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Acetoin Production by Wild-Type Strains and a Lactate Dehydrogenase-Deficient Mutant of *Streptococcus mutans*

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Eleven different laboratory strains of *Streptococcus mutans* representing the various serogroups were found to produce an average of 6.0 ± 4.8 mM acetoin when grown in glucose-containing medium under aerobic conditions. None of the strains produced detectable acetoin when grown anaerobically. A lactate dehydrogenase-deficient mutant produced acetoin both aerobically and anaerobically and in substantially greater amounts than the wild-type strains did. Substitution of mannitol for glucose resulted in decreased acetoin production by wild-type strains and the lactate dehydrogenase-deficient mutant, indicating a role for $NADH_2$ in the regulation of the acetoin pathway. Pyruvate incorporated into the growth medium of a wild-type strain caused acetoin to be produced anaerobically and stimulated acetoin production aerobically. Cell extracts of a wild-type *S. mutans* strain were capable of producing acetoin from pyruvate and were (partly) dependent on thiamine PP_i. Extracts prepared from aerobically grown cells had approximately twice the acetoin-producing activity as did extracts prepared from anaerobically grown cells. The results indicate that acetoin production by *S. mutans* may represent an auxiliary reaction of pyruvate dehydrogenase in this organism.

Streptococcus mutans appears to be an important etiologic agent in dental caries (11, 12). Its pathogenic potential depends in large part on its ability to produce acid from the metabolism of dietary carbohydrates. Mutants of *S. mutans* that have reduced ability to produce acids because of defects in intracellular polysaccharide metabolism (22) or in lactic acid production (17) have significantly reduced cariogenic potential.

Early studies of *S. mutans* characterized it as a homolactic fermenter (9, 23). Lactate dehydrogenase (LDH) was found to be produced constitutively and to utilize fructose 1,6-diphosphate as an essential cofactor (3). More recently, substantial amounts of ethanol, formate, and acetate were observed in culture liquors of *S. mutans* grown anaerobically under conditions in which fructose 1,6-diphosphate levels and, hence, LDH activity were low (2, 25). These and subsequent studies led to the discovery of a pyruvate formate-lyase pathway in this organism (26). Carlsson and coworkers (6) also have reported the presence of a pyruvate dehydrogenase activity in *S. mutans* when this organism is grown under aerobic conditions.

We previously reported that culture liquors of LDH-deficient mutants of *S. mutans* contain significant amounts of the neutral four-carbon-compound acetoin (15). Acetoin is produced as a fermentation end product or as a metabolic intermediate by several different pathways, depending on the organism being studied; *Aerobacter aerogenes* produces acetoin from pyruvate by the combined activities of acetolactate-forming enzyme and acetolactate decarboxylase (20). *Bacillus subtilis* (18) and *Streptococcus diacetilactis* (13, 14) form acetoin from 2,3-butanediol and citrate, respectively. Pyruvate dehydrogenase from pigeon breast muscle (1) and pyruvate decarboxylase from yeast (7) and mammalian liver (10) have also been shown to catalyze the formation of acetoin. The present paper describes the conditions for promoting acetoin production by wild-type strains of *S. mutans* and some characteristics of this pathway. Also, acetoin is shown to be a major end product of glucose fermentation by an LDH-deficient mutant of *S. mutans*, suggesting that this pathway can largely account for its decreased acidogenic and cariogenic potential.

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MATERIALS AND METHODS

Microorganisms and growth conditions. Strains of *S. mutans* used in these studies are listed in Table 1. Strain JH145 is an LDH-deficient mutant of strain BHT-2 that has been previously described (17). Strains were maintained in glycerol stabs prepared by mixing equal volumes of broth-grown cultures with sterile glycerol and storing them at −20°C. The chemically defined medium (3 ml in tubes [16 by 125 mm]) of Socransky et al. (19) supplemented with 55.5 mM glucose or mannitol was used throughout these studies. The medium was modified by substituting 0.1 M potassium phosphate (pH 7.1) for HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer and by omitting cysteine hydrochloride as a reducing agent. Aerobic cultures were incubated at 37°C on a New Brunswick TC-7 Rollordrum operating at 50 rpm. Anaerobic cultures were prepared by using prereduced media and were incubated at 37°C in a glove box containing an atmosphere of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen.

Analysis of fermentation end products. After incubation for the indicated times, 1-ml samples of the cultures were used to determine cell density, measured by turbidity at 550 nm. The remainder of the cultures was centrifuged at 15,000 × *g* for 10 min, and the cell-free liquors were collected. Ethanol and acetoin concentrations were measured by using a Hewlett-Packard 5880 gas-liquid chromatograph operated isothermally. Chromatography conditions with Chromosorb 101 were those described by Carlsson (5), except that the oven temperatures for ethanol and acetoin were 150 and 190°C, respectively.

Acetoin production by cell extracts. Overnight anaerobic cultures of bacteria were diluted 1:100 in fresh medium (200 ml) and grown in the glove box to early stationary phase. Aerobic cultures (200 ml) were incubated at 37°C in air in 1-liter Erlenmeyer flasks on a reciprocating shaker operating at 30 cycles/min. Cells were harvested by centrifugation at

TABLE 1. Acetoin and ethanol production by *S. mutans* strains during growth with glucose

Strain (serogroup)	Concn (mM)[a]			
	Aerobic		Anaerobic	
	Acetoin	Ethanol	Acetoin	Ethanol
E49 (a)	2.8	<1.0	<1.0	5.2
FA1 (b)	16.0	<1.0	<1.0	6.5
BHT-2 (b)	5.4	<1.0	<1.0	6.7
JH1001 (c)	2.9	<1.0	<1.0	16.0
H12 (c)	4.2	<1.0	<1.0	8.3
MT3 (c)	3.5	<1.0	<1.0	3.5
10449 (c)	7.8	<1.0	<1.0	8.1
Ingbritt (c)	3.8	<1.0	<1.0	6.9
OMZ176 (d)	3.1	<1.0	<1.0	<1.0
6715 (d/g)	1.9	<1.0	<1.0	5.6
LM7 (e)	14.3	<1.0	<1.0	4.2
JH145-*ldh*[b] (b)	37.0	10.6	17.3	44.5

[a] Cultures (48-h) were analyzed for their content of acetoin and ethanol by gas-liquid chromatography.
[b] From a previous study by Johnson et al. (17).

4°C, washed twice by centrifugation with 0.1 M potassium phosphate buffer, pH 7, and suspended in 1 ml of buffer. Cell extracts were prepared by treating the cell suspension at 4°C in a French press at 15,000 lb/in^2. Cell debris was removed by centrifugation at 15,000 × *g* for 10 min. Acetoin production by the cell extracts was measured under aerobic conditions of incubation in a standard reaction mixture containing 100 mM potassium phosphate buffer, pH 7, 100 mM sodium pyruvate, and 20 μg of thiamine PP_i per ml. The reaction was initiated by the addition of 0.6 ml of cell extract to give a final volume of 4 ml and incubated in a 37°C water bath. At indicated times, 0.2-ml samples were treated with 5 μl of 10 NaOH to stop the reaction and analyzed for their acetoin content by gas-liquid chromatography. The protein concentration of the cell extracts was determined by using the Bio-Rad protein assay kit.

RESULTS

Forty-eight-hour cultures of 11 *S. mutans* strains representing the various serogroups had consumed over 99% of the added (55.5 mM) glucose. Cell-free culture liquors were analyzed by gas-liquid chromatography for their content of neutral fermentation end products (Table 1). When grown with aeration, all of these strains were found to produce a compound that cochromatographed with authentic acetoin. Its retention time of 3.34 min differed from those of other common fermentation end products, including pyruvate, formate, lactate, acetate, ethanol, diacetyl, butanediol, and butanol. The colorimetric assay of Westerfield (24) further confirmed its identity as acetoin. The concentrations of acetoin in these cultures ranged from 1.9 mM for strain 6715 to 16.0 mM for strain FA1, with an average value and standard deviation of 6.0 ± 4.8 mM. As previously reported (26, 27), culture liquors from aerobically grown *S. mutans* contained little or no detectable ethanol. During anaerobic growth, the opposite situation prevailed, in which ethanol production increased substantially and little or no acetoin was observed.

In five independent trials, the amount of acetoin produced by strain JH1001 in aerobic culture varied from 1.6 to 4.5 mM with a mean value of 2.9 mM. Similar variation was observed for strain BHT-2, in which values ranged from 3.0 to 8.3 mM with a mean of 5.4 mM. The possibility that the variation in acetoin concentration was due to further metabolism of this compound was studied by analyzing culture liquors of strain BHT-2 as a function of time. Figure 1 shows that acetoin production by strain BHT-2 began in the lag phase and continued throughout the exponential phase of growth and into early stationary phase. Production ceased when the glucose in the medium became exhausted. Prolonged incubation of the culture resulted in no significant decrease of acetoin concentrations, indicating that further metabolism of this compound did not occur and was not responsible for the observed variability in the levels of acetoin found in independent trials. Similar results were obtained with cultures of JH1001.

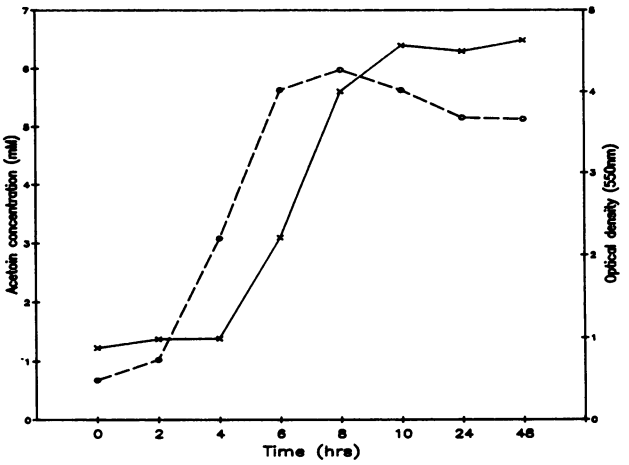

FIG. 1. Acetoin production (○) by strain BHT-2 in relationship to cell numbers (×) during growth in glucose-containing medium. An overnight aerobic culture of BHT-2 was diluted 1 to 7 with fresh medium, and 2-ml aliquots were incubated on a Rollordrum. Samples were taken at the indicated times and analyzed for their turbidity at 550 nm and acetoin content by gas-liquid chromatography.

When mannitol was substituted for glucose in the culture medium, strains BHT-2, JH1001, and JH145 produced significantly less acetoin during aerobic growth (Table 2). Acetoin production by JH145 also decreased during anaerobic growth, while ethanol production substantially increased in all three strains.

Cell extracts of strain JH1001 prepared from aerobically and anaerobically grown cultures were found to produce acetoin from pyruvate (Table 3). The rate of reaction was linear over a 2-h period. Acetoin formation was totally dependent on added pyruvate but only partially dependent (35%) on added thiamine PP_i. This incomplete dependence may be due to endogenous thiamine PP_i in the extracts. The activity was slightly greater at pH 7.0 than at pH 6.5 or 6.1

TABLE 2. Acetoin and ethanol production during growth on mannitol

Strain	Concn (mM)[a]			
	Aerobic		Anaerobic	
	Acetoin	Ethanol	Acetoin	Ethanol
JH1001	<1.0	2.7	<1.0	28.6
BHT-2	<1.0	<1.0	<1.0	29.4
JH145	1.46	24.2	2.6	47.5

[a] Cultures (48-h) were analyzed for their content of acetoin and ethanol by gas-liquid chromatography.

TABLE 3. Acetoin synthesis activity of JH1001 extracts[a]

Reaction mixture	Enzyme activity[b] (nmol of acetoin formed min^{-1} mg of $protein^{-1}$)	
	Aerobic	Anaerobic
Standard (pH 7.0)	227	115
Pyruvate	0	0
Thiamine PP_i	148	NT
Cells grown with 80 mM pyruvate	285	261

[a] Cell extracts were prepared from cultures of JH1001 grown aerobically or anaerobically in chemically defined medium containing 1% glucose.
[b] Data are the averages of two trials. NT, Not tested.

(data not shown). Extracts prepared from aerobically grown cells had an approximately twofold-higher specific activity than extracts prepared from anaerobically grown cells.

Citrate (8, 13, 14) and acetate (4, 16, 20) incorporated into the medium at concentrations up to 80 mM did not stimulate acetoin production in *S. mutans*, as has been reported for certain other bacteria. It was found that acetoin production (7.1 mM) could be stimulated in anaerobic cultures of JH1001 by incorporation of sodium pyruvate into the growth medium at a concentration of 80 mM. The amount of acetoin produced during aerobic cultivation was also increased about threefold (11.0 mM) by the presence of 80 mM pyruvate in the medium. Cell extracts prepared from cultures grown in the presence of pyruvate possessed increased specific acetoin-producing activities both aerobically and anaerobically (Table 3).

The LDH-deficient mutant, JH145, grew in glucose- or mannitol-containing medium like its parent, BHT-2, in both growth rate and cell yield under aerobic and anaerobic conditions. With glucose as the carbon source, JH145 differed from its parent and other wild-type *S. mutans* strains in producing both acetoin and ethanol when grown either aerobically or anaerobically. JH145 produced substantially more acetoin than the wild-type strains when grown aerobically (Table 1). In this strain, acetoin production accounted for 44.3 and 20.8% of the carbon metabolized aerobically and anaerobically, respectively. Under the same growth conditions. JH145 produced 10.6 ± 2.6 mM ethanol aerobically and 44.5 ± 6.6 mM ethanol anaerobically, accounting for 6.0 and 26.7% of the metabolized carbon, respectively.

DISCUSSION

The microenvironment of the tooth surface where *S. mutans* resides must undergo substantial changes over time with regard to a number of factors, including nutritional content, pH, oxidation-reduction potential, and atmosphere. To efficiently colonize this environment, *S. mutans* has evolved a versatile array of metabolic pathways for the conversion of pyruvate into fermentation end products. The constitutive L-(+)-lactate dehydrogenase of *S. mutans* is completely dependent on fructose 1,6-diphosphate for activity (3) and is largely inactive when the carbon supply is low or slowly metabolized (25). Pyruvate formate-lyase activity is high under anaerobic conditions when the supply of carbon is low, but it is rapidly inactivated by oxygen (21, 27). Pyruvate dehydrogenase appears to complement pyruvate formate-lyase by being most active in cells growing in the presence of oxygen (6).

The present demonstration of acetoin synthesis by *S. mutans* under conditions of aerobic growth reveals another alternative pathway for pyruvate metabolism. Like the pyruvate dehydrogenase activity of *S. mutans* (6), the enzyme activity responsible for acetoin synthesis was found to be present in extracts of both aerobically and anaerobically grown cells but was somewhat more active in the former. Also like pyruvate dehydrogenase, acetoin synthesis was promoted by the presence of pyruvate in the growth medium. Extracts prepared from cells grown under these conditions showed only a slight increase in acetoin formation activity. The increase in acetoin production, therefore, is probably due to increased substrate concentration. Wild-type strains of *S. mutans* and the LDH-deficient mutant were found to produce significantly less acetoin during aerobic growth when mannitol, which yields an additional mole of $NADH_2$ per mole of substrate utilized, was substituted for glucose. The LDH-deficient mutant also produced less acetoin anaerobically with mannitol as the carbon source. Carlsson et al. (6) have demonstrated that, in vitro, the pyruvate dehydrogenase complex of *S. mutans* is inhibited by $NADH_2$. As in the case of pigeon breast muscle (1), the results of these studies indicate that acetoin production by *S. mutans* may be the result of an auxiliary reaction performed by pyruvate dehydrogenase. Further analysis of this possibility must await purification of the enzyme or isolation of the appropriate mutants.

In short-term (30-min) fermentation studies, Yamada et al. (27) reported that the LDH-deficient mutant, JH145, hardly metabolized glucose in the presence of oxygen. They concluded that pyruvate formate-lyase was the sole enzyme responsible for metabolism of pyruvate in this strain. In the present study, we have found that JH145 does metabolize glucose and grow in the presence of oxygen by making extensive use of acetoin and ethanol production for the dissimilation of pyruvate. The production of these neutral end products in large amounts can account for the much-reduced acidogenic and cariogenic potential observed (15, 17) for the mutant.

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