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Citrate Metabolism by *Pediococcus halophilus*

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Several strains of non-citrate-metabolizing *Pediococcus halophilus* have previously been isolated from soy sauce mash or moromi. The factors controlling the metabolism of citrate in soy pediococci were studied. All the soy pediococcal strains tested which failed to decompose citrate did not possess citrate lyase [citrate (*pro*-3*S*)-lyase; EC 4.1.3.6] activity. In *P. halophilus*, citrate lyase was an inducible enzyme, and the optimum pH for activity was 7.0. The metabolism of citrate in *P. halophilus* was different from that observed in lactic streptococci. The main products from citrate were acetate and formate, and this bacterium produced no acetoin or diacetyl. Formate production from citrate was greatly reduced in the presence of glucose. *P. halophilus* 7117 (Cit$^+$) was proved to contain citrate lyase, pyruvate formate-lyase (EC 2.3.1.54) phosphotransacetylase (phosphate acetyltransferase; EC 2.3.1.8), and acetate kinase (EC 2.7.2.1), i.e., all the enzymes necessary to convert citrate to acetate and formate.

The soy pediococcus, *Pediococcus halophilus* (22, 30), is a salt-tolerant, homofermentative, lactic acid bacterium used in the brewing of soy sauce. Recently, we reported that soy pediococci metabolized citrate and malate during lactic acid fermentation of soy sauce brewing, but we found some strains that could not utilize these organic acids (14).

There are many reports on the organic acid metabolism of lactic acid bacteria. Citrate and malate are the acids that lactic acid bacteria encounter most frequently in their environment (28). In the manufacture of fermented dairy products, the citrate-utilizing lactic acid bacteria are important, because products of their metabolism of citrate, especially acetoin and diacetyl, are regarded as being favorable in enriching the flavor of butter, cheese, and other dairy products (2, 3, 6, 11, 16, 25).

In soy sauce brewing, citric acid is the main organic acid occurring in the early stages of moromi fermentation, but little information is available on citrate metabolism in *P. halophilus*. In the present work, citrate metabolism in several strains of soy pediococci was studied. It was observed that citrate-negative (Cit$^-$) strains were missing the inducible citrate lyase [citrate (*pro*-3*S*)-lyase; EC 4.1.3.6], and pathways of citrate degradation in *P. halophilus* differed largely from those in lactic streptococci.

MATERIALS AND METHODS

Organisms and culture conditions. The *P. halophilus* strains used were isolated from moromi several years ago and stocked in this laboratory, and *Streptococcus lactis* subsp. *diacetilactis* DRC1 was obtained from R. Irie, National Institute of Animal Industry, Ibaraki, Japan. *P. halophilus* was cultured in a basal medium containing the following (percent [weight/volume]): yeast extract (Difco Laboratories, Detroit, Mich.), 0.3; Polypepton (Daigo Eiyo Chemical Co., Osaka, Japan), 1.0; K_2HPO_4, 1.0; sodium thioglycolate, 0.1; and NaCl, 10. The medium was supplemented with 1.0% glucose (YPG), 0.5% citrate (YPC), 1% glucose plus 0.5% citrate (YPGC), or 0.5% pyruvate (YPP) as carbon sources. The medium was sterilized at 120°C for 15 min or by passage through a membrane filter system (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) after the pH was adjusted to 7.0. Pediococci were precultured in test tubes containing 2.5 ml of YPG at 30°C for 15 h, inoculated into 2.5 liters of the above-described media, and grown in stationary phase at 30°C.

S. lactis subsp. *diacetilactis* was maintained in sterile skim milk (11% [wt/vol] solution of skim milk powder autoclaved at 120°C for 10 min). This streptococcus was grown for 20 h at 30°C in lactose broth with 0.5% sodium citrate · $2H_2O$ added as described by Harvey and Collins (8).

The cells were harvested in the late-log phase by centrifugation (12,000 × *g* for 10 min) at 4°C. Dry weights were determined with washed cells after heating at 110°C for 20 h.

Preparation of resting and toluene-treated cells. Harvested cells were washed twice with 50 mM potassium phosphate buffer (pH 7.0), and the suspensions were kept for 90 min at 30°C. After starvation, the cells were washed and resuspended in the same buffer (resting cells). Toluene treatment (15) was carried out by the addition of 0.1 ml of a toluene-acetone mixture (1:9 [vol/vol]) to 5 ml of the cell suspension for 10 min at 0°C.

Preparation of cell extracts. Washed cells were resuspended in the same buffer as that described above (40 ml) containing 2 mM dithiothreitol and 150 mM sodium glutamate. The cells were disrupted with glass beads (diameter, 0.25 to 0.5 mm; MK-2GX) in a grinding container of a DYNO-MILL (Willy A. Bachofen Manufacturing Engineers, Basel, Switzerland) for 6 min at 0°C. The debris was removed by centrifugation (25,000 × *g* for 30 min), and after ultracentrifugation (105,000 × *g* for 2 h), the supernatant fluids were used as crude cell extracts.

Enzyme assays. Unless otherwise indicated, all assays were carried out at 30°C, and the reaction was started by the addition of the cell extract to the assay mixture (3 ml). Citrate lyase was assayed by determining spectrophotometrically the rate of formation of oxalacetate from citrate (17, 27). The assay mixture (pH 7.0) contained 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–NaOH, 10 mM $MgCl_2$, 0.16 mM NADH, 7.5 mM sodium citrate, 10 U of lactate dehydrogenase, and 10 U of malate dehydrogenase. In the citrate lyase assays, one unit

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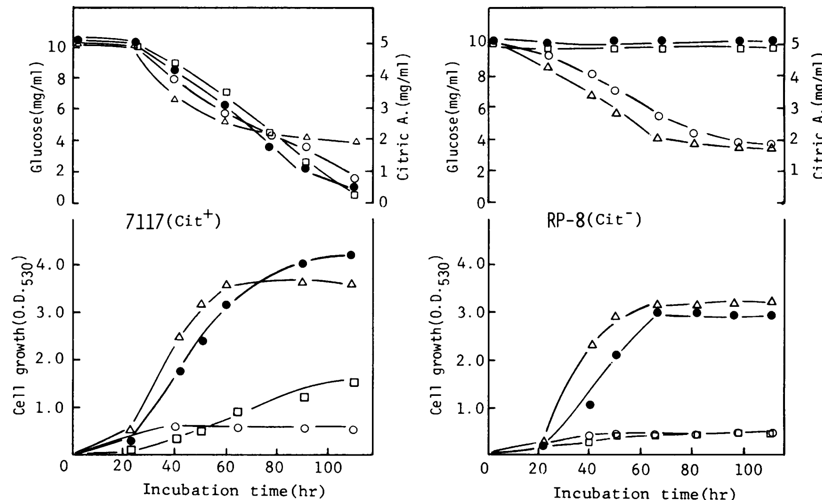

FIG. 1. Time course of citrate (Citric A.) decomposition and growth of *P. halophilus* 7117 (Cit$^+$) and RP-8 (Cit$^-$). The two strains of *P. halophilus* were grown at 30°C in YPG, YPC, and YPGC media under the conditions described in Materials and Methods. Symbols for cell growth: △, YPG; □, YPC; ●, YPGC; ○, basal medium. Symbols for citrate decomposition: □, YPC; ●, YPGC. Symbols for glucose consumption: △, YPG; ○, YPGC. O.D.$_{530}$, Optical density at 530 nm.

of enzyme activity was expressed as micromoles of substrate utilized per minute per milligram of protein and was calculated by using the absorption coefficient of NADH (ε = 6.22 cm^{-1} $mmol^{-1}$). Phosphotransacetylase (phosphate acetyltransferase; EC 2.3.1.8) and acetate kinase (EC 2.7.2.1) were assayed by published methods (32, 37). Storage of the cell extracts at −20°C for less than 4 weeks had little effect on the specific activities of these enzymes.

The pyruvate-formate exchange activity of pyruvate formate-lyase (EC 2.3.1.54) was assayed with intact cells. The reaction mixture contained the following in a total volume of 2.0 ml: 50 mM potassium phosphate buffer (pH 7.0), 20 mM sodium pyruvate, and cell suspension. After incubation for 20, 40, and 60 min at 30°C, the reactions were terminated by the addition of HCl, and the cells were removed by centrifugation. The amounts of formate produced and pyruvate consumed were determined with a carboxylic acid analyzer (23). The reaction rate increased linearly with time, and activity was proportional to cell mass.

Analysis of D-glucose, carboxylic acids, and other fermentation products. Citrate was determined by using an assay kit (catalog no. 139076; Boehringer GmbH, Mannheim, Federal Republic of Germany). Carboxylic acids were analyzed with a carboxylic acid analyzer (model S-14; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). D-Glucose was determined enzymatically by the GOD-POD method (40). Acetoin and diacetyl were assayed with a gas chromatograph (model 4BMPF; Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector as described by Nunomura et al. (24).

Other methods. Growth rates were determined by measuring turbidity (optical density at 530 nm) at low cell densities, and the protein concentration in the cell extract was estimated by the method of Lowry et al. (19) with bovine serum albumin as the standard. Sugar fermentation was tested by the method of Uchida (41).

Chemicals. All enzymes were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. All other reagents used were of analytical grade and were obtained from Sigma Chemical Co., St. Louis, Mo., or Wako Pure Chemical Industries, Tokyo, Japan.

RESULTS

Growth and citrate utilization of *P. halophilus* 7117 (Cit$^+$) and RP-8 (Cit$^-$). Most strains of *P. halophilus* metabolize citrate but strains unable to utilize citrate have also been found (14). To clarify the difference between Cit$^+$ and Cit$^-$ strains, citrate utilization during growth was measured with representative strains 7117 (Cit$^+$) and RP-8 (Cit$^-$).

Both strains showed slight growth in the basal medium. Figure 1 shows that the growth pattern of 7117 was similar to that of RP-8 when each strain was grown in YPG medium. However, a marked difference was observed when these strains were cultured in either YPC or YPGC medium. Strain 7117 could grow with citrate as the sole carbon source (YPC medium), although the growth was limited. This utilization of citrate as an energy source has been reported previously for other lactic acid bacteria (6, 10). Cells grown in YPC or YPGC medium almost completely decomposed citrate without the glucose effect. A similar response was observed in other Cit$^+$ strains (data not shown). On the other hand, strain RP-8 was unable to grow on citrate and did not decompose citrate even in the presence of fermentable carbohydrates.

Citrate utilization by intact and toluene-treated cells. *S. lactis* subsp. *diacetilactis* has been shown to possess two enzymes, citrate permease and citrate lyase, which transport citrate into the cell and split it into oxalacetate and acetate, respectively (8, 9). Oxalacetate is decarboxylated to pyruvate. Citrate-negative strains of *S. lactis* subsp. *diacetilactis* were shown to be missing citrate permease but not citrate lyase by Collins and Harvey (4). Therefore, it was of interest to compare the nature of Cit$^+$ and Cit$^-$ soy pediococcal strains by using intact cells or cells that had their permeability barrier removed by toluene treatment. Figure 2 shows that intact and toluene-treated cells of 7117 utilized all of the citrate in the reaction mixture within 60 min, whereas neither intact nor toluene-treated cells of RP-8 could utilize citrate. These results suggested that the inability of RP-8 to utilize citrate was due to the absence of citrate lyase.

Determination of citrate lyase activity. The citrate lyase activity responsible for the degradation of citrate to

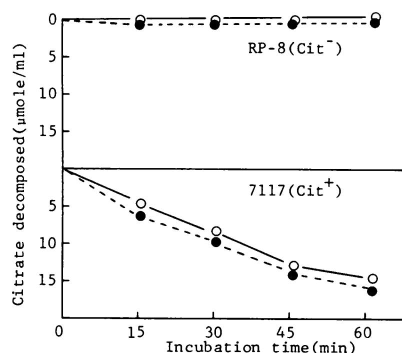

FIG. 2. Citrate decomposition by intact ○ and toluene-treated (●) cells of *P. halophilus* 7117 (Cit^+) and RP-8 (Cit^-). Reaction mixtures (2.0 ml, pH 7.0) containing 100 mM sodium phosphate, 36 μmol of sodium citrate, 5 μmol of $MgCl_2$, and 20 mg of cells were incubated at 30°C. At periodic intervals, samples were removed and analyzed for citrate with a Boehringer Mannheim assay kit.

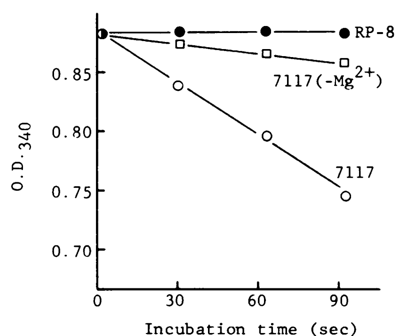

FIG. 3. Citrate lyase activity in cell extracts of *P. halophilus* 7117 (Cit^+) and RP-8 (Cit^-) grown in YPGC medium. O.D.$_{340}$, Optical density at 340 nm.

oxalacetate and acetate was detected in cell extracts of *P. halophilus* 7117 (Fig. 3). The optimum pH for enzyme activity was 7.0, and the enzyme required divalent cations for activity. Maximal enzyme activity was obtained with 10 mM Mg^{2+}. Magnesium ions could be partially replaced by other divalent ions, such as Mn^{2+} or Co^{2+}. The enzyme did not require L-glutamate for activity, but it was stabilized by the presence of L-glutamate (data not shown). Six strains of Cit^+ and Cit^- soy pediococci and *S. lactis* subsp. *diacetilactis* DRC1 were compared for their citrate lyase activities in cell extracts. The activities of the enzyme in Cit^+ soy pediococcal strains 220, 7116, and 7117 grown in YPGC medium were 0.117, 0.212, and 0.169 U/mg of protein, respectively (Table 1). It was also observed that the levels of citrate lyase activity were very low in YPG-grown cells, as compared with those grown in the presence of citrate. These results indicated that citrate lyase was an inducible protein in soy pediococci, in contrast to the enzyme in *S. lactis* subsp. *diacetilactis* DRC1. On the other hand, no enzyme activity could be found in any of the Cit^- strains (23, 34, and RP-8). There was a significant difference in citrate lyase activities between the two groups.

Fermentation products of *P. halophilus* 7117 growing in batch cultures with various substrates. Figure 4 shows the end products produced by *P. halophilus* 7117 from various substrates. The product from glucose was primarily L-lactate, and about 1.9 mol of lactate was formed per mol of glucose fermented. Acetate and formate were also found in small amounts. In contrast, acetate and formate were major products when this organism was grown on citrate or pyruvate. The molar ratios of the products (acetate to formate) were about 2:1 on citrate and 1:1 on pyruvate. The amount of lactate produced was very small. Furthermore, this organism was found to form neither acetoin nor diacetyl from citrate alone.

In the presence of glucose, formate produced from citrate was greatly reduced. This results was perhaps due to the inhibition of pyruvate formate-lyase by some intermediates of glucose metabolism (7, 34, 38). In addition to the data shown, the same results were also obtained with the other Cit^+ soy pediococci.

Citrate metabolism by resting cells of *P. halophilus* 7117. YPGC-grown cells of *P. halophilus* 7117 produced a lower level of formate than did those grown in YPC medium (Fig. 4). YPC-, YPG-, and YPGC-grown cells were therefore examined for citrate metabolism in buffer to clarify the effect of the culture medium. YPC-grown cells of *P. halophilus* produced acetate, formate, and lactate from citrate (Table 2). Pyruvate was also detected as an intermediate of citrate metabolism. In contrast, YPGC-grown cells produced equimolar acetate and pyruvate only. Formate was not found. Citrate was not metabolized by YPG-grown cells. We

TABLE 1. Citrate lyase activities in cell extracts of several strains of *P. halophilus* grown in the absence or presence of citrate

Strain	Fermentation of[a]:						Growth on:		Citrate lyase activity[b] in indicated cells grown in:	
	A	L	B	S	M	G	Citrate	Pyruvate	YPG	YPGC
P. halophilus										
220	−	+	+	−	−	+	+	+	0.016	0.117
7116	+	+	+	+	+	+	+	+	0.028	0.212
7117	+	−	+	−	+	+	+	+	0.001	0.169
23	−	−	−	−	−	+	−	+	ND[c]	ND
34	+	−	+	+	−	+	−	+	ND	ND
RP-8	+	−	−	−	−	+	−	+	ND	ND
S. lactis subsp. *diacetilactis* DRC1	−	+	−	−	−	+	−	−	0.900[d]	0.845[d]

[a] A, Arabinose; L, lactose; B, melibiose; S, sorbitol; M, mannitol; G, glucose.
[b] Expressed as micromoles of substrate utilized per minute per milligram of protein.
[c] ND, Not detected.
[d] *S. latis* subsp. *diacetilactis* DRC1 was grown in lactose broth in the absence or presence of citrate.

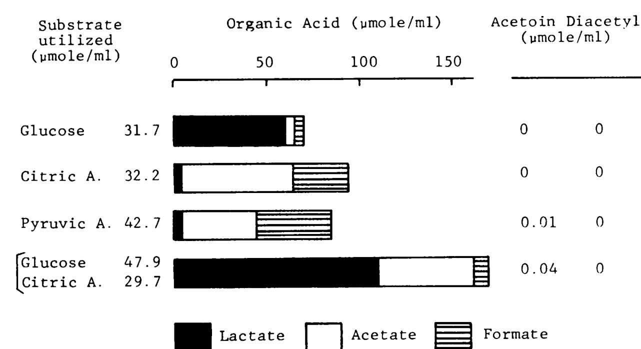

FIG. 4. Amounts of fermentation end products produced by *P. halophilus* 7117 grown in stationary culture in YPG, YPC, YPP, and YPGC media. A., Acid.

concluded that citrate lyase was not induced in the YPG culture.

Activities of enzymes involved in citrate metabolism. The activities of the four enzymes involved in the pathway of citrate metabolism (Fig. 5) were determined in cell extracts of *P. halophilus* 7117 under atmospheric conditions (Table 3). Citrate lyase activity in strain 7117 grown in YPGC medium was twice that in cells grown in YPC medium. Phosphotransacetylase was present at a higher level of activity in YPC-grown cells than in YPGC-grown cells. The levels of acetate kinase in both types of cells were not significantly different.

Pyruvate formate-lyase activities were significantly different between YPC- and YPGC-grown cells. In YPC-grown cells, the in vivo activity of pyruvate formate-lyase was detected, but this activity was not found in YPGC-grown cells. It was assumed that in YPGC-grown cells there exist, at high levels, some pyruvate formate-lyase inhibitors, e.g., D-Glyceraldehyde 3-phosphate, possibly explaining why only a small amount of formate was produced from citrate in the presence of glucose.

DISCUSSION

In general, citrate-utilizing soy pediococci are desirable, since acetate, a product of their metabolism of citrate, is one of the important constituents of soy sauce.

According to several studies of *P. halophilus*, this bacterium was considered to be able to utilize citrate (13, 35). In fact, most soy pediococci we studied were able to grow with citrate as an energy source or to decompose it in conjunction with carbohydrate fermentation. Recently, we isolated some strains lacking the ability to decompose citrate from naturally brewed soy sauce mash or moromi (14). Their properties, other than citrate utilization, were consistent with those of the typical members of *P. halophilus*. It has been already confirmed that soy pediococci are actually quite heterogeneous with regard to carbohydrate fermentation (41). The difference in citrate utilization among soy pediococci was also considered as evidence of heterogeneity within the species *P. halophilus*.

It was the main object of this study to explain why Cit$^-$ strains cannot ferment citrate. The results indicated that the Cit$^-$ strain RP-8 was missing citrate lyase. The same result was obtained with the other Cit$^-$ soy pediococcal strains we examined.

There are several reports regarding the instability of citrate utilization by *S. lactis* subsp. *diacetilactis*. Collins and Harvey (4) stated that a variant of *S. lactis* subsp. *diacetilactis* DRC1-X that could not utilize citrate contained citrate lyase but not citrate permease. Kempler and McKay (15, 20) reported that Cit$^-$ mutants GK10 and GK82 were not only missing citrate permease but also appeared to be defective in citrate lyase.

Harvey et al. (8) showed that citrate lyase was a constitutive enzyme in *S. lactis* subsp. *diacetilactis*, and we also confirmed this with a strain of the same species. The citrate lyase of soy pediococci was, however, inducible, as in *Enterobacter cloacae* (27) and *Klebsiella aerogenes* (26).

The biochemical details of citrate metabolism in several starter lactic acid bacteria have been studied (2, 3, 5, 11, 12,

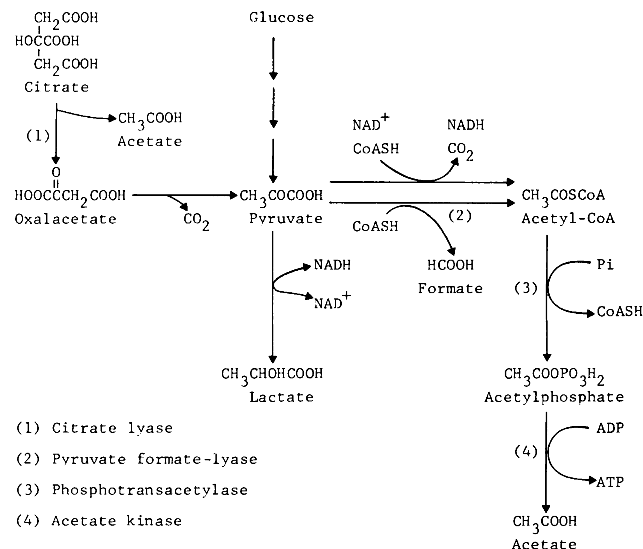

FIG. 5. Proposed pathway for citrate metabolism in soy pediococci.

TABLE 2. Amounts of end products produced from citrate by resting cells of *P. halophilus* 7117[a]

Culture medium	Amt (nmol/mg of dry cells) of: Substrate utilized	L-Lactate produced	Acetate produced	Formate produced	Pyruvate produced
YPC	810	140	1,300	330	220
YPG	20	ND[b]	20	ND	10
YPGC	450	ND	430	ND	410

[a] Each test tube (3.0 ml, pH 7.0) contained 100 mM sodium phosphate, 25 μmol of sodium citrate, 7.5 μmol of $MgCl_2$, and 30 mg (dry weight) of resting cells. The reaction was carried out at 30°C for 20 min and terminated by the addition of HCl.

[b] ND, Not detected.

TABLE 3. Activities of enzymes involved in citrate metabolism in *P. halophilus* 7117

Medium	Activity[a] of: Citrate lyase	Pyruvate formate-lyase[b]	Phosphotransacetylase	Acetate kinase
YPC	0.059	9.0	2.02	7.96
YPGC	0.118	ND[c]	0.77	9.78

[a] Unless otherwise indicated, expressed as micromoles of substrate utilized per minute per milligram of protein.

[b] Expressed as nanomoles of formate produced from pyruvate per minute per milligram (dry weight) of bacteria.

[c] ND, Not detected.

16, 21, 25, 31, 36, 39). Most strains were reported to produce increased amounts of acetoin and diacetyl when grown in the presence of citrate. Little information has been available about citrate metabolism in *P. halophilus*. The results of this study indicate that the products of citrate fermentation in soy pediococci differ significantly from those in lactic streptococci. *P. halophilus* fermented 1 mol of citrate into 2 mol of acetate, 1 mol of formate, and a smaller amount of lactate. The formation of acetoin or diacetyl was not observed (Fig. 4). The production of formate suggested the presence of pyruvate formate-lyase, although this enzyme had not been demonstrated previously in pediococci. Attempts to detect this enzyme in cell extracts were unsuccessful, probably because this enzyme is immediately inactivated by exposure to air (1, 34, 43).

Pyruvate formate-lyase is known to be present in citrate-grown *S. faecalis* (18), glucose-limited *S. mutans* (1, 34, 42, 43), and sorbitol-fermenting *S. sanguis* and *S. mitior* (33). Recently, the regulation of pyruvate formate-lyase and lactate dehydrogenase has been suggested to be involved in the shift from homolactic to heterolactic fermentation in lactic streptococci (37, 38) and *Lactobacillus bulgaricus* (29).

The products expected from the cleavage of pyruvate by pyruvate formate-lyase were equimolar formate and acetyl coenzyme A. The acetyl coenzyme A could be converted to acetate, providing the energy for growth. However, when the pediococcal cells were grown in YPGC medium, the amount of formate produced was less than that expected. It is known that intracellular triose phosphates (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate), intermediates of glycolysis, inhibit pyruvate formate-lyase activity in *S. mutans* (34) as well as in lactic streptococci (7, 38). Our observations with soy pediococci suggest that the inhibition of pyruvate formate-lyase activity might be responsible for the decrease in formate produced from pyruvate. The fact that the amounts of acetate were not so decreased in YPGC cultures could be explained in the following way: acetyl coenzyme A could also be produced through a separate pathway, e.g., a pyruvate dehydrogenase system, without the production of formate. In metabolizing glucose to pyruvate, a cell generates NADH from NAD and, to recover the NAD, must convert pyruvate to lactic acid. On citrate alone, no NADH is formed with the formation of pyruvate, so pyruvate can be metabolized through the acetyl coenzyme A branch. If CO_2 fixation can occur (Fig. 5), then some pyruvate can be metabolized to lactate, with the recovery of the NAD. This speculation is coincident with the results observed when glucose and citrate were both present (Fig. 4).

P. halophilus 7117 has now been proved to contain citrate lyase, pyruvate formate-lyase, phosphotransacetylase, and acetate kinase, i.e., all the enzymes necessary for the conversion of citrate to acetate and formate, producing ATP during acetate production. However, with excess glucose (the normal situation in soy sauce fermentation), the strain preferentially metabolizes pyruvate to acetate without the production of formate.

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