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Involvement of Oxygen-Sensitive Pyruvate Formate-Lyase in Mixed-Acid Fermentation by *Streptococcus mutans* Under Strictly Anaerobic Conditions

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Streptococcus mutans JC2 produced formate, acetate, ethanol, and lactate when suspensions were incubated with an excess of galactose or mannitol under strictly anaerobic conditions. The galactose- or mannitol-grown cell suspensions produced more formate, acetate, and ethanol than the glucose-grown cells even when incubated with glucose. The levels of lactate dehydrogenase and fructose 1,6-bisphosphate were not significantly different in these cells, but the level of pyruvate formate-lyase was higher in the galactose- or mannitol-grown cells, and that of triose phosphate was lower in the galactose-grown cells. This suggests that the regulation of pyruvate formate-lyase may play a major role in the change of the fermentation patterns. The cells of *S. mutans* grown on glucose produced a significant amount of volatile products even in the presence of excess glucose under strictly anaerobic conditions. However, when the anaerobically grown cells were exposed to air, only lactate was produced from glucose. When cells were anaerobically grown on mannitol and then exposed to air for 2 min, only trace amounts of fermentation products were formed from mannitol under anaerobic conditions. It was found that the pyruvate formate-lyase in the cells was inactivated by exposure of the cells to air.

Streptococcus mutans is considered one of the most important cariogenic agents among the microorganisms in dental plaque (6, 8, 21). Therefore, the regulation of acid production in this microorganism attracts considerable attention in connection with the initiation of dental caries.

When *S. mutans* is grown in an excess of glucose, the main fermentation product is lactate. However, this organism produces formate, acetate, and ethanol when grown either under glucose limitation (5, 37) or at a low dilution rate (9–11, 25) in continuous culture. *S. mutans* also ferments mannitol or sorbitol to a large amount of ethanol (1). *Streptococcus lactis* and *Streptococcus cremoris* produce volatile products during galactose fermentation (36). Thus, streptococci produce a variety of acids under several conditions, even though they are classified as lactic acid bacteria.

The present study suggests that the regulation of pyruvate formate-lyase (formate acetyltransferase, EC 2.3.1.54), which catalyzes the first step of conversion of pyruvate into formate, acetate, and ethanol, plays an important role in the change of fermentation patterns of *S. mutans* and that the exposure of the intact cells to air greatly influences the fermentation patterns.

MATERIALS AND METHODS

Microorganism and growth conditions. *S. mutans* JC2 (4) was grown at 35°C in a medium containing, in 1 liter of 0.1 M potassium phosphate buffer (pH 6.8): sugar (glucose, galactose, or mannitol), 9.9 g; NH_4HCO_3, 2 g; sodium-L-glutamate, 1 g; dried extract of yeast (Daigo Eiyokagaku Ltd., Osaka, Japan), 2 g; L-cysteine-HCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.01 g; $MnSO_4 \cdot 4H_2O$, 0.01 g; and $FeSO_4 \cdot 7H_2O$, 0.01 g. The cells were harvested in the logarithmic growth phase and washed three times with 0.04 M potassium phosphate buffer (pH 6.8). A portion of the cells was used for cell suspension experiments, and the others were stored at −20°C for assays of enzymatic activities. Unless otherwise noted, all procedures were carried out under strictly anaerobic conditions with an anaerobic glove box (N_2, 80%; H_2, 10%; CO_2, 10%) as described previously (37). The purity of the culture was checked by culturing on mitis-salivarius agar (Difco Laboratories, Detroit, Mich.) and blood agar plates.

Sugar fermentation. The fresh washed cells were suspended in 0.04 M potassium phosphate buffer (pH 6.8), incubated for 10 min at 35°C, and washed again. The cell suspension contained 2.5 mmol of potassium phosphate buffer (pH 6.8), 75 μmol of $MgCl_2$, and cells (44 mg [dry weight]) in 12 ml. The reaction was started by the addition of 3 ml of 0.15 M sugar (glucose, galactose, or mannitol) and was run at 35°C for 20 min under constant stirring. Anaerobically grown cells

were incubated with sugar under three different atmospheric conditions. (i) All procedures were carried out under strictly anaerobic conditions. (ii) The cell suspensions (88 mg [dry weight] in 15 ml of phosphate buffer) in 100-ml Erlenmeyer flasks were removed from the anaerobic glove box, shaken for 2 min under air, and returned to the anaerobic glove box through the entry box; the air was removed with vacuum four times. The cell suspensions were then incubated with sugar under strictly anaerobic conditions. (iii) Anaerobically grown cells were incubated with sugar aerobically under constant stirring. The pH in the reaction mixtures was not changed during the 20-min incubation under these experimental conditions, and no more than one-third of the glucose added was consumed during the incubations. The reaction was stopped by the addition of 1.5 ml of 6.6 N perchloric acid at 0°C, and the mixture was kept at 0°C for 20 min. The mixture was then neutralized with potassium carbonate at 0°C, and the precipitate was removed by centrifugation. The supernatants were stored at −20°C until the assay of fermentation products and glycolytic intermediates. The samples for the analysis of glycolytic intermediates were not stored for more than 24 h.

Analysis of fermentation products. L-Lactate was estimated with L-lactate dehydrogenase (rabbit muscle; EC 1.1.1.27) (13), and formate was estimated with formate dehydrogenase (yeast; EC 1.2.1.2) as described by Quayle (28). Acetate and ethanol were determined by gas chromatography with a hydrogen flame detector as described previously (37).

Assay of glycolytic intermediates. Glycolytic intermediates were estimated by following the change in absorption at 340 nm after addition of an enzyme (16). They were assayed according to the method of Minakami et al. (26), except 3-phosphoglycerate, which was assayed by the method of Czok (7).

Since rapid sampling and extraction are reported to be necessary for the accurate estimation of the levels of glycolytic intermediates (35), the reactions were immediately stopped by the addition of perchloric acid to extract the intermediates. It should be noted, however, that the intracellular levels of glucose 6-phosphate and pyruvate cannot be determined by this method, because these metabolites are also found outside the cells (17, 35).

Preparation of cell-free extract and assays of enzymatic activities. The frozen cell paste was thawed and suspended in 0.04 M potassium phosphate buffer (pH 6.8) and 20 mM dithiothreitol. The cells were disrupted by sonic oscillation for 18 min at 0°C (200 W; 2 A), and cell debris was removed by centrifugation at 17,500 × *g* for 30 min at 4°C. The supernatant fluid was designated cell-free extract.

The activities of lactate dehydrogenase (EC 1.1.1.27), pyruvate formate-lyase, and alcohol dehydrogenase (EC 1.1.1.1) were estimated spectrophotometrically by recording the change in absorption at 340 nm. Each enzyme-assay mixture was prepared in a quartz cuvette with a side arm (Thunberg tube T-26; Nihon Sekiei Glass Co., Tokyo, Japan) in the anaerobic glove box. The Thunberg tubes were tightly stoppered before they were removed from the glove box. The reactions were started by the addition of the solution in the side arm and were run at 35°C. The assay system of lactate dehydrogenase contained 20 mM sodium pyruvate, 0.24 mM NADH, 0.5 mM fructose 1,6-bisphosphate, and cell-free extract in 50 mM morpholine propanesulfonic acid-NaOH buffer (pH 6.8) (37). The assay system of pyruvate formate-lyase contained 20 mM sodium pyruvate, 0.08 mM coenzyme A (CoA), 1 mM NAD, 6 mM sodium DL-malate, 2 mM dithiothreitol, 1.1 U of citrate synthase (pig heart; EC 4.1.3.7) per ml, 22 U of malate dehydrogenase (pig heart; EC 1.1.1.37) per ml, and cell-free extract in 100 mM potassium phosphate buffer (pH 7.2) (32). The reaction mixture for the assay of alcohol dehydrogenase contained 25 mM acetaldehyde, 0.24 mM NADH, 2 mM dithiothreitol, and cell-free extract in 50 mM potassium phosphate buffer (pH 6.9) (36).

The reactions for phosphate acetyltransferase (EC 2.3.1.8), acetate kinase (EC 2.7.2.1), and aldehyde dehydrogenase (EC 1.2.1.10; CoA dependent) were run at 35°C in the anaerobic glove box. The activities of phosphate acetyltransferase and acetate kinase were estimated by the determination of the amounts of acetyl phosphate used. The assay system for phosphate acetyltransferase contained 2 mM acetyl phosphate, 0.05 mM CoA, 50 mM sodium arsenate, 50 mM NH_4Cl, 2 mM dithiothreitol, and cell-free extract in 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH buffer (pH 7.2) (30). The assay mixture for acetate kinase contained 2 mM acetyl phosphate, 5 mM ADP, 5 mM $MgCl_2$, 0.5 mM NADP, 10 mM glucose, 2 mM dithiothreitol, 2 U of hexokinase (yeast; EC 2.7.1.1) per ml, 1 U of glucose-6-phosphate dehydrogenase (yeast; EC 1.1.1.49) per ml, and cell-free extract in 100 mM HEPES-NaOH buffer (pH 7.6) (36). The aldehyde dehydrogenase activity was assayed by the determination of the amount of acetyl phosphate formed. The reaction mixture for the assay of aldehyde dehydrogenase contained 100 mM acetaldehyde, 1 mM NAD, 0.05 mM CoA, 2 mM dithiothreitol, 10 U of phosphate acetyltransferase (*Clostridium kluyveri*; EC 2.3.1.8) per ml, 10 U of alcohol dehydrogenase (yeast; EC 1.1.1.1) per ml, and cell-free extract in 20 mM HEPES-NaOH–100 mM potassium phosphate (pH 7.2) (31). Acetyl phosphate was estimated by the method of Lipmann and Tuttle (23).

Storage of the cells at −20°C for less than 1 week had little effect on the specific activities of these enzymes in the cell-free extract.

Other analytical methods. The protein concentration in the cell-free extract was estimated by the biuret method (22). Cell densities were measured at 660 nm, and the dry weight of cells was calculated from the relationship between dry weight and absorption (*A*) of streptococcal cells (0.532 mg of cells [dry weight]/*A*) (16).

RESULTS

Fermentation by cells under strictly anaerobic conditions. Table 1 shows the end products produced by the cells from various sugars during the strictly anaerobic incubations. Even in the presence of excess glucose, the glucose-grown cells produced an appreciable amount of volatile products (32% of the total end products) under strictly anaerobic conditions.

Only 2% of the total end products were lactate when galactose was fermented by the galactose-

TABLE 1. Fermentation end products from glucose, galactose, or mannitol by cells of *S. mutans* under strictly anaerobic conditions

Carbon source	Sugar fermented	Amt (nmol/mg of cells [dry wt]) per 10 min of incubation					Relative rate of sugar fermentation[b]
		L-Lactate	Formate	Acetate	Ethanol	L-Lactate per total products[a]	
Glucose	Glucose	1,720	488	123	199	0.68	1.0
Galactose	Galactose	36.5	982	445	405	0.02	0.44
	Glucose	1,120	1,170	433	622	0.34	1.1
Mannitol	Mannitol	261	1,610	196	1,410	0.08	0.89
	Glucose	1,060	816	296	597	0.38	0.92

[a] Total products = amount of L-lactate + amount of formate + amount of acetate + amount of ethanol.
[b] The relative rate of sugar fermentation was calculated from the carbon recovery, and the value in the glucose fermentation by the glucose-grown cells was regarded as 1.0.

grown cells. The molar ratio of the products (formate, acetate, and ethanol) was almost 2:1:1. Thus, these volatile products seem to be produced by the pyruvate formate-lyase.

A large amount of volatile products (92% of the total end products) was produced from mannitol by the mannitol-grown cells. The amount of ethanol produced was about seven times that of acetate. Since the amount of formate produced was equimolar with acetate plus ethanol, these volatile products were also considered to result from the metabolism of pyruvate by the pyruvate formate-lyase. The calculations, based on the total amounts of end products, indicate that the rate of galactose metabolism was less than one-half the rate of glucose metabolism (Table 1).

The galactose- and mannitol-grown cells did not metabolize mannitol and galactose, respectively, and neither galactose nor mannitol was fermented by the glucose-grown cells.

Glycolytic intermediates in resting cells. Fructose 1,6-bisphosphate, an activator of lactate dehydrogenase, was predominant among the glycolytic intermediates during the fermentation of glucose or mannitol (Table 2). The intracellular level of fructose 1,6-bisphosphate during galactose metabolism was lower than that during glucose or mannitol metabolism. This, however, does not necessarily mean that the level of the activator of lactate dehydrogenase is low during galactose catabolism, since some of the galactose is expected to be catabolized through the tagatose 6-phosphate pathway, and another activator of lactate dehydrogenase, tagatose 1,6-bisphosphate, may be accumulated (15, 34, 36). The level of D-glyceraldehyde 3-phosphate, a potent inhibitor of pyruvate formate-lyase, during galactose metabolism was also less than that during glucose or mannitol metabolism.

The level of pyruvate during galactose metabolism was the lowest and that of 3-phosphoglycerate was the highest among the intermediates during the metabolism of the three sugars studied.

Activities of enzymes involved in pyruvate metabolism. The activities of the six enzymes involved in the alternative pathway of pyruvate metabolism (Fig. 1) were detected in the cell-free extracts of *S. mutans* JC2 when assayed

TABLE 2. Glycolytic intermediates during glucose, galactose, or mannitol metabolism of *S. mutans* under strictly anaerobic conditions

Intermediate	Amt of intermediate (nmol/mg of cells [dry wt])					
	Glucose[a]	Galactose[a]		Mannitol[a]		
	Glucose[b]	Galactose[b]	Glucose[b]	Mannitol[b]	Glucose[b]	
Glucose 6-phosphate[c]	6.3	1.4	5.6	2.6	8.6	
Fructose 1,6-bisphosphate	73	29	60	90	80	
Dihydroxyacetone phosphate	12	6.0	8.9	12	13	
D-Glyceraldehyde 3-phosphate	5.6	1.0	3.8	9.5	20	
3-Phosphoglycerate	10	42	6.5	9.5	10	
2-Phosphoglycerate	1.2	4.5	ND[d]	2.2	1.8	
Phosphoenolpyruvate	3.3	2.2	6.0	0.70	3.4	
Pyruvate[c]	13	2.9	8.9	12	19	

[a] Carbon source.
[b] Sugar fermented.
[c] Including intracellular and extracellular intermediates.
[d] ND, Not detectable.

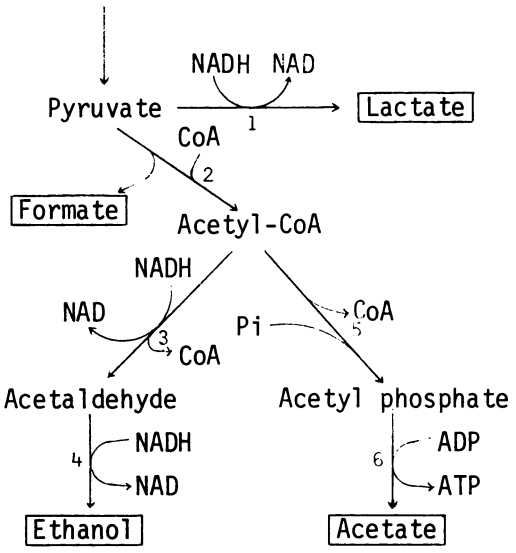

FIG. 1. Proposed pathway for pyruvate metabolism in *S. mutans*. Enzymes: 1, lactate dehydrogenase (EC 1.1.1.27); 2, pyruvate formate-lyase (EC 2.3.1.54); 3, aldehyde dehydrogenase (acylating) (EC 1.2.1.10); 4, alcohol dehydrogenase (EC 1.1.1.1); 5, phosphate acetyltransferase (EC 2.3.1.8); 6, acetate kinase (EC 2.7.2.1).

under strictly anaerobic conditions (Table 3). Lactate dehydrogenase activity (with 0.5 mM fructose 1,6-bisphosphate) in *S. mutans* grown on glucose, galactose, or mannitol was of the same order of magnitude (Table 3), although the activity in galactose-grown cells was a little smaller.

Pyruvate formate-lyase activities were different in cells grown on different sugars. In the galactose-grown cells, it was three times higher than in the glucose-grown cells (Table 3). This high level of pyruvate formate-lyase together with the low level of the inhibitor, D-glyceraldehyde 3-phosphate, during galactose metabolism (Table 2) may ensure a high in vivo activity and explain why a large part of the fermentation end products of galactose was comprised of volatile products.

The cells grown on different sugars also contained various levels of alcohol dehydrogenase; the highest specific activity was found in the mannitol-grown cells (Table 3).

The levels of phosphate acetyltransferase, aldehyde dehydrogenase, and acetate kinase in the cells grown on the three sugars were not significantly different.

Glycolytic intermediates and fermentation end products from glucose by cells grown on glucose, galactose, or mannitol. Cells grown on galactose or mannitol were exposed to glucose to clarify the effect of different levels of enzymes on the end products (Tables 1 and 3). Lactate comprised 68% of the fermentation products when the glucose-grown cells were exposed to glucose. However, the galactose- and the mannitol-grown cells with higher levels of pyruvate formate-lyase produced less lactate (34 and 38% of the total end products, respectively) and more volatile products (66 and 62%, respectively) from glucose (Table 1). During glucose fermentation, the levels of fructose 1,6-bisphosphate and lactate dehydrogenase activity were not significantly different in the cells grown on the three sugars (Tables 2 and 3). Thus, the differences in the end products of glucose among these cells did not seem to arise from the regulation of lactate dehydrogenase activity through changes in intracellular concentrations of fructose 1,6-bisphosphate, as shown in cells grown in continuous culture (37).

During glucose metabolism, the level of D-glyceraldehyde 3-phosphate in the mannitol-grown cells was about four times that in the glucose-grown cells, but the levels of dihydroxyacetone phosphate were not significantly different (Table 2). The galactose-grown cells maintained lower levels of triose phosphates than the glucose-grown cells during glucose fermentation, but the difference was not large enough to explain the difference in the end products of glucose between these cells (Table 1).

Thus, the regulation of the enzyme activities through the change in intracellular levels of glycolytic intermediates alone did not seem to be attributable to the shift of pyruvate metabolism under these experimental conditions.

Effect of oxygen on glucose metabolism by glucose- or mannitol-grown cells. As described above, anaerobically grown cells of *S. mutans*

TABLE 3. Activities of enzymes involved in the pyruvate metabolism of *S. mutans*[a]

Carbon source	Lactate dehydrogenase	Pyruvate formate-lyase	Phosphate acetyl-transferase	Acetate kinase	Aldehyde dehydrogenase	Alcohol dehydrogenase
Glucose	9.5	0.42	0.40	7.7	0.24	0.07
Galactose	7.7	1.2	0.52	12	0.26	0.24
Mannitol	10.3	0.80	0.62	7.2	0.26	0.90

[a] All the activities were measured under strictly anaerobic conditions and are expressed as micromoles of substrate or product per milligram of protein per minute.

TABLE 4. Effect of oxygen on fermentation end products from glucose by cells of *S. mutans*

Atmospheric conditions[a]	Carbon source	Sugar fermented	Amt (nmol/mg of cells [dry wt]) per 10 min of incubation					Relative rate of glucose fermentation[c]
			L-Lactate	Formate	Acetate	Ethanol	L-Lactate per total products[b]	
Strictly anaerobic	Glucose	Glucose	1,720	488	123	199	0.68	1.0
	Mannitol	Glucose	1,060	816	297	597	0.38	0.92
Strictly anaerobic (after exposure to air)	Glucose	Glucose	2,200	20	38	43	0.96	1.1
Aerobic	Glucose	Glucose	1,870	ND[d]	ND	ND	1	0.89
	Mannitol	Glucose	2,140	ND	ND	ND	1	1.0

[a] Atmospheric conditions are described in the text.
[b] Total products = amount of L-lactate + amount of formate + amount of acetate + amount of ethanol.
[c] The relative rate of glucose fermentation was calculated from the carbon recovery, and the value in the glucose fermentation by the glucose-grown cells was regarded as 1.0.
[d] ND, Not detectable.

produced formate, acetate, and ethanol, as well as lactate, from glucose under strictly anaerobic conditions. In contrast, when the cells were incubated aerobically with glucose, only lactate was formed (Table 4). The anaerobically grown cells produced lactate almost exclusively when they had been exposed to air for only 2 min and incubated anaerobically again. This increase in lactate production was accompanied by a decrease in the formation of volatile products. Therefore, the total rate of glucose metabolism was not changed by exposure to air. Thus, exposure to air did not seem to affect the activities of the enzymes involved in the Embden-Meyerhof pathway. The level of pyruvate was raised when the cells were incubated aerobically (Table 5).

Analogous effects of oxygen were observed when the mannitol-grown resting cells were exposed to glucose (Tables 4 and 5).

Effect of oxygen on mannitol metabolism by mannitol-grown cells. The mannitol-grown cells fermented mannitol to produce large amounts of formate and ethanol in addition to small amounts of lactate and acetate under strictly anaerobic conditions. However, the anaerobically grown cells fermented only a small amount of mannitol under aerobic conditions and produced trace amounts of volatile products and lactate (Table 6). The amount of lactate produced was similar to that produced during the strictly anaerobic fermentation. Only 2 min of exposure of the cells to air minimized the anaerobic mannitol metabolism, and only trace amounts of fermentation products were produced (Table 6). After a 2-min exposure of the mannitol-grown cells to air, a cell-free extract was prepared anaerobically, and it was confirmed that most (94% of the original activity) of the pyruvate formate-lyase was inactivated by exposure to air. During the

TABLE 5. Effect of oxygen on the levels of glycolytic intermediates during glucose metabolism of *S. mutans*[a]

Intermediate	Amt of intermediate (nmol/mg of cells [dry wt])				
	Strictly anaerobic		Strictly anaerobic (after exposure to air)	Aerobic	
	Glucose[b]	Mannitol[b]	Glucose[b]	Glucose[b]	Mannitol[b]
Glucose 6-phosphate[c]	6.3	8.6	6.6	6.2	3.6
Fructose 1,6-bisphosphate	73	80	85	74	62
Dihydroxyacetone phosphate	12	13	14	12	12
D-Glyceraldehyde 3-phosphate	5.6	20	12	17	46
3-Phosphoglycerate	10	10	12	14	20
2-Phosphoglycerate	1.2	1.8	2.7	2.6	4.3
Phosphoenolpyruvate	3.3	3.4	3.6	8.0	6.4
Pyruvate[c]	13	19	28	87	98

[a] Atmospheric conditions are described in the text.
[b] Carbon source.
[c] Including intracellular and extracellular intermediates.

TABLE 6. Effect of oxygen on fermentation products from mannitol by mannitol-grown cells of *S. mutans*

Atmospheric conditions[a]	Amt (nmol/mg of cells [dry wt]) per 10 min of incubation					Relative rate of mannitol fermentation[c]
	L-Lactate	Formate	Acetate	Ethanol	L-Lactate per total products[b]	
Strictly anaerobic	261	1,610	196	1,410	0.08	0.89
Strictly anaerobic (after exposure to air)	28	44	49	82	0.14	0.062
Aerobic	216	13	67	98	0.56	0.16

[a] Atmospheric conditions are described in the text.
[b] Total products = amount of L-lactate + amount of formate + amount of acetate + amount of ethanol.
[c] The relative rate of mannitol fermentation was calculated from the carbon recovery, and the value in the glucose fermentation by the glucose-grown cells was regarded as 1.0.

aerobic fermentation or the anaerobic fermentation after the short exposure to air, the levels of most glycolytic intermediates, including fructose 1,6-bisphosphate and D-glyceraldehyde 3-phosphate, were significantly lower, and the levels of pyruvate were much higher than during the strictly anaerobic fermentation (Table 7). Thus, the high proportion of lactate in the end products of the aerobic mannitol metabolism (Table 6) could not be ascribed to the regulation of the enzyme activities by the change in glycolytic intermediates, but were ascribed to the inactivation of pyruvate formate-lyase by oxygen.

DISCUSSION

Since the pathway of pyruvate conversion by pyruvate formate-lyase into formate, acetate, and ethanol in oral streptococci was proposed (5), only the activities of pyruvate formate-lyase (32, 38) and alcohol dehydrogenase (1) have been detected in *S. mutans*. All the enzymes involved in this pathway, except aldehyde dehydrogenase, are reported to be present in lactic streptococci (36). In this study, all the enzyme activities, including aldehyde dehydrogenase, involved in the conversion of pyruvate into formate, acetate, and ethanol were detected in *S. mutans* under strictly anaerobic conditions (Table 3). Thus, the presence of the metabolic pathway from pyruvate into these end products in *S. mutans* was confirmed (Fig. 1). The level of pyruvate formate-lyase and of alcohol dehydrogenase in *S. mutans* varied depending on the sugar on which the organism was grown. However, the regulation of the synthesis of alcohol dehydrogenase does not seem to be important in the change of the fermentation patterns, because even the glucose-grown cells, which had less alcohol dehydrogenase activity than galactose- or mannitol-grown cells, produced more ethanol than acetate, and there was no significant difference in the level of acetate kinase. Thus, the change in the level of pyruvate formate-lyase, but not in that of the other enzymes, seemed to determine the shift of fermentation patterns under these experimental conditions (Table 1).

The regulation of lactate dehydrogenase synthesis has been considered to be important in the change of the fermentation patterns of lactic streptococci (35, 36). However, the levels of lactate dehydrogenase among the *S. mutans* cells grown on glucose, galactose, or mannitol were not significantly different (Table 3). The

TABLE 7. Effect of oxygen on the levels of glycolytic intermediates during mannitol metabolism by mannitol-grown cells of *S. mutans*[a]

Intermediate	Amt of intermediate (nmol/mg of cells [dry wt])		
	Strictly anaerobic	Strictly anaerobic (after exposure to air)	Aerobic
Glucose 6-phosphate[b]	2.6	0.53	0.26
Fructose 1,6-bisphosphate	90	2.6	6.1
Dihydroxyacetone phosphate	12	2.0	2.9
D-Glyceraldehyde 3-phosphate	9.5	ND[c]	0.45
3-Phosphoglycerate	9.5	5.9	5.3
2-Phosphoglycerate	2.2	0.28	0.56
Phosphoenolpyruvate	0.70	0.56	1.4
Pyruvate[b]	12	51	83

[a] Atmospheric conditions are described in the text.
[b] Including intracellular and extracellular intermediates.
[c] ND, Not detectable.

mannitol-grown cells had the highest activity but produced less lactate and more volatile products from glucose than the glucose-grown cells (Table 1). Thus, the regulation of lactate dehydrogenase synthesis does not seem to play an important role in the shift of fermentation products in *S. mutans*, at least under these conditions.

In this study, when the galactose-grown cells of *S. mutans* were incubated with glucose, the cells produced much more volatile products and less lactate than the glucose-grown cells (Table 1). The intracellular levels of fructose 1,6-bisphosphate and lactate dehydrogenase in these cells were, however, similar (Tables 2 and 3). On the other hand, these cells had different levels of pyruvate formate-lyase and the triose phosphates. Thus, the differences in the end products of glucose fermentation between these cells were due to the regulation of the activity and the synthesis of pyruvate formate-lyase, rather than of lactate dehydrogenase.

The mannitol-grown cells produced more volatile products and less lactate from glucose than the glucose-grown cells (Tables 1 and 2), even though the former had a higher intracellular level of D-glyceraldehyde 3-phosphate, an inhibitor of pyruvate formate-lyase, than the latter. The unique properties of pyruvate formate-lyase may provide a solution to this discrepancy. As previously reported (32), the inhibition of the pyruvate formate-lyase of *S. mutans* by D-glyceraldehyde 3-phosphate is largely dependent on the concentration of the enzyme. The higher the concentration of pyruvate formate-lyase, the more D-glyceraldehyde 3-phosphate is required for 50% inhibition. During glucose metabolism by the mannitol-grown cells, the enzyme at high concentration is presumed to overcome the inhibitory effect of D-glyceraldehyde 3-phosphate.

Galactose-grown cells produced much more volatile products from galactose than from glucose (Table 1). This may arise from the lower levels of fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate, and dihydroxyacetone phosphate in the galactose-grown cells metabolizing galactose than in the cells metabolizing glucose. The low levels of these intermediates have been observed when streptococci metabolize sugar slowly, for example, when grown under glucose limitation (37). The galactose-grown cells also seem to metabolize galactose more slowly than they do glucose (Table 1).

The level of 3-phosphoglycerate was remarkably high when the galactose-grown cells were metabolizing galactose (Table 2), but the reason was not clarified. This high level of 3-phosphoglycerate does not seem to have any special effect on the shift of the fermentation pattern, because 3-phosphoglycerate has no significant effect on the activities of pyruvate formate-lyase (32) or lactate dehydrogenase (2, 37) of *S. mutans*.

An important role of pyruvate formate-lyase in the fermentation shift was also clearly demonstrated by the effect of oxygen. In contrast to many previous reports (12, 14, 18, 27, 33, 37), the glucose-grown cells of *S. mutans* produced a significant amount of volatile products even in the presence of excess glucose under strictly anaerobic conditions (Table 1). However, when the cells were exposed to air, the pyruvate formate-lyase in the cells was inactivated, and the streptococcus produced lactate exclusively. In the previous studies, cell suspensions were apparently exposed to air or cells were not incubated under strictly anaerobic conditions; thus, the cells produced lactate exclusively.

The remarkable decrease in mannitol metabolism by the exposure of the cells to air (Table 6) suggested that pyruvate formate-lyase was essential for anaerobic mannitol metabolism, since 1 mol more of NADH is produced from mannitol than from glucose (3, 24). The pathway from pyruvate to lactate does not have enough capacity to oxidize the excess NADH to regenerate NAD. Therefore, the reoxidation of NADH in mannitol metabolism should be accomplished by means of NAD-linked aldehyde dehydrogenase and NAD-linked alcohol dehydrogenase; that is, during the conversion of acetyl-CoA into ethanol, 2 mol of NADH are regenerated per mol of ethanol formed. This assumption may also explain why the mannitol-grown cells produced much more volatile products from mannitol than from glucose, that is, why the lactate dehydrogenase reaction was not accelerated despite the high level of fructose 1,6-bisphosphate in the cells during anaerobic mannitol metabolism.

The redox potential in the deeper layers of dental plaque is expected to be so low that strictly anaerobic conditions seem to prevail in vivo (19, 20, 29). These observations are of interest in connection with dental caries and evaluation of acidogenicity of sugar substitutes, because the fermentation of sugars in the deeper layers of dental plaque seems to be more important in the initiation of dental caries than that in the surface layers of dental plaque. Sugar alcohols like mannitol, sorbitol, or Lycasin are used as low cariogenic sweeteners, and the evaluation of the acidogenicity of these sugar alcohols is performed in air, or cell suspensions or dental plaque suspensions are at least exposed to air during preparation. It is probable that the acidogenicity of these sugars was underestimated when microorganisms were exposed to air and their pyruvate formate-lyase was inactivated. Cautious treatment of the microorganisms is necessary to elucidate their anaerobic metabolisms.

It was concluded that the regulation of pyruvate formate-lyase, as well as that of lactate dehydrogenase, played an important role in the shift of fermentation patterns during the sugar metabolism of *S. mutans*, especially under strictly anaerobic conditions.

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