APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1988, p. 3053–3056 Vol. 54, No. 12
0099-2240/88/123053-04$02.00/0

Production of Succinic Acid from Citric Acid and Related Acids by *Lactobacillus* Strains

CHOJI KANEUCHI,[1†*] MASAKO SEKI,[1] AND KAZUO KOMAGATA[2]

Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01,[1] *and Institute of Applied Microbiology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113,*[2] *Japan*

Received 20 June 1988/Accepted 9 September 1988

A number of *Lactobacillus* strains produced succinic acid in de Man-Rogosa-Sharpe broth to various extents. Among 86 fresh isolates from fermented cane molasses in Thailand, 30 strains (35%) produced succinic acid; namely, 23 of 39 *Lactobacillus reuteri* strains, 6 of 18 *L. cellobiosus* strains, and 1 of 6 unidentified strains. All of 10 *L. casei* subsp. *casei* strains, 5 *L. casei* subsp. *rhamnosus* strains, 6 *L. mali* strains, and 2 *L. buchneri* strains did not produce succinic acid. Among 58 known strains including 48 type strains of different *Lactobacillus* species, the strains of *L. acidophilus, L. crispatus, L. jensenii,* and *L. parvus* produced succinic acid to the same extent as the most active fresh isolates, and those of *L. alimentarius, L. collinoides, L. farciminis, L. fructivorans* (1 of 2 strains tested), *L. malefermentans,* and *L. reuteri* were also positive, to lesser extents. Diammonium citrate in de Man-Rogosa-Sharpe broth was determined as a precursor of the succinic acid produced. Production rates were about 70% on a molar basis with two fresh strains tested. Succinic acid was also produced from fumaric and malic acids but not from DL-isocitric, α-ketoglutaric, and pyruvic acids. The present study is considered to provide the first evidence on the production of succinic acid, an important flavoring substance in dairy products and fermented beverages, from citrate by lactobacilli.

During the study of lactic acid bacteria in fermented cane molasses in Thailand, a number of *Lactobacillus* strains were isolated which produced succinic acid when grown in MRS (de Man-Rogosa-Sharpe) broth. There are a few papers on the production of succinic acid by lactobacilli. Radler and Yannissis (17, 18) reported the conversion of one-third of the tartaric acid added to a medium to succinic acid through oxaloacetic acid, malic acid, and fumaric acid by a *Lactobacillus brevis* strain. Whiting (20) observed the production of an appreciable amount of succinic acid from malate by a few *Lactobacillus* strains isolated from ciders and perries. Cato et al. (3) reported the production of a small amount of succinic acid in glucose broth by *L. crispatus*.

Succinic acid has a strong salty bitter flavor in addition to its acidic taste and becomes detrimental to the flavor of various fermented beverages at different concentrations (20). In ciders and wines, 0.1% succinic acid is commonly found, while 0.03% in beers would be considered a high concentration (20). At high concentrations, the succinic acid flavor may well become objectionable. It is well known that succinic acid is an important flavoring substance in a fermented beverage, sake, in Japan (14). Lactobacilli are widely distributed in fermented beverages and dairy products (19). If lactobacilli produce succinic acid, the presence of lactobacilli may be closely related to the flavors of the products. Whether production of succinic acid is a positive or negative trait of lactobacilli as starter cultures or spoilage microorganisms depends on the type of product. Therefore, we were interested in succinic acid production by lactobacilli and its substrate.

This paper deals with the determination of succinic acid production from presumed precursors by *Lactobacillus* strains.

* Corresponding author.

† Present address: Azabu University, Sagamihara, Kanagawa 229, Japan.

MATERIALS AND METHODS

Strains used. A total of 144 *Lactobacillus* strains were used. Of these, 86 (2 *L. buchneri*, 10 *L. casei* subsp. *casei*, 5 *L. casei* subsp. *rhamnosus*, 18 *L. cellobiosus*, 6 *L. mali*, 39 *L. reuteri*, and 6 unidentified strains) were freshly isolated from fermented cane molasses in Thailand, and 58 were known strains, mainly the type strains of various *Lactobacillus* species, obtained from Japan Collection of Microorganisms (JCM), The Institute of Physical and Chemical Research, Wako, Saitama, Japan (Table 1).

The known non-succinic acid-producing *Lactobacillus* strains used were as follows: *L. agilis* JCM 1187^T, *L. amylophilus* JCM 1125^T, *L. amylovorus* JCM 1126^T, *L. bavaricus* JCM 1129^T, *L. bifermentans* JCM 1094^T, *L. brevis* subsp. *brevis* JCM 1059^T, *L. brevis* subsp. *gravesensis* JCM 1102, *L. brevis* subsp. *otakiensis* JCM 1183, *L. buchneri* JCM 1115^T, *L. casei* subsp. *alactosus* JCM 1133^T, *L. casei* subsp. *casei* JCM 1134^T, *L. casei* subsp. *pseudoplantarum* JCM 1181^T, *L. casei* subsp. *rhamnosus* JCM 1136^T, *L. casei* subsp. *tolerans* JCM 1171^T, *L. catenaforme* JCM 1121^T, *L. cellobiosus* JCM 1137^T, *L. confusus* JCM 1093^T, *L. coryniformis* subsp. *coryniformis* JCM 1164^T, *L. coryniformis* subsp. *torquens* JCM 1166^T, *L. curvatus* JCM 1096^T, *L. delbrueckii* subsp. *bulgaricus* JCM 1002^T, *L. delbrueckii* subsp. *delbrueckii* JCM 1012^T, *L. delbrueckii* subsp. *lactis* JCM 1248^T and JCM 1148, *L. fermentum* JCM 1173^T, *L. fructivorans* JCM 1117^T, *L. fructosus* JCM 1119^T, *L. gasseri* JCM 1131^T, *L. halotolerans* JCM 1114^T, *L. hilgardii* JCM 1155^T, *L. homohiochii* JCM 1199^T, *L. mali* JCM 1116^T and JCM 1153, *L. maltaromicus* JCM 1154^T, *L. minor* JCM 1168^T, *L. minutus* JCM 1118^T, *L. murinus* JCM 1717^T, *L. pastorianus* JCM 1113, *L. ruminis* JCM 1152^T, *L. sake* JCM 1157^T, *L. salivarius* subsp. *salicinius* JCM 1150^T, *L. salivarius* subsp. *salivarius* JCM 1231^T, *L. sharpeae* JCM 1186^T, *L. vaccinostercus* JCM 1716^T, *L. viridescens* JCM 1174^T, *L. vitulinus* JCM 1143^T, and *Lactobacillus* sp. strain JCM 1162.

Media used. MRS broth (pH 6.5) (6), Briggs broth (pH 6.8) (1), and peptone-yeast extract (PY) broth (pH 6.8) (11) were

TABLE 1. Production of succinic acid in MRS broth by known *Lactobacillus* strains

Strain	Relative amt of succinic acid produced[a]
None (uninoculated medium)	0.4
L. crispatus JCM 1185[T]	6.7
L. crispatus JCM 1024	6.7
L. crispatus JCM 1037	6.0
L. jensenii JCM 1146[T]	5.7
L. parvus JCM 1189	4.9
L. acidophilus JCM 1132[T]	3.9
L. malefermentans JCM 1167	1.9
L. alimentarius JCM 1095[T]	1.7
L. collinoides JCM 1123[T]	1.3
L. farciminis JCM 1097[T]	1.3
L. fructivorans JCM 1198	1.3
L. reuteri JCM 1112[T]	1.0
L. reuteri M9[b]	11.3
L. reuteri M4[b]	7.4

[a] Estimated as the ratio of a peak area for succinic acid to a peak area for an internal standard (malonic acid, 32 mM) in gas chromatograms; values are averages of three separate tests.

[b] Fresh strains from fermented cane molasses, which were among the most active in succinic acid production.

prepared according to methods described previously. Tomato juice extract for Briggs broth was prepared by using commercial canned tomato juice. Modified MRS broths were prepared by omitting individual components to determine which component is responsible for the succinic acid production. Fumaric acid, DL-isocitric acid, α-ketoglutaric acid, DL-malic acid, pyruvic acid, and sodium citrate were tested for production of succinic acid by adding them individually to MRS broth at 2 g/1,000 ml in place of diammonium citrate. Broth media were distributed in test tubes (3 ml each) and sterilized at 115°C for 15 min.

Detection and determination of succinic acid. Strains were grown in MRS broth or modified MRS broths at 35°C, usually for 48 h. Succinic acid was detected by the method of Holdeman et al. (11). The broth cultures were centrifuged at 1,500 × *g*, and 0.5 ml of the supernatant was mixed with 0.1 ml of 0.2% malonic acid (as an internal standard), 0.2 ml of 50% H_2SO_4, and 1 ml of methanol. The mixture was heated in a water bath at 60°C for 30 min for methylation of acids, and then 0.5 ml of distilled water and 0.5 ml of chloroform were added and mixed by gentle inversion of the tube 20 times to extract methylated acids. If an emulsion was formed, the tube was briefly centrifuged to break the emulsion. The chloroform extract (1 μl) was subjected to gas chromatography (model GC-7A; Shimadzu Ltd., Kyoto, Japan) with a hydrogen flame ionization detector, a column packed with FAL-M (Wako Chemical Industries, Ltd., Osaka, Japan), and a data processor (Chromatopac, model C-RlA; Shimadzu). A standard curve of the peak area for quantitative analysis of succinic acid was prepared from chloroform extracts of a series of known concentrations of methylated succinic acid. The peak area was plotted against the original concentration in uninoculated medium.

RESULTS

Determination of succinic acid produced. One of the methylated acids in MRS broth cultures showed exactly the same retention time as did the methylated standard succinic acid in gas chromatograms, and thus it was clearly identified as succinic acid.

***Lactobacillus* strains which produced succinic acid in MRS medium.** Of 86 fresh strains isolated from fermented cane molasses, 30 (35%) produced succinic acid in MRS broth to various extents. They were 6 (33%) of 18 *L. cellobiosus* strains, 23 (59%) of 39 *L. reuteri* strains, and 1 (17%) of 6 unidentified *Lactobacillus* strains. None of the fresh strains of *L. buchneri, L. casei* subsp. *casei, L. casei* subsp. *rhamnosus*, and *L. mali* produced succinic acid. All succinic acid-producing strains isolated from the fermented cane molasses were heterofermentative except for an unidentified strain. The amounts of succinic acid produced varied from strain to strain.

Among 58 known strains, 12 strains of 10 species produced succinic acid in MRS broth (Table 1). The amounts of succinic acid produced varied from species to species, and three *L. crispatus* strains were among the most active. Both homofermentative and heterofermentative species were included among the producing strains.

Production of succinic acid in different media. The production of succinic acid in MRS, Briggs, and PY broths with and without glucose was tested by using representative fresh isolates. The relative amounts of succinic acid produced were estimated by the ratio of a peak area for succinic acid to a peak area for an internal standard (malonic acid, 32 mM) in gas chromatograms. *L. reuteri* M4 and M9 and *L. cellobiosus* M6 produced relatively large amounts of succinic acid in MRS broth with and without glucose, moderate amounts in Briggs broth, and small amounts in PY broth. In MRS broth with glucose, production of succinic acid reached almost the maximum level after incubation for 2 days, but without glucose, it attained the maximum level only after incubation for 7 days. Succinic acid was not produced by *L. casei* subsp. *casei* M44 in the three media tested.

Components responsible for the production of succinic acid in MRS broth. Components in MRS broth responsible for the production of succinic acid were investigated with succinic acid-producing *L. reuteri* M4 and M9 and non-succinic acid-producing *L. casei* subsp. *casei* M44. These strains were grown in a series of modified MRS broths. Diammonium citrate was clearly found to be responsible for the most production of succinic acid with *L. reuteri* M4 and M9.

Production of succinic acid from substances related to diammonium citrate. Sodium citrate and DL-isocitric, fumaric, DL-malic, α-ketoglutaric, and pyruvic acids were examined for the production of succinic acid by using *L. reuteri* M4 and M9 and *L. casei* subsp. *casei* M44. Of the substances tested, sodium citrate, fumaric acid, and DL-malic acid were converted to succinic acid at the percentages of 30 to 57% (on a molar basis) by *L. reuteri* M4 and M9 (Table 2). The percentages were a little lower than 67 to 74% with diammonium citrate. Percentages for DL-isocitric, α-ketoglutaric, and pyruvic acids were 0 to 8%. *L. casei* subsp. *casei* M44 did not convert any of the acids to succinic acid.

DISCUSSION

Diammonium citrate was found to be degraded to succinic acid by the *Lactobacillus* strains. The fermentation of citrate itself is a fairly general property within the strains in the genus *Lactobacillus*. Both homofermentative and heterofermentative citrate-fermenting lactobacilli have been isolated from silage (12), feces (10), lamb stomach (5), sheep milk (5), Pecorino Romano cheese (5), and dairy products (8). In some cases, the presence of lactobacilli has been correlated with "blowing" of the cheeses due to the production of carbon dioxide from citrate (4). However, the main end

TABLE 2. Production of succinic acid from various substances related to diammonium citrate[a]

Substance tested	Amt		mM (%)[b] succinic acid produced by:			
	g/liter	mM	Control (uninoculated)	*L. reuteri* M4	*L. reuteri* M9	*L. casei* subsp. *casei*
Basal medium[c]			0.5	2.5	2.6	1.1
Diammonium citrate (MRS)	2.0	8.8	0.5	8.4 (67.0)	9.1 (73.9)	1.2
Sodium citrate	2.0	6.8	0.5	6.0 (51.5)	5.8 (47.1)	1.1
Fumaric acid	2.0	17.2	0.4	11.2 (50.6)	12.4 (57.0)	1.1
DL-Malic acid	2.0	15.3	0.6	7.2 (30.7)	7.3 (30.7)	1.0
α-Ketoglutaric acid	2.0	13.7	0.7	3.6 (8.0)	3.2 (4.4)	1.6
DL-Isocitric acid	2.0	10.4	0.6	2.6 (1.0)	2.8 (1.9)	1.3
Pyruvic acid	2.0	22.7	0.5	2.5 (0.0)	2.6 (0.0)	1.3

[a] Medium was incubated for 2 days.
[b] Percent production of succinic acid was calculated as: [(mM produced − mM produced in basal medium)/mM of test substance added] × 100.
[c] MRS broth from which diammonium citrate was omitted.

products in the citrate breakdown by lactobacilli reported so far are acetate (4, 20), formate (8), diacetyl (2, 8, 9, 13), and carbon dioxide (4), depending on the strain or species. *Streptococcus diacetilactis* and *Leuconostoc* species also ferment citrate but characteristically produce diacetyl and acetoin (4, 9). Therefore, the present work is considered to provide the first evidence on the production of succinic acid from citrate by lactic acid bacteria, although there are a few reports about the production of succinic acid from tartarate (17), malate (20), and fumarate (20) by some *Lactobacillus* strains.

The importance of succinic acid as a flavoring compound in dairy products is well known (17). For a fermented beverage, sake, in Japan, succinic acid is an important flavoring substance (14). In ciders and wines, 0.1% succinic acid is commonly found, while 0.03% in beers is considered a rather high concentration (20). At a high concentration, the strong bitter flavor of succinic acid may become objectionable. Citric acid is distributed in a wide variety of raw materials for dairy products (4, 7) and fermented beverages (16, 17). Lactobacilli also are widely found in dairy products and fermented beverages (19). Accordingly, if the citric acid is converted to succinic acid by lactobacilli during the fermentation process, the flavor of the fermented products will greatly be influenced.

Among the *Lactobacillus* strains isolated from fermented cane molasses, the strains of *L. reuteri* and *L. cellobiosus* produced succinic acid. The type strain of *L. reuteri* also produced succinic acid, but that of *L. cellobiosus* did not. The type strain and two other strains of *L. crispatus*, by which Cato et al. (3) reported the production of a slight amount of succinic acid in glucose broth, also produced succinic acid from citric acid. The type strain of *L. brevis* did not degrade citric acid to succinic acid, although Radler (17) observed succinic acid production from tartartic acid by a strain of *L. brevis*.

The presence of fermentable carbohydrates such as glucose and lactose may influence the extent of citric acid utilization (5). Evidence presented by Campbell and Gunsalus (2) indicates that lactobacilli may utilize citric acid in the absence of carbohydrates as an energy source. In the present work, the production of succinic acid from citric acid was slightly enhanced in the presence of glucose at 2 days of incubation but not at 7 days of incubation. The slight enhancement of succinic acid production with glucose after incubation for 2 days seemed to be ascribable to faster growth of the *Lactobacillus* strains using glucose as an energy source. Accordingly, the presence of glucose did not actually influence the production of succinic acid, and citric acid did not always seem to be utilized as an energy source.

The production rates of succinic acid from diammonium citrate were approximately 70% on a molar basis with the two *L. reuteri* strains, and those from malic and fumaric acids were approximately 60% (Table 2). These rates are considered to be relatively high, compared with the production rates of succinic acid from tartarate (17, 18) and malate (20) and in glucose broth (3) by some *Lactobacillus* strains.

Radler and Yannissis (18) reported that tartaric acid is converted to succinic acid through malic and fumaric acids by an *L. brevis* strain. In the present work, malic and fumaric acids also were converted to succinic acid by the strains isolated from fermented cane molasses. It is, however, unknown whether citric acid is metabolized to succinic acid through the same biochemical pathway as that used by the *L. brevis* strain. The mechanism of succinic acid production from citric acid by lactic acid bacteria remains to be investigated. It seems interesting to study the mechanism in connection with the tricarboxylic acid cycle (15) because citric, fumaric, malic, and succinic acids are all members of the tricarboxylic acid cycle.

LITERATURE CITED

1. **Briggs, M.** 1953. An improved medium for lactobacilli. J. Dairy Res. **20:**36–40.
2. **Campbell, J. J. R., and I. C. Gunsalus.** 1942. Citric acid fermentation by streptococci and lactobacilli. J. Bacteriol. **44:**71–76.
3. **Cato, E. P., W. E. C. Moore, and J. L. Johnson.** 1983. Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. Int. J. Syst. Bacteriol. **33:**426–428.
4. **Christensen, M. D., and C. S. Peterson.** 1956. Factors affecting diacetyl production by lactic acid bacteria. Appl. Microbiol. **6:**319–322.
5. **Dellaglio, F., S. Arrizza, and A. Ledda.** 1981. Classification of citrate fermenting lactobacilli isolated from lamb stomach, sheep milk, and Pecorino Romano cheese. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. A **C2:**349–356.
6. **de Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:**130–135.
7. **Faulkner, A., E. Y. Brechany, R. M. Mabon, and H. T. Pollock.** 1986. Seasonal changes in the fat composition and concentration of citrate and related metabolites in cow's milk. J. Dairy Res. **53:**223–227.
8. **Fryer, T. F.** 1970. Utilization of citrate by lactobacilli isolated from dairy products. J. Dairy Res. **37:**9–15.
9. **Fryer, T. F., M. E. Sharpe, and B. Reiter.** 1970. Utilization of milk citrate by lactic acid bacteria and "blowing" of film-

wrapped cheese. J. Dairy Res. **37**:17–28.

10. **Gasser, F.** 1964. Identification des *Lactobacillus* fécaux. Ann. Inst. Pasteur (Paris) **106**:778.
11. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Materials and methods, p. 117–149. *In* Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
12. **Keddie, R. M.** 1959. The properties and classification of lactobacilli isolated from grass and silage. J. Appl. Bacteriol. **22**:403–416.
13. **Keenan, T. W., and R. C. Lindsay.** 1986. Diacetyl production and utilization by *Lactobacillus* species. J. Dairy Sci. **51**:188–191.
14. **Kodama, K.** 1970. Saké yeast, p. 225–282. *In* A. H. Rose, and J. S. Harrison (ed.), The yeast, vol. 3. Academic Press, Inc. (London), Ltd., London.
15. **London, J.** 1976. The ecology and taxonomic status of the lactobacilli. Annu. Rev. Microbiol. **30**:279–301.
16. **Pilone, G. J.** 1975. Control of malo-lactic fermentation in table wines by addition of fumaric acid, p. 121–138. *In* J. G. Carr, C. V. Cutting, and G. C. Whiting (ed.), Lactic acid bacteria in beverages and food. Academic Press, Inc. (London), Ltd., London.
17. **Radler, F.** 1975. The metabolism of organic acids by lactic acid bacteria, p. 17–27. *In* J. G. Carr, C. V. Cutting, and G. C. Whiting (ed.), Lactic acid bacteria in beverages and food. Academic Press, Inc. (London), Ltd., London.
18. **Radler, F., and C. Yannissis.** 1972. Weinsäureabbau bei Milchsäurebakterien. Arch. Mikrobiol. **82**:219–239.
19. **Sharpe, M. E.** 1981. The genus *Lactobacillus*, p. 1635–1679. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes, vol. 1. Springer-Verlag KG, Berlin.
20. **Whiting, G. C.** 1975. Some biochemical and flavour aspects of lactic acid bacteria in ciders and other alcoholic beverages, p. 69–85. *In* J. G. Carr, C. V. Cutting, and G. C. Whiting (ed.), Lactic acid bacteria in beverages and food. Academic Press, Inc. (London), Ltd., London.