCC 31989, NCTC 10449, and NCIB 11723 by 86, 26, and 0%, respectively. The activities of the xylitol : phosphoenolpyruvate phosphotransferase system (PEP-PTS) relative to those of glucose : PEP-PTS were 120, 16, and 3%, respectively. In ATCC 31989 and NCTC 10449, intracellular accumulation of xylitol 5-phosphate and decreases of fructose 1,6-bisphosphate and glucose 6-phosphate were observed. Furthermore, in the presence of xylitol (30 mM), glucose : PEP-PTS activities decreased by 34, 17, and 0%, respectively. These findings indicated that the higher the xylitol : PEP-PTS activity was and the more effectively xylitol decreased glucose : PEP-PTS activity, the more sensitive the strain was to xylitol. These results suggest that the following inhibitory mechanisms are active in the xylitol-sensitive mutans streptococci: direct inhibition of glycolytic enzymes by xylitol 5-phosphate derived from xylitol : PEP-PTS and, possibly, indirect inhibition through competition for the phosphoryl donor, HPr-P, between glucose and xylitol : PEP-PTSs.

Key words: acid production; phosphoenolpyruvate-sugar phosphotransferase system; *Streptococcus mutans*; xylitol

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Xylitol is widely used as a noncariogenic sugar substitute because it is not fermented by oral bacteria (6). Xylitol has been reported to inhibit the growth of mutans streptococci in the presence of glucose, galactose, mannose, lactose, maltose, sucrose, sorbitol or mannitol as a carbon source *in vitro* (1, 5, 7, 8, 16, 24, 25), and the acid production from glucose by resting cells of *Streptococcus mutans* (7, 13, 25). Xylitol is also known to selectively inhibit the growth of *S. mutans* in mixed culture using a chemostat (3, 15).

The major route of sugar transport by microorganisms is via the phosphoenolpyruvate phosphotransferase system (PEP-PTS). Two sugar-nonspecific proteins, enzyme-I and histidine-containing phosphocarrier protein (HPr), and a sugar-specific protein, enzyme-II are required for PEP-PTSs. PEP phosphorylates enzyme-I to phospho-enzyme-I, which in turn transfers the phosphoryl group to HPr. In many cases, phospho-HPr (HPr-P) generated from phospho-enzyme-I, transfers the phosphoryl group directly to enzyme-II, which in turn phosphorylates incoming sugar (10, 14).

Bacterial cells are thought to incorporate xylitol as xylitol 5-phosphate through xylitol : PEP-PTS and the xylitol 5-phosphate inhibits the enzyme activity of sugar metabolism, resulting in the inhibition of both bacterial growth and acid production (18). In addition, the futile cycle, in which xylitol 5-phosphate is dephosphorylated to xylitol with waste of PEP potential, can also retard the growth of *S. mutans* (18).

However, some strains are xylitol sensitive whereas others are resistant (19), and the degree of inhibition varies among strains (25). Thus, we studied the biochemical mechanism of the variable inhibition, using three strains of *S. mutans* previously characterized as highly sensitive, moderately sensitive, and resistant to xylitol under strictly anaerobic conditions such as those found in deep layers of dental plaque.

Material and methods

Bacterial strains and growth conditions

We used the following strains of *S. mutans*: NCTC 10449, ATCC 31989, and NCIB 11723. *S. mutans* NCTC 10449 was a gift as a xylitol-sensitive strain from Prof. L. Trahan (Université Laval, Québec, Canada) (20). Each strain was inoculated into a complex medium containing 1.7% tryptone (Difco Laboratories, Detroit, MI), 0.3% yeast extract (Difco), 85 mM NaCl, and 11 mM glucose as described (25) under strictly anaerobic conditions in an anaerobic chamber (N_2, 80%; H_2, 10%; CO_2, 10%, NHC-type, Hirasawa Works, Tokyo, Japan) and incubated at 35°C overnight. Cell cultures were transferred into the same complex medium and precultured overnight at 35°C. The cell cultures were again transferred into the same complex medium (5% inoculum size) and grown at 35°C. The bacterial cells were harvested by centrifugation ($7000 \times g$ for 15 min at 4°C) at an early logarithmic phase of growth (optical density at 660 nm [OD_{660}] ≈ 0.3) under anaerobic conditions as described previously (17). Bacterial purity was regularly confirmed by culturing on blood agar plates.

Acid production from glucose in the presence of xylitol

The following experiments were conducted in a different type of anaerobic chamber (N_2, 90%; H_2, 10%, NH-type, Hirasawa Works). Cells were washed twice with cold 2 mM potassium phosphate buffer (pH 7.0) containing 150 mM KCl and 5 mM $MgCl_2$, and suspended in the same buffer. The optical density of the cell suspension at 660 nm was adjusted to 3.5 (1.9 mg of cells [dry weight] per ml).

The cell suspensions were agitated with a magnetic stirrer at 35°C. The reaction was started by adding a mixture of 10 mM glucose and 0 or 30 mM xylitol to the cell suspensions. The rate of acid production by the cells was monitored at pH 7.0 using an automatic pH titrator (model AUT-211S, Toa Electronics Ltd, Kobe, Japan) with 50 mM KOH. The rate of acid production at 2 min after adding glucose or the glucose-xylitol mixture was calculated as μmol of protons per min per mg dry weight of cells.

Before and after the incubation for 10 min, cell suspensions (0.9 ml) were sampled and mixed immediately with 0.1 ml of 6 N perchloric acid. The mixtures were filtered (pore size 0.20 μm, polypropylene; ADVANTEC, Toyo Roshi Ltd, Tokyo, Japan) and cell-free filtrates were diluted with 0.2 N hydrochloric acid and stored at 4°C for the assay of acidic end products.

Analysis of acidic end products

Amounts of acidic end products, lactic, acetic, formic and pyruvic acids were quantified using a carboxylic acid analyzer (model EYELA S-3000X; Tokyo Rikakikai Co., Ltd, Tokyo, Japan) in stored cell-free filtrates, as described previously (17).

PEP-PTS activities for glucose, xylitol and fructose (glucose, xylitol and fructose : PEP-PTS activities)

The PEP-PTS activities were estimated by a modification of the method of Kornberg & Reeves (9) as described previously (13). Cells were harvested, washed twice as described above and stored at −20°C. After thawing, the cells were suspended in 2 mM potassium phosphate buffer (pH 7.0) containing 150 mM KCl and 5 mM $MgCl_2$ (OD_{660} ≈ 5.0). Toluene was added at a final concentration of 1% to the cell suspension, and mixed vigorously for 1 min. After centrifugation ($1200 \times g$ for 3 min), the cells were suspended in the same buffer (OD_{660} ≈ 50). The PEP-PTS activities for glucose, xylitol or fructose at pH 7.0 were estimated as a decrease of reduced nicotinamide adenine dinucleotide (NADH) in reaction mixtures containing 0.1 mM NADH, 53 μg of cells (dry weight)/ml, 1 mM phosphoenolpyruvate, 11 U/ml lactate dehydrogenase (EC 1.1.1.27, rabbit muscle; Roche Diagnostics, Indianapolis, IN) and 100 mM potassium phosphate buffer (pH 7.0) at 35°C. The reaction was started by adding 5, 10, 30, 60 or 120 mM glucose, xylitol or fructose. The decrease of NADH was monitored using a dual wavelength spectrophotometer (model 557; Hitachi Ltd, Tokyo, Japan) at 340 nm.

Inhibition of glucose : PEP-PTS activity in the presence of xylitol

Glucose : PEP-PTS activity in the presence of xylitol was also determined. Toluene-treated cells as described above were suspended in a reaction mixture containing 1 mM NADP, 53 μg of cells [dry weight]/ml, 1 mM phosphoenolpyruvate, 3.5 U/ml glucose 6-phosphate dehydrogenase (EC 1.1.1.49, yeast; Roche Diagnostics) and 100 mM potassium phosphate buffer (pH 7.0) at 35°C. The reaction was started by adding 10 mM glucose and 0, 10, 30, 60 or 120 mM xylitol to the cell suspensions. The increase of NADPH was monitored spectrophotometrically at 340 nm.

Assays of glycolytic intermediates and xylitol 5-phosphate

Cell suspensions were reacted with 10 mM glucose containing 0 or 30 mM xylitol as described above for the experiment of acid production. After the incubation for 2 min, the cells were collected by passing the reaction mixture through a membrane filter (pore size 0.45 μm, polyethersulfone; Acrodisc, Pall Gelman Laboratory, Ann Arbor, MI). Glycolytic intermediates and xylitol 5-phosphate in the cells were immediately extracted in 0.6 N perchloric acid, and neutralized with 5 M K_2CO_3 in air. The neutralized extracts were stored at 4°C for subsequent assays of the glycolytic intermediates and xylitol 5-phosphate, and at −20°C for 3-phosphoglycerate assays.

The glycolytic intermediates in the cell extracts were enzymatically determined at 35°C by a modification of the enzymatic method of Minakami et al. (12). Xylitol 5-phosphate was estimated as described previously (13). The assay mixture for xylitol 5-phosphate contained 1.1 mM NAD, 5 mM $MgCl_2$, 0.1 mM EDTA, and the extracts in 50 mM Tris-HCl buffer (pH 8.5) at 35°C. The reaction was started by the addition of 4.2 U/ml polyol dehydrogenase (EC 1.1.1.14, sorbitol dehydrogenase, sheep liver; Roche Diagnostics) and 50 U/ml alkaline phosphatase (EC 3.1.3.1, calf intestine; Roche Diagnostics). The increase of NADH was monitored spectrophotometrically at 340 nm.

Statistical methods

Differences in rates of acid production, rates of glucose : PEP-PTS activities with xylitol and in profiles of glycolytic intermediates were analyzed by the Mann–Whitney *U*-test. Differences in amounts of

Table 1. Relative rate of acid production and the formation of acidic end products from 10 mM glucose (G) and 10 mM glucose plus 30 mM xylitol (G +X).

S. mutans strain	Substrate	Relative rate of acid production	Acidic end products		
			Lactate	Acetate	Formate
ATCC 31989	G	100 [a]	1.44 ± 0.28[b]	0.34 ± 0.09	0.34 ± 0.06
	G +X	14 ± 1[#]	0.07 ± 0.01*	0.25 ± 0.04	0.27 ± 0.04
NCTC 10449	G	100	1.07 ± 0.07	0.92 ± 0.04	0.90 ± 0.05
	G +X	74 ± 9[#]	0.42 ± 0.03	1.00 ± 0.07	1.11 ± 0.13
NCIB 11723	G	100	1.72 ± 0.21	0.67 ± 0.08	0.75 ± 0.10
	G +X	102 ± 6	1.62 ± 0.22	0.69 ± 0.09	0.78 ± 0.12

[a] Relative rate of acid production (mean ± standard deviation, %) obtained from six independent experiments. Significant difference between relative rates of acid production in the presence and absence of xylitol: #$P < 0.01$.
[b]Amounts of acidic end products (mean ± standard deviation, μmol/mg cells) obtained from three independent experiments. Significant difference between amounts of acidic end products in the presence and absence of xylitol: *$P < 0.05$.

acidic end products were analyzed by the Dunn test.

Results

Inhibitory effect of xylitol on acid production

Xylitol significantly inhibited the acid production from glucose of *S. mutans* ATCC 31989 and NCTC 10449. In the presence of 30 mM xylitol, the acid production rates of ATCC 31989 and NCTC 10449 were decreased by 86 ± 1% ($P < 0.01$) and 26 ± 9% ($P < 0.01$), respectively, whereas that of NCIB 11723 was not inhibited (Table 1).

The total amounts of acidic end products generated by ATCC 31989 and NCTC 10449 cells decreased in the presence of xylitol. The reduction of lactic acid was remarkable and it was significant in ATCC 31989 ($P < 0.05$), resulting in an end product shift to formate-acetate-dominant (Table 1). Xylitol had no effect on NCIB11723.

Glucose, xylitol and fructose : PEP-PTS activities

All *S. mutans* strains had PEP-PTS activities for glucose, xylitol, and fructose (Fig. 1). In ATCC 31989, fructose : PEP-PTS activity at 5–120 mM fructose and xylitol : PEP-PTS activity at 30–120 mM xylitol were higher than glucose : PEP-PTS activity. In particular, xylitol : PEP-PTS activity at 30 mM xylitol was 120 ± 14% of glucose : PEP-PTS activity at 10 mM glucose. In NCTC 10449, both fructose and xylitol : PEP-PTS activities were lower than glucose : PEP-PTS activity. Xylitol : PEP-PTS activity at 30 mM xylitol was 16 ± 6% of glucose : PEP-PTS activity at 10 mM glucose. Both fructose and xylitol : PEP-PTS activities were low in NCIB 11723, and xylitol : PEP-PTS at 30 mM xylitol activity was only 3 ± 1% of glucose : PEP-PTS activity at 10 mM glucose.

Decrease in glucose : PEP-PTS activity in the presence of xylitol

In the presence of added xylitol, glucose : PEP-PTS activities of ATCC 31989 and NCTC 10449 were decreased (Fig. 2). The presence of 30 mM xylitol decreased glucose : PEP-PTS activity to 67 ± 6 and 83 ± 5%, respectively. As the xylitol concentration increased, the decrease became larger and statistically significant over 60 mM xylitol. However, little inhibition was observed in NCIB 11723.

Effect of xylitol on the profile of glycolytic intermediates during glucose metabolism

When metabolizing glucose only, all of the *S. mutans* strains had large amounts of fructose 1,6-bisphosphate, but the profiles of glycolytic intermediates downstream of

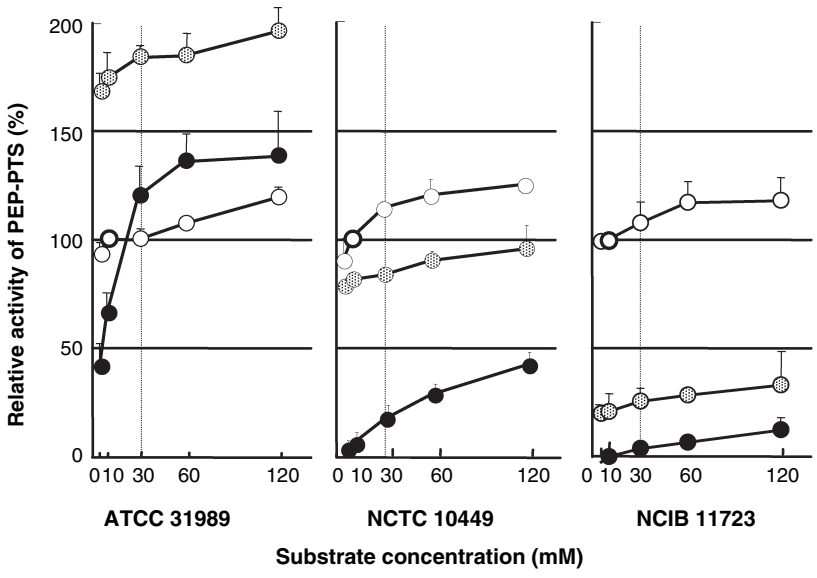

Fig. 1. PEP-PTS activities for glucose (○), xylitol (●) and fructose (◍) of *Streptococcus mutans* ATCC 31989, NCTC 10449, and NCIB 11723. Vertical bars indicate standard deviations from three independent experiments. PEP-PTS activity for 10 mM glucose was regarded as 100%.

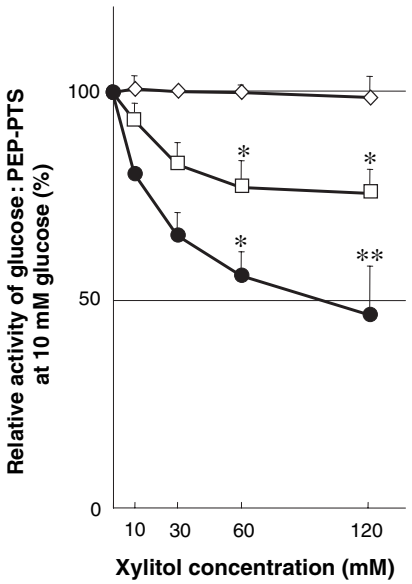

Fig. 2. PEP-PTS activities for 10 mM glucose of *Streptococcus mutans* in the presence of xylitol. ATCC 31989 (●), NCTC 10449 (□) and NCIB 11723 (◇). Significant difference between the PEP-PTS activities in the presence and absence of xylitol: *$P < 0.05$, **$P < 0.01$. Vertical bars indicate standard deviations from three independent experiments.

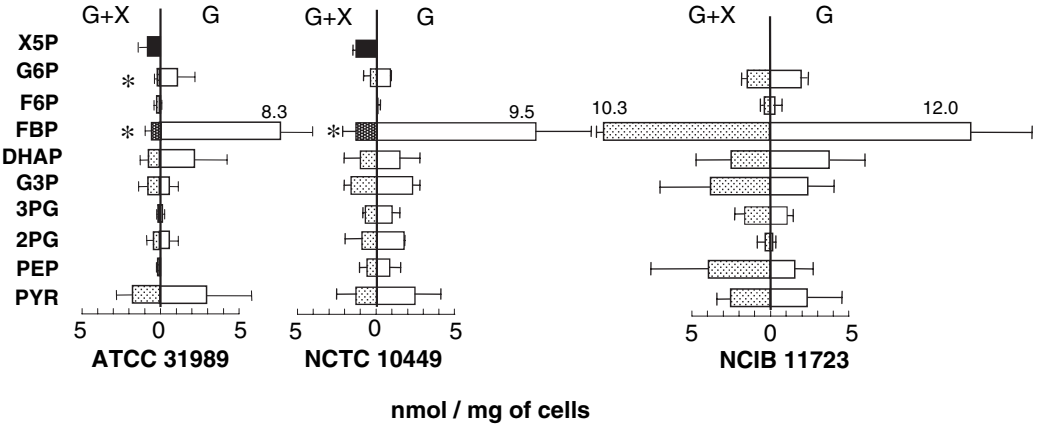

Fig. 3. Glycolytic intermediates and xylitol 5-phosphate of *S. mutans* ATCC 31989, NCTC 10449, and NCIB 11723 at 2 min after adding glucose (10 mM) or a mixture of glucose (10 mM) and xylitol (30 mM). Glycolytic intermediates (G, □) in the absence of xylitol. Glycolytic intermediate (G +X, ▨) and xylitol 5-phosphate (X5P, ■) in the presence of xylitol. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate. Significant difference between intermediate levels in the presence and absence of xylitol: $*P = 0.05$. Horizontal bars indicate standard deviations from four independent experiments.

dihydroxyacetone phosphate were slightly different among strains (Fig. 3).

When glucose was being metabolized by the ATCC 31989 and NCTC 10449 strains in the presence of xylitol, xylitol 5-phosphate accumulated and the amounts of glycolytic intermediates decreased, particularly those of fructose 1,6-bisphosphate ($P = 0.05$) in ATCC 31989 and NCTC 10449 and glucose 6-phosphate (G6P) ($P = 0.05$) in ATCC 31989. However, such inhibition was not evident in NCIB 11723.

Discussion

As previously reported (13, 25), xylitol inhibited the acid production of *S. mutans* strains and decreased the lactate production in strains ATCC 31989 and NCTC 10449 (Table 1), but the degree of these inhibitions varied between strains. On the other hand, in strain NCIB 11723, xylitol did not inhibit acid production or shift the end product profile.

Trahan (18) proposed an inhibitory mechanism of xylitol in which *S. mutans* transports xylitol as xylitol 5-phosphate through the activity of xylitol : PEP-PTS and, consequently, the xylitol 5-phosphate inhibits phosphoglucose isomerase and phosphofructokinase, the glycolytic enzymes for G6P conversion to fructose 1,6-bisphosphate, resulting in a decrease in intracellular levels of fructose 1,6-bisphosphate and the entire glycolytic rate. Miyasawa et al. (13) and Maehara et al. (11) then confirmed this notion, and found the decrease in lactate production was due to the decrease in fructose 1,6-bisphosphate, an absolute activator of streptococcal lactate dehydrogenase. The present study found that higher xylitol : PEP-PTS activity indicated more inhibition of acid production by xylitol (Table 1 and Fig. 1). This observation supports the notion that xylitol 5-phosphate produced by xylitol : PEP-PTS activity is responsible for glycolytic inhibition and suggests that *S. mutans* strains with higher xylitol : PEP-PTS activity are more sensitive to xylitol inhibition. It has been proposed that xylitol is transported via a constitutive fructose : PEP-PES and that xylitol : PEP-PTS activity appears as part of the constitutive fructose : PEP-PTS activity (18, 19, 22). We found here that high fructose : PEP-PTS activities in *S. mutans* were accompanied by high xylitol : PEP-PTS activities (Fig. 1), supporting this notion. Furthermore, it is reported that the *fxpC* gene of the constitutive fructose : PEP-PTS was located in the genomes of xylitol-sensitive streptococci and the *fxpC*-defective mutant was resistant to xylitol (2), although no information is available about *fxpC* gene in the strains used in our study.

Analyses of metabolic intermediates revealed that the xylitol-sensitive strains ATCC 31989 and NCTC 10449 accumulated xylitol 5-phosphate and decreased fructose 1,6-bisphosphate in the presence of xylitol (Fig. 3). These results support the notion that xylitol inhibits the glycolytic enzymes required for G6P conversion to fructose 1,6-bisphosphate. Despite the powerful inhibitory effect of xylitol (Table 1) and high xylitol : PEP-PTS activity (Fig. 1), the accumulation of xylitol 5-phosphate in ATCC 31989 seemed to be smaller than that in NCTC 10449. It is suggested that the glycolytic enzymes of ATCC 31989 are more sensitive to xylitol and a small amount of xylitol 5-phosphate is enough to inhibit the entire glycolytic metabolism.

Not only fructose 1,6-bisphosphate but also G6P significantly decreased in a highly xylitol-sensitive strain ATCC 31989 (Fig. 3), indicating that xylitol itself can inhibit the glucose uptake system (e.g. glucose : PEP-PTS) and result in a decrease in intracellular G6P. This was confirmed by the observation that the presence of xylitol decreased the glucose : PEP-PTS activity in xylitol-sensitive strains, ATCC 31989 and NCTC 10449 (Fig. 2). This could be due to competition for the phosphoryl donor, HPr-P, between the glucose and the xylitol : PEP-PTSs. In the ATCC 31989 strain, with high xylitol : PEP-PTS activity and powerful xylitol inhibition of glucose : PEP-PTS activity, HPr-P could phosphorylate xylitol to xylitol 5-phosphate efficiently and result in a direct inhibition by xylitol 5-phosphate on glycolytic enzymes and an indirect inhibition on glucose phosphorylation by HPr-P.

NCIB 11723 isolated from human dental plaque by Carlsson (4) has natural xylitol resistance like other xylitol-resistant strains isolated from xylitol consumers (21, 23). It has been suggested that the xylitol resistance is due to the absence of a constitutive fructose : PEP-PTS by which xylitol is also incorporated, thus preventing xylitol-resistant strains from incorporating xylitol (19, 21). In the present study, NCIB 11723 had little xylitol : PEP-PTS

activity (Fig. 1) and did not accumulate xylitol 5-phosphate (Fig. 3) in the presence of a low concentration of xylitol, supporting this notion. As the xylitol concentration increased, however, xylitol : PEP-PTS activities of NCIB 11723 appeared (Fig. 1). In the presence of 120 mM xylitol, the xylitol : PEP-PTS activity compared with that of glucose : PEP-PTS reached 12 ± 4%. However, 120 mM xylitol did not inhibit glucose : PEP-PTS activity (Fig. 2) and negligibly inhibited acid production from glucose (data not shown). These findings indicate that xylitol has less affinity for HPr-P than glucose in NCIB 11723. Thus, the strain cannot incorporate xylitol as xylitol 5-phosphate in the presence of both xylitol and glucose.

In conclusion, xylitol sensitivity varies among *S. mutans* strains: the higher the xylitol : PEP-PTS activity, and the more effectively xylitol decreases glucose : PEP-PTS activity, the more sensitive the strain is to xylitol. Xylitol has two inhibitory mechanisms:

- direct inhibition of glycolytic enzymes by xylitol 5-phosphate derived from xylitol : PEP-PTS;
- possibly, indirect inhibition of sugar uptake through competition for the phosphoryl donor, HPr-P, between the glucose and the xylitol : PEP-PTS.

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