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Effects of Citrate on the Composition and Metabolism of *Lactobacillus casei*

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Lactobacillus casei ATCC 393 converted small amounts of citrate to diacetyl, other volatile compounds, and lipids. Citrate was accumulated passively by the organism. The presence of citrate in the growth medium decreased the uptake of acetate and its conversion to cellular lipids. Cells grown in citrate media contained more protein per cell than did controls. This increased protein content was reflected mainly in the soluble fraction when cells were subjected to sonic lysis. Soluble fractions from cells cultured in the presence of citrate contained more total protein as well as more individual proteins than these fractions from control cells. The presence of citrate caused extensive flocculation and increased the susceptibility of cells to lysis.

Microbial metabolism of citric acid and its salts has been investigated extensively. With lactic acid bacteria, citrate is known to stimulate both growth rate and diacetyl production (5, 9). The metabolism of citrate has been studied more extensively with *Streptococcus diacetilactis* than with any other lactic acid bacteria. Although this organism possesses the necessary permease system, it does not catabolize citrate by the ordinary citric acid cycle (6, 11). Instead, citrate is cleaved by citrate lyase to acetate and oxalacetate; the oxalacetate formed is subsequently decarboxylated to pyruvate, which can then be reduced to lactate or converted to acetoin and diacetyl (9, 11, 20).

Campbell and Gunsalus (4) found that citrate could be utilized by *Lactobacillus casei* in the absence of fermentable carbohydrate, and it was thus conceivable that citrate could stimulate *L. casei* by acting as an additional energy source. Citrate is known to influence the metabolism of acetate (24), a compound which also stimulates growth of *L. casei* (22). In conjunction with studies on diacetyl metabolsm by *L. casei*, we observed that addition of citrate to the fermentation medium markedly enhanced the growth of this organism (3). Addition of citrate increased both the rate of diacetyl synthesis (4) and the level of diacetyl reductase (2) in *L. casei* cultures. Citrate also profoundly influenced other aspects of the metabolism as well as the composition of *L. casei*. The present communication details some of these effects.

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MATERIALS AND METHODS

Organism and culturing conditions. *L. casei* 393, obtained from the American Type Culture Collection, was propagated routinely in Elliker broth (Difco) with or without the addition of 14 μmoles of sodium citrate per ml. Growth rates were determined by relating absorbance at 660 nm to dry weight of cells by using standard curves prepared under the experimental conditions.

Determination of cellular composition. Two-liter portions of the citrate-containing and control sterile broths were inoculated and incubated for 48 hr at 30 C. Cells were harvested by centrifugation at 10,000 × *g* for 20 min at 4 C, washed once with distilled water, and resuspended in distilled water. Lipids were recovered by extraction of cell suspensions three times with 6 to 8 volumes of chloroform-methanol (2:1, v/v). The chloroform-rich phases were combined, washed (8), and evaporated to dryness under reduced pressure at room temperature. Lipid residues were redissolved in accurately measured volumes of chloroform, and the quantities of lipid obtained were determined by the dichromate method of Bragdon (1). Protein (15) and carbohydrate (19) contents of cells and lipid-depleted residues were determined with crystalline bovine serum albumin and glucose, respectively, as reference standards. Nucleic acids were extracted from the lipid-depleted residues with trichloroacetic acid, and the amounts of deoxyribonucleic acid (DNA; reference 5) and ribonucleic acid (RNA; reference 23) recovered were determined.

Cell-free extracts were prepared by sonic oscillation of cells suspended in 20 ml of 0.1 M potassium phosphate buffer, *p*H 7.0, for 10 min in a Branson B110 Sonicator operated at maximum output. During this treatment, the suspension was cooled in an ice-water bath. Cellular debris was removed by centrifugation at 10,000 × *g* for 20 min at 5 C and was resuspended

in distilled water. Protein contents of debris and soluble fractions were determined. Portions of the soluble fractions were applied to 4 by 50 cm columns of Sephadex G-200. Elution was accomplished in a 5 C cold room with a 0.1 M potassium phosphate buffer (*p*H 7.0); 5-ml fractions were collected, and the absorbance at 280 nm was determined. Portions of soluble fractions were subjected to disc electrophoresis in both large- and small-pore polyacrylamide gels by the method of Davis (7).

Accumulation of citrate and acetate. The ability of cells harvested from control and citrate-containing media to accumulate citrate and acetate was evaluated by the method described by Harvey and Collins (10) or by this method modified to permit the use of radioactive substrates. Sodium citrate-*1,5-*^{14}C or sodium acetate-*1-*^{14}C was added to sufficient amounts of the respective cold salt in 0.1 M potassium phosphate buffer, final *p*H 4.5, to yield a concentration of 2.5 mmoles in 20 ml. To this was added 0.5 ml of cell suspension in 0.1 M potassium phosphate buffer and 2.5 mmoles of magnesium sulfate. These mixtures were incubated at 30 C with constant agitation, and 2-ml samples were taken from each at intervals throughout the incubation period. Cells were removed from these samples by membrane filtration (0.45 μm pore size, Millipore Corp., Bedford Mass.) and were washed with 1.0 ml of distilled water. Filters containing the cells, and also the supernatant fluids and washings, were dissolved separately in 20-ml quantities of xylene-dioxane-cellosolve (1:3:3 by volume) containing 1% 2,5-diphenyloxazole (PPO), 0.05% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 8% naphthalene, and counted in a Packard Tri-Carb liquid-scintillation-spectromoter.

Utilization of citrate and acetate. To determine utilization of citrate and acetate, 1 μCi of sodium citrate-*1,5-*^{14}C or sodium acetate *1-*^{14}C was added to 50 ml of sterile Elliker broth with or without 14 μmoles of sodium citrate per ml. These broths were inoculated with 1% by volume of an actively growing *L. casei* culture and were incubated for 72 hr at 30 C. Lipids were extracted from 25-g portions of the well mixed cultures and were washed as described. Total neutral lipid and polar lipid fractions were obtained by silicic acid column chromatography (13). Polar lipid was defined as that fraction retained during elution with diethyl ether containing 0.5% formic acid, but eluted with methanol. Free fatty acids were separated from the neutral lipid fraction by the method of McCarthy and Duthie (16). Known portions of lipid fractions were counted in a liquid scintillation spectrometer in toluene fluid (5 g of PPO and 100 mg of POPOP per liter).

Diacetyl and other volatile compounds were purged from other portions of the 72-hr broth cultures by using a modification of the method of Pack et al. (18). The apparatus was the same, except that a second graduated centrifuge tube containing 1 ml of 10% aqueous KOH was connected to the tube containing hydroxylamine by means of a glass U-tube. This centrifuge tube was cooled in ice water. The culture was held at 65 C for 2 hr, and nitrogen was bubbled through at the recommended flow rate (18). After purging, accurately measured volumes of the hydroxylamine and KOH solutions were added to XDC scintillation fluid and counted. Portions of the purged broth from which lipids had previously been extracted were also taken for counting. The utilization of citrate was followed by determining citrate content of cultures at intervals during incubation by using the method of Marier and Boulet (17).

Microscopic examination. A few drops of culture or cell suspension were spotted on slides and examined with a phase-contrast microscope.

Storage stability of cells. To determine the effect of citrate on the storage stability of cells, cells were isolated from both types of media, washed, and resuspended in 0.1 M potassium phosphate buffer, at *p*H 4.5 or 7.0, to a concentration of cells equal to that in the original culture. Suspensions thus obtained were dispensed into tubes and held at 4 C. At different intervals, duplicate tubes were removed and small portions were used to inoculate sterile 10% nonfat milk, which was then incubated for 24 hr at 30 C. Acid production was measured by titration of milk cultures to the phenolphthalein end point with 0.1 N NaOH. Cells were assayed for their ability to produce diacetyl plus acetoin from pyruvate by recovering cells from suspensions by centrifugation, resuspending them in 3 ml of 0.1 M phosphate buffer (*p*H 4.5), adding 100 μmoles of sodium pyruvate, and incubating the mixture at 30 C for 2 hr. Diacetyl plus acetoin was quantified by the method of Hill et al. (12). Protein content of the cell-free, spent buffer was determined (15).

Chemicals. All solvents used were of reagent grade quality, and those used for lipid extraction and fractionation were redistilled in glass. DNA and RNA were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Bovine serum albumin was from Sigma Chemical Co., St. Louis, Mo. Sodium citrate-*1,5-*^{14}C (1.9 mCi/mmole) was from Tracerlab, Waltham, Mass., and sodium acetate-*1-*^{14}C (52.9 mCi/mmole) was from Amersham/Searle, Des Plaines, Ill.

RESULTS

Only small amounts of labeled citrate were incorporated into diacetyl, other volatile compounds, and lipids (Table 1). By far the greatest amount of radioactivity was present in the lipid-depleted, purged broth. Analysis revealed that 20 to 30% of the original citrate disappeared from the medium during incubation. Acetate was incorporated primarily into lipids; small amounts of label were recovered also in diacetyl and other volatile compounds (Table 1). The presence of citrate in the medium reduced significantly the amount of labeled acetate which was incorporated into lipids and volatile compounds.

Optimum uptake of citrate occurred at *p*H 4.5, as determined by the method of Harvey and Collins (10). By using labeled citrate, low levels of uptake were observed over 60 min with cells cultured in the presence of citrate (Table 2). Con-

TABLE 1. *Utilization of citrate-1,5-^{14}C and acetate-1-^{14}C by Lactobacillus casei 393 in broth cultures*

Labeled compound[a]	Medium	Per cent of total radioactivity recovered in						
		Diacetyl	Other volatiles[b]	Neutral lipid	Free fatty acids	Phospho-lipids	Total lipids	Water soluble
Citrate	Elliker broth + 14 μ-moles of citrate per ml	0.06	0.02				0.56	99.36
Acetate	Elliker broth	0.71	0.17	11.4	52.2	17.2	80.8	18.2
Acetate	Elliker broth + 14 μ-moles of citrate per ml	0.39	0.25	8.0	42.4	15.7	66.1	33.3

[a] A 1-μCi amount added per 50 ml of broth.
[b] Compounds trapped from the purge gas in cold aqueous potassium hydroxide.

TABLE 2. *Accumulation of citrate-1,5-^{14}C and acetate-1-^{14}C by Lactobacillus casei 393 whole cells suspended in buffer*

Type of cells[a]	Labeled compound	Radioactivity associated with cells (as per cent of total added)					
		Control[b]			Plus citrate[c]		
		0 min	60 min	Net change	0 min	60 min	Net change
Control	Citrate-1,5-^{14}C				2.06	1.60	−0.46
Citrate	Citrate-1,5-^{14}C				2.18	3.07	+0.89
Control	Acetate-1-^{14}C	38.8	40.0	+1.2	26.3	21.6	−4.7
Citrate	Acetate-1-^{14}C	33.6	41.5	+7.9	22.0	28.8	+6.8

[a] Cells harvested from Elliker broth with 14 μM/ml or without (control) citrate.
[b] Assay mixture consisted of 0.2 μCi of label, 2.5×10^{-3} M $MgSO_4$, 0.5 ml of cell suspension, and 0.1 M (*p*H 4.5) phosphate buffer to 20 ml.
[c] Same assay mixture as above plus 2.5×10^{-3} M sodium citrate.

TABLE 3. *Effect of citrate on the composition of Lactobacillus casei 393 whole cells*

Medium	Concn of cell component (μg per μg of DNA)			
	Protein	RNA	Carbohydrate	Lipid
Elliker broth	5.31 ± 0.44	4.70 ± 0.30	3.58 ± 0.45	1.26 ± 0.46
Elliker broth + 14 μmoles of sodium citrate per ml	7.53 ± 0.29[a]	4.30 ± 0.50	3.03 ± 0.48	1.20 ± 0.03

[a] Highly significantly different from control cells.

trol cells did not accumulate citrate although some citrate was associated with cells. The low *p*H necessary for optimum uptake, together with the observation that only small amounts of citrate were accumulated, indicated passive uptake of citrate by this organism.

Since the presence of citrate decreased utilization of exogenous acetate, the uptake of acetate was studied also. When labeled acetate was added, an almost immediate association of acetate with *L. casei* cells was found, and the quantity of bound acetate increased during 60 min of incubation. There was little difference between citrate-grown and control cells (Table 2). Addition of citrate to the assay mixture reduced the initial uptake of acetate by 10 to 20% with both control and citrate-grown cells.

Based on DNA content, cells cultured in the presence of citrate contained more protein than did control cells (Table 3). When tested by the new multiple-range test of Duncan, this increase in protein was significant at the 0.01 level of probability (21). Although not statistically significant, citrate-grown cells had smaller quantities of RNA, carbohydrate, and lipid than did control cells. On a dry weight basis, cells cultured in the

TABLE 4. *Effect of growth in the presence of citrate on the protein content and susceptibility to sonic treatment of Lactobacillus casei 393 whole cells*

Incubation time (hr)	Protein (μg/μg of cells × 100)					
	Whole cells		Cell material[a]		Cell debris[b]	
	Control	Citrate[c]	Control	Citrate	Control	Citrate
15	18.35	21.74	32.00	30.70	4.30	5.41
24	16.85	21.51	28.20	32.40	14.35	14.39
39	15.52	18.89	33.40	37.10	14.80	12.10
48	16.57	18.63	33.00	39.20	12.70	8.80

[a] Soluble protein after 10 min of sonic treatment.

[b] Cellular debris removed by centrifugation after sonic treatment.

[c] Cells grown in Elliker broth plus 14 μmoles of sodium citrate per ml.

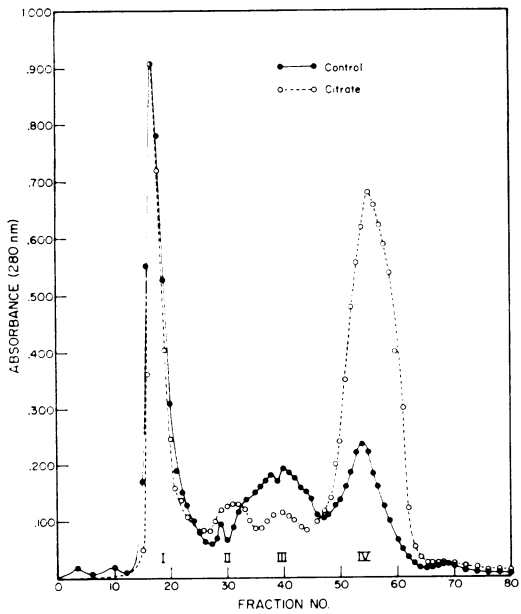

FIG. 1. *Sephadex G-200 protein elution profiles of the soluble fractions from Lactobacillus casei 393 cultured in the presence or absence (control) of citrate. Elution was with 0.1 M potassium phosphate buffer, pH 7.0; 5-ml fractions were collected*

presence of citrate had higher amounts of protein than did control cells throughout 48 hr of incubation (Table 4). Fractionation revealed that this increased protein content was reflected in the soluble fraction; cellular debris from citrate grown cells generally contained smaller quantities of protein than did that from control cells on a dry weight basis (Table 4). This increase in protein released by sonic treatment was noted first after 24 hr of incubation and was maintained throughout the 48-hr period. By 24 hr, growth stimulation by citrate was also evident (2), indicating that this stimulation is paralleled by an increase in protein which can be released by sonic treatment.

Comparison of protein elution profiles of soluble fractions derived from cells after 48 hr of incubation showed some pronounced differences between cells grown under the two sets of conditions (Fig. 1). Soluble fractions from cells cultured in the presence of citrate showed a much higher protein peak IV and slightly lower peaks II and III than those from control cells. Peak I was found to be very similar when the two cells types were compared. Elution profiles of soluble fractions from 24-hr cultures were essentially identical to those of 48-hr cultures (Fig. 1), indicating that the increase in peak IV with citrate-grown cells may be due to increased susceptibility of these cells to sonic treatment. Care was taken to subject cells to sonic oscillation in exactly the same manner, and these observations (Table 4, Fig. 1) were replicated in several trials. Under the conditions of sonic treatment, virtually all cells were disrupted.

Gel electrophoresis of the soluble fractions confirmed the differences between the two cell types (Fig. 2). On both large and small-pore polyacrylamide gels, soluble fractions from cells cultured in the presence of citrate showed more protein bands than did these fractions from control cells. Increased protein content of both cells and cell-free extracts could result from conversion

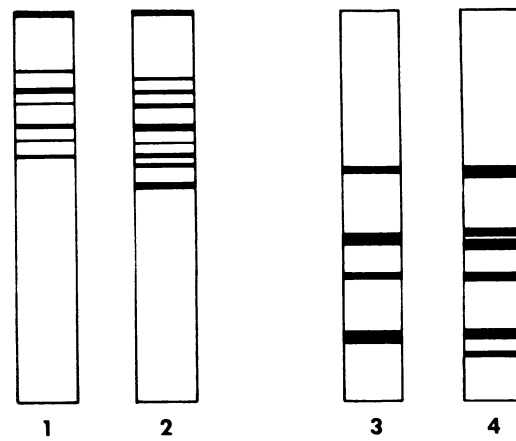

FIG. 2. *Traces of gel electrophoresis patterns of soluble fractions derived from Lactobacillus casei 393 cultured in media with or without citrate. (1) Control soluble fraction, small-pore gel; (2) citrate soluble fraction, small-pore gel; (3) control soluble fraction, large-pore gel; and (4) citrate soluble fraction, large-pore gel.*

TABLE 5. *Effect of citrate on the buffer storage stability of Lactobacillus casei 393 whole cells*

Medium	*p*H of buffer	Time of storage at 4 C (day)	Diacetyl production[a]	Acid production[b]	Soluble protein in spent buffer[c]
Control	4.5	0	54	11.1	8.2
	4.5	2	25	13.4	12.0
	4.5	12	5	10.0	15.4
	7.0	0	55	14.2	14.7
	7.0	2	39	11.0	19.4
	7.0	12	26	5.4	23.9
Citrate	4.5	0	51	14.9	14.7
	4.5	2	6	10.9	10.9
	4.5	12	5	8.7	28.7
	7.0	0	53	12.5	27.7
	7.0	2	25	9.7	31.6
	7.0	12	14	4.1	64.6

[a] Diacetyl plus acetoin produced from pyruvate in 30 min (μg/ml).

[b] Acid produced by cells in 10 ml of milk in 24 hr. Value expressed as milliliters of 0.1 N NaOH necessary to neutralize to the phenophthalein end point.

[c] Protein in buffer after removal of cells (μg).

of citrate to amino acids. However, the increased number of proteins indicates that this is only part of the answer.

When stored in buffer, the loss of soluble protein was greater from cells harvested from citrate-containing media than from control cells. These cells had decreased ability to produce diacetyl and acid compared to control cells (Table 5). Storage for 48 hr in buffer with a *p*H of 4.5 resulted in a 90% loss in ability of citrate-grown cells to produce diacetyl compared to 54% loss with control cells. Storage at *p*H 7.0 resulted in 54 and 30% loss of ability of citrate and control cells to produce diacetyl, respectively. Citrate-grown cells also produced less acid during incubation after buffer storage (Table 5).

Microscopic examination revealed that citrate-grown cells had a greater tendency to clump together than did control cells. This was true for both cells stored in buffer and cells in actively growing cultures.

DISCUSSION

Citrate is a good chelating agent and the presence of this ion adsorbed on the cell surface may enhance flocculation (14). An earlier observation on the effect of citrate on cell growth indicated that this may be the case (2). Although citrate enhances growth during incubation for 24 hr or more, quite early in the growth period it had an inhibitory effect. Magnesium and calcium overcame this early citrate inhibition (3). These metal ions may act by neutralizing the charges on citrate, thus preventing flocculation of the cells. Flocculation would decrease the surface area for entrance of substrates into cells.

Results obtained in this study demonstrate that the presence of citrate has profound effects on both the metabolism and physiology of *L. casei*. It decreases uptake of acetate and reduces the amount of exogenous acetate incorporated into lipids. Cells grown in medium containing citrate have a significantly higher protein content than do control cells. The presence of citrate causes flocculation of cells, and these cells are less stable to cold storage and undergo lysis to a greater extent than do control cells. Citrate itself is accumulated passively by the organism. Small amounts of citrate are utilized in the formation of volatile compounds and lipids.

The metabolical changes noted when citrate was present in the medium can be explained in part by its physiological effects on cells. Citrate-induced flocculation which was observed in both growing cultures and isolated, resuspended cells would decrease the surface area available for entry of metabolites. Citrate also appeared to induce lability to sonic treatment and to lysis during buffer storage. This suggests that the presence of the ion in some manner weakens the cell wall, the cell membrane, or both. Increased lysis could also explain the decreased metabolical activity of citrate cells.

Decreased utilization of exogenously supplied acetate in the presence of citrate may also be explained by assuming that *L. casei* catabolizes citrate by cleaving it to oxalacetate plus acetate. The acetate thus produced would dilute the exogenously supplied pool and result in a net decrease of incorporation of radioactivity from acetate-*1*-^{14}C into diacetyl plus acetoin and cell materials. This explanation is supported by the observation that radioactivity from either citrate-*1,5*-^{14}C or acetate-*1*-^{14}C was incorporated into diacetyl and lipids. It thus appears that *L. casei* formed acetyl CoA from the radioactive acetate which was produced by cleavage of citrate or added to the medium and then incorporated the acetyl CoA into cellular lipids and diacetyl plus acetoin.

Based on DNA content, growth in the presence of citrate increased the quantity of protein per cell. This can be explained by assuming that citrate was converted to amino acids, thus allowing for more protein synthesis. Most of the increased protein content was accounted for in the soluble fraction after sonic treatment of cells, again suggesting that citrate increases release of bound protein. More protein bands were observed on electrophoresis of soluble fractions from citrate

cells, and the gel filtration profiles of these soluble fractions were altered compared to those from control cells. Although proof of this will require characterization of the additional proteins observed in soluble fractions, it appears that citrate weakens the cell wall complex, thus permitting the release of the additional proteins from this complex.

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