

# Expulsion mechanism of xylitol 5-phosphate in *Streptococcus mutans*

ANNE PIHLANTO-LEPPÄLÄ<sup>1</sup>, EVA SÖDERLING<sup>1</sup> AND KAUKO K. MÄKINEN<sup>2</sup>

<sup>1</sup>Department of Biochemistry, Institute of Dentistry, University of Turku, Turku, Finland, and

<sup>2</sup>School of Dentistry, The University of Michigan, Ann Arbor, MI, USA

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**Abstract** - The expulsion mechanism of xylitol 5-phosphate in *Streptococcus mutans* ATCC 25175 was studied using resting cells incubated in the presence of <sup>14</sup>C-xylitol. The expulsion appeared to be a two-step process: xylitol 5-phosphate was first hydrolyzed to xylitol and inorganic phosphate, and the xylitol was subsequently expelled from the cells. The dephosphorylation step appeared to be energy-requiring and it was most likely associated with a phosphatase which was active on xylitol 5-phosphate. Two to three successive cultivations of the cells in the presence of 6% xylitol increased this enzyme activity 4.3-fold. These results are in accordance with the presence of an energy-dependent xylitol 5-phosphate cycle in *S. mutans*, which is regulated by exogenous xylitol.

**Key words:** *S. mutans*; xylitol.

Eva Söderling, Institute of Dentistry, University of Turku, SF-20520 Turku, Finland.

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Several clinical studies have shown that xylitol, a naturally occurring five-carbon sugar alcohol, can be used as an effective caries-preventive agent (1-4). The cariostatic and the therapeutic (4) effects of xylitol have been attributed to both microbiologic and physicochemical actions of xylitol (for reviews, see 5, 6).

The majority of oral bacteria cannot utilize xylitol (7, 8) and no adaptation to use xylitol has been detected in long-term in vivo studies (9, 10). On the contrary, acid production and growth of most oral streptococci

are inhibited by xylitol (11, 12). This inhibition has been attributed to the intracellular formation of xylitol 5-phosphate (xylitol 5-P), which is toxic to the organism, but not to humans (11, 13). Xylitol can be transported into the cells of *Streptococcus mutans* and *S. sobrinus* via a phosphoenolpyruvate:fructose phosphotransferase system (13).

Regulatory mechanisms for sugar transport in Gram-positive bacteria have been studied intensively during the last years (for review, see 14). Especially the components involved in the uptake of various sugars have

received interest (14–18). Less, however, is known about the mechanisms involved in the regulation of the levels of the intracellular sugar phosphates (14). Regulation of the sugar phosphate accumulation by expulsion was first demonstrated in *S. pyogenes* (19). An expulsion mechanism based on intracellular dephosphorylation and efflux of the free sugar has also been demonstrated in *Lactobacillus casei*, the sugar phosphate being xylitol 5-P (20). Indirect evidence has been presented on the existence of such an efflux mechanism in some oral streptococci as well (21–23).

The aim of the present study was to verify the existence of an expulsion mechanism for xylitol 5-P in *S. mutans* 25175 and to study the nature and the number of steps as well as the energy dependence of the expulsion system.

## Material and methods

### CULTIVATION OF BACTERIA

*S. mutans* ATCC 25175 cells were cultured in a medium containing 5 g trypticase, 5 g yeast extract, 5 g  $K_2HPO_4$ , 4 g glucose, 80 mg  $MgSO_4 \cdot 7H_2O$ , 4 mg  $FeSO_4 \cdot 7H_2O$ , 2 mg  $MnCl_2 \cdot 4H_2O$  and 5 ml Tween-80 in 1 liter of distilled water. The pH of the medium was adjusted to 7.2 and the medium was autoclaved for 10 min at 120°C. The bacteria were pregrown overnight at 37°C in the basal medium followed by one cultivation in the same medium (8 h). The growth was followed by measuring the turbidity with a Klett-Summerson colorimeter (filter 62).

For preparation of xylitol-cultured cells, the basal medium was supplemented with xylitol. The concentrated xylitol solution was sterilized by filtration through a Millipore filter (Millex HA 0.45  $\mu m$ ) and added aseptically to the medium at the appropriate concentration. The cells obtained after two to three successive cultivations (8 h each) were called "xylitol-cultured cells". Between each cultivation the cells (collected by centrifugation) were stored as a pellet (overnight at 8°C) in the presence of 6% xylitol. Control cells were cultivated successively in the basal medium without added xylitol.

### XYLITOL 5-P EXPULSION

The cells for expulsion studies were harvested from cultures at the end of the mid-log phase of growth ( $12\,000 \times g$ , 4°C, 10 min) and washed twice with 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM  $MgCl_2$ . The cells were finally suspended in the above buffer to a final concentration of 1.0–1.5 mg (dry weight) per ml. The suspensions were preincubated for 10 min at 37°C using agitation whereafter [ $^{14}C$ ]-xylitol (Amersham, Buckinghamshire, England) was added to the mixtures (final specific activity 0.1 mCi/mmol, total concentration of xylitol 1 mM). After 15 min incubation at 37°C the mixtures were chilled to 0°C and the resulting "preloaded cells" were harvested ( $12\,000 \times g$ , 4°C, 2 min). The cell pellets were resuspended by rapid vortexing in the above buffer (37°C).

To study the expulsion mechanism the following compounds were added to the above cell suspensions containing preloaded cells: D-glucose, D-fructose, xylitol, L-arginine, NaF, iodoacetic acid (IAA), and Na-arsenate. The final concentrations are given in figure legends. In some experiments a preincubation time of 5 min was used before triggering the expulsion by the addition of glucose. All incubations were performed at 37°C.

The total label of the cells and the levels of intracellular  $^{14}C$ -xylitol and  $^{14}C$ -xylitol 5-P were determined from duplicate samples withdrawn at proper time intervals from the cell suspensions. For the determination of total label (sample size 0.4 ml) the cells were collected on membrane filters (Millex HA 0.45  $\mu m$ ). The filters were rinsed twice with 4 ml 0.9% NaCl and transferred to scintillation vials. Five ml of scintillation cocktail (ACS, Amersham) was added to each vial and the radioactivity of the cells was determined with a LKB 1210 Ultrabeta Liquid Scintillation Counter (LKB-Wallac, Turku, Finland). For the determination of intracellular  $^{14}C$ -xylitol and  $^{14}C$ -xylitol 5-P the cells (sample size 0.4 ml) were collected by centrifugation ( $12\,000 \times g$ , 4°C, 2 min). Each cell pellet was treated for 10 min in 3 ml boiling water. The resulting suspensions were cleared by centrifugation ( $12\,000 \times g$ , 4°C, 10 min). The supernatants were freeze-dried and the residues were finally dissolved in 0.5 ml of distilled water.  $^{14}C$ -Xylitol and  $^{14}C$ -xylitol 5-P in the solutions were determined by ion exchange chromatography on an AG 1-X2 resin (50–100 mesh, BioRad Labora-

tories, Richmond, CA) as previously described (24).

### PHOSPHATASE ACTIVITY OF CELL EXTRACTS

The phosphatase activities were determined from cell extracts of *S. mutans* grown in the basal medium only, and from xylitol-cultured cells.

The cell extracts were prepared as follows. The cells were harvested from the late logarithmic growth phase by centrifugation and washed twice with 50 ml of 0.02 M [2-(N-morpholino)ethanesulfonic acid]-buffer (MES) (pH 6.2) containing 5 mM MgCl<sub>2</sub> and 10 mM 2-mercaptoethanol, to remove phosphate. The cells were then suspended to the above MES-buffer to a final concentration of 0.25 mg (wet weight) of cells per ml. The final cell-free extracts were prepared from these suspensions by treating them twice (6 min at a time) with an ultrasonic disintegrator (MSE disintegrator; amplitude 6, end diameter of the probe 3 mm) at 4°C. The sonicates were cleared by centrifugation (50 000 × g, 4°C, 30 min).

The phosphatase activity of the resulting supernatants was studied using the following substrates: D-glucose 1-phosphate (P), D-glucose 6-P, D-mannitol 1-P, D-ribose 5-P, D-ribulose 5-P, D-fructose 6-P, D-fructose 1,6-diP, D-galactose 6-P, glucosamine 6-P, xylitol 5-P, adenosine triphosphate (ATP) as sodium salt, phosphoenolpyruvic acid (PEP), D-sorbitol 6-P and 6-P-gluconic acid and 3-P-glyceric acid. Xylitol 5-P was synthesized from the corresponding ketosugar by a sodium borohydride reduction procedure described by MARZLUF & METZENBURG (25) as modified by LONDON & HAUSMAN (26). The enzyme assay was based on the colorimetric method of HORDER (27). The reaction time was 60 min at 37°C, under which conditions the enzyme reaction was linear.

The protein concentrations of the cell extracts were measured with the Folin method (28).

## Results

### ENERGY DEPENDENCE OF XYLITOL 5-P EXPULSION

The presence of D-glucose and D-fructose (5 mM) increased the rate of expulsion of the label from *S. mutans* preloaded with <sup>14</sup>C-xylitol 5-P (Fig. 1), but the presence of 5 mM

xylitol had no effect on the rate of expulsion (Fig. 1). The expulsion of the label in the presence of 4 mM D-glucose was inhibited by inhibitors of glycolysis, IAA (inhibitor of glyceraldehyde 3-phosphate dehydrogenase) and NaF (inhibitor of enolase) (10 mM, Fig. 2). When 2 mM L-arginine (a non-glycolytic source of ATP) was added to the incubation mixtures simultaneously with IAA (not shown) or NaF (Fig. 2), the inhibitory effect was abolished. L-Arginine alone, without D-glucose, did not induce expulsion of preaccumulated xylitol 5-P. Thus the expulsion of xylitol 5-P appeared to be an energy-dependent process (involving possibly ATP).

### NUMBER OF STEPS INVOLVED IN THE EXPULSION OF XYLITOL 5-P

Two mechanisms may account for the expulsion of xylitol 5-P by *S. mutans*: either the xylitol 5-P is dephosphorylated intracellularly followed by efflux of free xylitol (two-step mechanism) or both the dephosphorylation and efflux occur simultaneously (one-step mechanism).

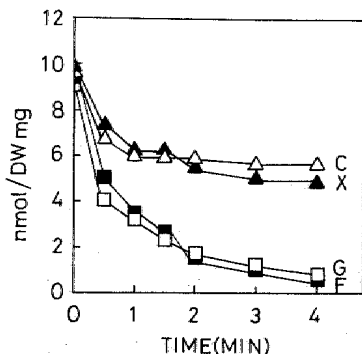


Fig. 1. Effect of D-glucose, D-fructose and xylitol on xylitol expulsion by preloaded cells of *S. mutans*, given in nmol of expelled xylitol per mg cells (dry weight). The expulsion was followed at 37°C in the presence of 5 mM xylitol (▲-▲), 5 mM D-glucose (□-□), 5 mM D-fructose (■-■), or with no additions (△-△).

The first set of experiments designed to reveal the connection between the dephosphorylation of xylitol 5-P and the efflux of this compound involved Na-arsenate, an inhibitor of intracellular dephosphorylation.

Preincubation with Na-arsenate inhibited expulsion of  $^{14}\text{C}$ -xylitol 5-P by preloaded *S. mutans* cells (Fig. 3A). Na-arsenate did not, however, inhibit the efflux of  $^{14}\text{C}$ -xylitol, formed before the addition of the inhibitor (Fig. 3B and C). Thus enzymatic dephosphorylation of xylitol was necessary before the expulsion of xylitol 5-P could take place.

The second set of experiments was performed with cells preincubated with 10 mM NaF and 5 mM L-arginine essentially according to REIZER *et al.* (29). Fig. 4A shows the efflux of xylitol 5-P as triggered by 10 mM D-glucose (control). In theory, the addition of unlabeled xylitol to this reaction mixture caused an uptake of the unlabeled xylitol and resulted in competition for efflux (29). In fact, for a high concentration (100

mM) of external xylitol, the amount of  $^{14}\text{C}$ -xylitol within the cells was clearly higher than in the controls (Fig. 4A and D). Even a ten-fold lower external xylitol concentration (10 mM) resulted in elevated  $^{14}\text{C}$ -xylitol levels in *S. mutans* (Fig. 4C). The external xylitol however, had no effect on the levels of intracellular  $^{14}\text{C}$ -xylitol 5-P (Fig. 4). Thus, only the efflux of  $^{14}\text{C}$ -xylitol was inhibited, not the dephosphorylation of xylitol 5-P. This result together with the Na-arsenate experiment described above speaks for a two-step expulsion mechanism for xylitol 5-P: 1) enzymatic dephosphorylation of xylitol 5-P, and 2) subsequent efflux of free xylitol.

#### PHOSPHATASE ACTIVITY IN *S. MUTANS* CELL EXTRACTS

*S. mutans* cell extracts showed phosphatase activity towards phosphorylated sugars, sugar alcohols and hexonic acids. The best substrates were galactose 6-P and glucose 6-P (Fig. 5A). The following substrates examined were hydrolyzed with rates ranging from 40 to 60% as compared to galactose 6-P: xylitol 5-P, fructose 6-P, ribose 5-P, glucosamine 6-P, ATP, glucose 1-P, 3-phosphoglyceric acid, PEP, and fructose 1,6-diphosphate. The rest of the substrates mentioned above were hydrolyzed at even lower rates. Successive cultivations of the cells in the presence of 6% xylitol considerably increased the rates of hydrolysis of especially the sugar 5- and 6-phosphates (Fig. 5A, 5B, and 5C). For example, three successive cultivations of *S. mutans* in the presence of 6% xylitol increased the hydrolytic activity towards galactose 6-P (3.3-fold), fructose 6-P with 6.3-fold, xylitol 5-P (4.3-fold) and ribose 5-P (7.9-fold) (Fig. 5). The enzyme activities towards ATP, PEP, 3-phosphoglyceric acid, and fructose 1,6-diphosphate were, however, affected to a small extent by the successive cultivations in the presence of xylitol.

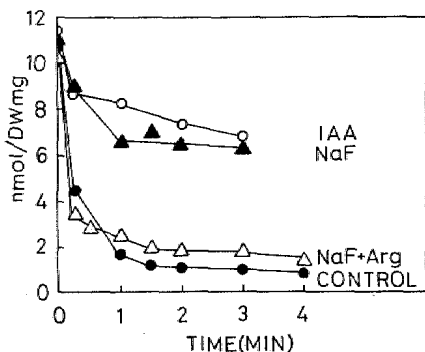


Fig. 2. Effect of IAA, NaF and L-arginine on expulsion of xylitol by preloaded cells of *S. mutans* at 37°C given in nmol of expelled xylitol per mg cells (dry weight). Cells were preincubated for 5 min (at 37°C) in the presence of 10 mM IAA (O-O), 10 mM NaF (▲-▲), or 10 mM NaF + 2 mM L-arginine (△-△). The control cells were preincubated without any additions (●-●). The expulsion was triggered by the addition of 5 mM glucose.

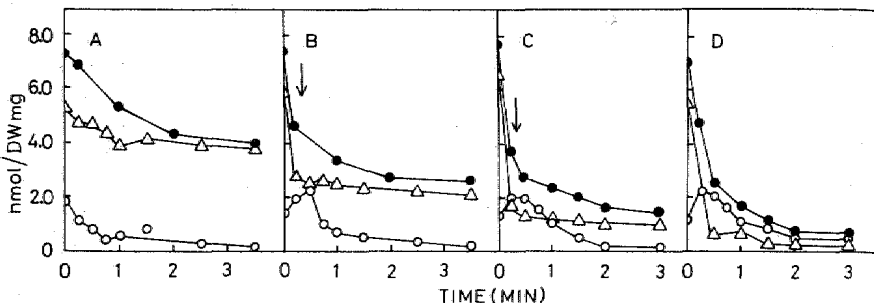


Fig. 3. Effect of 10 mM sodium arsenate on the expulsion of xylitol by preloaded cells of *S. mutans* at 37°C. Expulsion was elicited by addition 5 mM D-glucose. Na-arsenate was added (A) prior to D-glucose addition (preincubation at 37°C 5 min), (B) 10 s, or (C) 20 s after D-glucose addition (arrow). D, control without any additions. Duplicate samples were collected for the determination of total label (●-●),  $^{14}\text{C}$ -xylitol (○-○), and  $^{14}\text{C}$ -xylitol 5-P (Δ-Δ).

### Discussion

The key compound in the growth inhibition of streptococci by xylitol appears to be xylitol 5-P. Different streptococci, however, seem to differ in their handling of the toxic xylitol metabolite. Successive cultivations of *S. mutans* LG-1 led to a selection of "xylitol-

resistant" cells devoid of the PEP:fructose phosphotransferase needed for the uptake of xylitol (30). Successive cultivations of *S. sobrinus* in the presence of xylitol also led to a decrease in the uptake of xylitol. This phenomenon was, however, reversible and should thus not involve selection of xylitol-resistant cells (31). Evidence on the existence

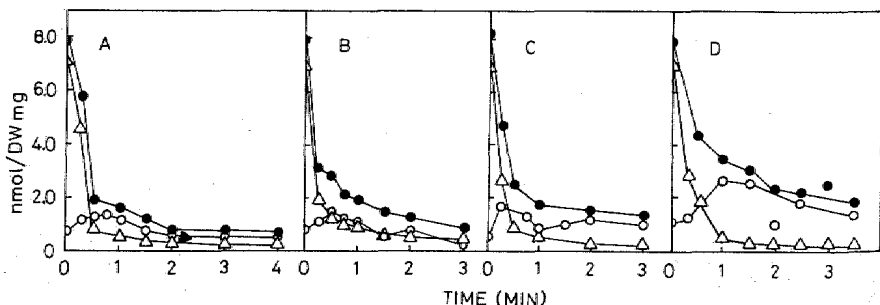


Fig. 4. Intracellular content of  $^{14}\text{C}$ -xylitol,  $^{14}\text{C}$ -xylitol 5-P and total label (xylitol plus xylitol 5-P) of cells during expulsion process. Preloaded *S. mutans* cells were suspended in buffer (37°C) containing (A) 10 mM NaF and 5 mM L-arginine, (B) 10 mM NaF, 5 mM L-arginine and 1 mM unlabeled xylitol, (C) 10 mM NaF, 5 mM arginine and 10 mM unlabeled xylitol, or (D) 10 mM NaF, 5 mM L-arginine and 100 mM unlabeled xylitol. Mixtures were preincubated for 5 min. Expulsion was then elicited by addition of 10 mM D-glucose. Duplicate samples were collected for determination of total label (●-●),  $^{14}\text{C}$ -xylitol (○-○), and  $^{14}\text{C}$ -xylitol 5-P (Δ-Δ). Results are given in nmol of labeled compound per dry weight of bacteria (in mg).

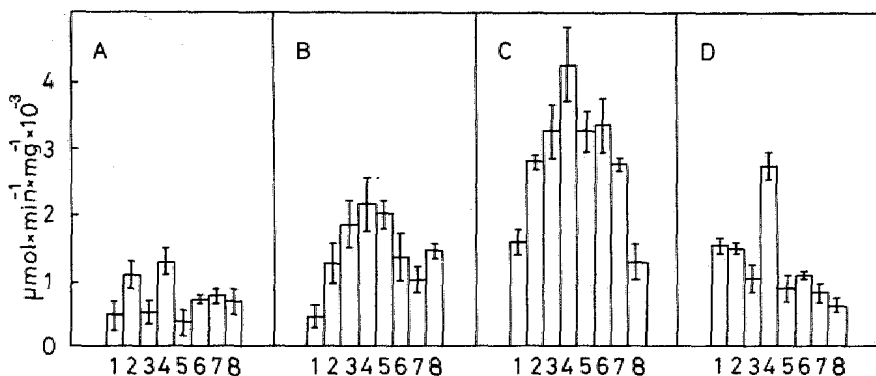


Fig. 5. Phosphatase activity of *S. mutans* cell extracts. Extracts of control cells grown in basal medium (A), or of cells obtained after two (B) or three (C) successive xylitol (6%) cultivations, or after three xylitol and one basal medium cultivation (D), were used in experiments. Phosphatase activities were determined using following substrates: 1: glucose 1-P. 2: glucose 6-P. 3: fructose 6-P. 4: galactose 6-P. 5: ribose 5-P. 6: xylitol 5-P. 7: glucosamine 6-P. 8: ATP. Values shown are means  $\pm$  SD ( $n=3-5$ ) of specific enzyme activities. Enzyme assays were performed in 0.1 M ES buffer, pH 6.0, containing 5 mM  $MgSO_4$ , final substrate concentration being 10 mM (for ATP 5 mM).

of a futile, energy-demanding xylitol 5-P cycle in *S. mutans* has also been presented (21-23). The intracellular xylitol 5-P has been proposed to inhibit glycolysis (32), but it could also act as an intracellular regulator. In fact, the level of xylitol 5-P in growing cells of *S. mutans* 25175 has been reported to be high (22).

The present study focused on the expulsion of xylitol 5-P by *S. mutans* 25175. The results speak for a two-step process in which xylitol 5-P is first dephosphorylated with subsequent expulsion of free xylitol. Such a mechanism has been demonstrated to be responsible for the expulsion of methyl- $\beta$ -D-thiogalactoside phosphate (TMG-P) by *S. pyogenes* and xylitol 5-P by *L. casei* (20, 29, 33). Expulsion of sugar phosphates appears to be one of the diverse mechanisms gram-positive bacteria use to regulate sugar accumulation (14, 34).

The energy dependency of the expulsion of xylitol 5-P by *S. mutans* 25175 was demonstrated by the experiments showing that rapid expulsion of xylitol was possible only in

the presence of a metabolizable substrate (D-glucose, D-fructose) but not a non-metabolizable one (xylitol). Furthermore, inhibitors of glycolysis inhibited the expulsion. Our experiments do not allow us to conclude which one of the two expulsion steps needed energy. In *S. pyogenes* the energy may be needed for the activation of the dephosphorylation mechanism (29). The results of the present experiments involving L-arginine, D-glucose, and glycolysis inhibitors resembled those reported for the expulsion of TMG-P by *S. pyogenes*: the results indicate that both ATP and a glycolytic intermediate are required for optimal dephosphorylation (29). In *S. pyogenes* phosphorylation of a protein is also involved in the expulsion process (29).

The cell extracts of *S. mutans* 25175 showed phosphatase activity with a broad substrate specificity. Hexose 6-phosphohydrolases have been detected in and purified from a variety of microorganisms (35-38). In *L. casei*, an inducible, membrane-regulated xylitol 5-P phosphatase has been characterized (38). The exact physiologic role of these phosphatase

tases is largely unknown. They may, however, detoxify certain sugar phosphates and regulate the concentrations of glycolytic intermediates. It has also been suggested that the sugar-phosphate hydrolase of *S. lactis* is involved in the expulsion of sugar phosphates (37). In the present study, successive cultivations of *S. mutans* in the presence of xylitol clearly increased the level of the five- and six-carbon sugar-P hydrolase(s), while the activities towards PEP and ATP, for example, were less affected. These reactions were reversible. This argues for the participation of an inducible sugar phosphatase in the regulation of the intracellular xylitol 5-P level in *S. mutans* 25175. Also our previous study indicated that the rate of expulsion of xylitol 5-P by *S. mutans* 25175 was increased by successive xylitol cultivations (20). Thus it appears that the first step of the expulsion of xylitol 5-P is its hydrolysis to xylitol by an inducible sugar phosphatase. The involvement of a phosphatase in the first efflux step was also suggested by the Na-arsenate inhibition experiments. The regulation of the efficiency of the xylitol 5-P efflux appeared to be connected to this step. The broad substrate specificity of the phosphatase indicates that the enzyme may function in several other dephosphorylation reactions as well.

As a conclusion, the present study suggests that the efflux of xylitol 5-P in *S. mutans* 25175 is a two-step, energy (ATP)-demanding process, the first step being the hydrolysis of the phosphate by an inducible sugar (C5 and C6) phosphatase.

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