

Relationship of Cellular Fatty Acid Composition to Survival of *Lactobacillus bulgaricus* in Liquid Nitrogen¹

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Concentrated cultures of *Lactobacillus bulgaricus* were prepared by resuspending cells grown in semisynthetic media in sterile 10% non-fat milk solids. The concentrated cultures were frozen in liquid nitrogen for 24 h. The cell suspensions exhibited decreased viability after storage, and the amount of death varied among the different strains tested. Storage stability of all strains examined was improved by supplementing the growth medium with sodium oleate. Radioisotopes were used to study the fate of sodium oleate with *L. bulgaricus* NCS1. [1-¹⁴C]sodium oleate was incorporated solely into the lipid portion of the cells, including both neutral and polar lipids. The fatty acid composition of *L. bulgaricus* NCS1, NCS2, NCS3, and NCS4 grown with and without sodium oleate was studied. The major fatty acids of strains NCS1, NCS2, and NCS3 grown without sodium oleate were dodecanoic, tetradecanoic, hexadecanoic, hexadecenoic, and octadecenoic acids. In addition to these, strain NCS4 contained C₁₉ cyclopropane fatty acid. The major fatty acids of all strains grown with sodium oleate were tetradecanoic, hexadecanoic, hexadecenoic, octadecenoic, and C₁₉ cyclopropane fatty acids. All strains grown in broth containing sodium oleate contained larger amounts of octadecenoic and C₁₉ cyclopropane fatty acid, and less saturated fatty acids than when grown without sodium oleate. Statistical analyses indicated that C₁₉ cyclopropane fatty acid was most closely related to stability of the lactobacilli in liquid nitrogen. A negative regression line that was significant at $P < 0.001$ was obtained when the cellular content of this fatty acid was plotted against death.

Previous work from this laboratory (25) has shown that cells of *Lactobacillus bulgaricus* grown in media containing Tween 80 (polyoxyethylene sorbitan monooleate) were more resistant to freezing than those grown without it. Although such an effect had not been previously reported for the lactobacilli, it has been well documented that non-ionic detergents containing oleic acid, and free oleic and *cis*-vaccenic acids are important in the metabolism of lactobacilli (22, 29, 30). Either oleic or *cis*-vaccenic acids can replace the requirement for biotin by the lactobacilli (4, 10, 29, 30). Tween 80, which is a non-toxic form of oleic acid, can also replace this requirement (3, 29, 30). In addition, these acids are intimately involved in the control of the fatty acid synthesis in *Lactobacillus plantarum* (1, 9, 28).

Oleic and *cis*-vaccenic acids are incorporated intact into the lipids of lactobacilli or converted to a C₁₉ cyclopropane fatty acid (17, 21). C₁₉

cyclopropane fatty acids are formed from oleic or *cis*-vaccenic acid by the addition of a methylene bridge carbon across the double bond. S-adenosylmethionine serves as the donor of the bridge carbon for this conversion (22). Although C₁₉ cyclopropane fatty acid(s) are found in the lipids of many different types of bacteria, their exact physiological function is unknown. It is believed to have a role in maintaining cell membrane flexibility (16).

This study was initiated to determine the mechanism(s) whereby Tween 80 imparted freezing stability to *L. bulgaricus*.

MATERIALS AND METHODS

Cultures. The strains of *L. bulgaricus* selected for this study are used commercially in the manufacture of yogurt and Italian cheese. They were propagated and stored as described previously (25).

A medium containing 2% Tryptone (Difco), 1% yeast extract (BBL), 2% lactose, and 0.2% Tween 20 (Nutritional Biochemicals Corp.) was used as the control medium to study the effects of sodium oleate on the lactobacilli. A sterile solution of sodium oleate

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was added to the medium to give a final concentration of 100 $\mu\text{g/ml}$. The sodium oleate solution and control were sterilized by autoclaving 15 min at 121 C. In experiments involving radioisotope tracer studies, the sodium oleate broth was additionally supplemented after autoclaving with 0.125 μg of [$1\text{-}^{14}\text{C}$]sodium oleate (ICN Pharmaceuticals, Inc., Cleveland, Ohio; specific activity 250 to 500 mCi/mmol) per ml. The solution of [$1\text{-}^{14}\text{C}$]sodium oleate was sterilized by membrane filtration (pore size 0.45 μm).

Preparation of concentrated cell suspension. Each strain of *L. bulgaricus* was grown in the control broth and was used to inoculate the test growth media using a 2% inoculum. The cultures were grown statically to the stationary phase (15 h) in the test media at 37 C. They were harvested, concentrated, and stored in liquid nitrogen, and viability was measured as described previously (25).

Incorporation of [$1\text{-}^{14}\text{C}$]sodium oleate. Cells from 200 ml of broth containing [$1\text{-}^{14}\text{C}$]sodium oleate and from 200 ml of broth containing unlabeled sodium oleate were washed three times with cold distilled water at a ratio of approximately 250 mg of cells (dry weight) to 20 ml of water. The cells were recovered between washings by centrifugation at 0 C and 17,300 $\times g$ for 10 min. After the final wash the cells from each broth were suspended in 10 ml distilled water, and the resulting suspensions were combined and stored in a freezer until analyzed. The spent medium, washings, and cells were analyzed for ^{14}C content by using a Packard model 574 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) to ascertain whether or not sodium oleate was incorporated into (or adsorbed onto) the cells during growth. The counting fluid contained Triton X-100, 333 ml; toluene, 167 ml; 1, 4-bis[2(5-phenyloxazolyl)]-benzene, 0.1 g; and 2, 5 diphenyloxazole, 5.5 g. Ten ml of the counting fluid was added to each sample, and the final volume was adjusted to 20 ml with toluene. Whole cells were kept in suspension with the aid of Thixotropic Gel Powder (Packard Instrument Co., Inc.). Counts were corrected for background and quenching.

Free lipids. Free lipids were extracted from washed whole cells by the method of Bligh and Dyer (2). The cells were extracted three times at 4 C. One part chloroform and 2 parts methanol were mixed with 0.8 parts of an aqueous cell suspension. The first extraction was for 1 h and 40 min, and the second and third were for 30 min each. After each allotted extraction time, 1 part chloroform and 1 part distilled water were added. The mixture was shaken and set aside for 20 min to allow the phases to separate. Between extractions the cells were recovered by centrifugation at 0 C and 17,300 $\times g$ for 10 min. The three extracts were pooled and condensed for analyses.

Bound lipids. Cell suspensions from which free lipids had been extracted were hydrolyzed for 1.5 h in 1 N HCl at 121 C. The hydrolysates were extracted for 10 min by using the Bligh and Dyer procedure (2). Whole cells (not previously extracted) were also hydrolyzed and extracted for 10 min to obtain information for comparing total and bound lipids.

Silicic acid column chromatography. Free lipids

were separated by silicic acid chromatography into neutral and polar lipid fractions by using procedures outlined by Dittmer and Wells (6).

Gravimetric analysis. Lipid fractions were evaporated to dryness by using a rotary flash evaporator at 42 C. They were redissolved in chloroform and quantitatively transferred to a tared evaporating flask. The chloroform was removed by evaporation, and the flask was dried and placed in a desiccator for at least 30 min before weighing.

Gas-liquid chromatography. The fatty acid methyl esters were prepared from the lipid fractions by the method of Metcalfe et al. (20) using boron trifluoride (BF_3)-methanol reagent (Fisher Scientific Co.). The methyl esters were separated on a Packard 800 series gas chromatograph (Packard Instrument Co., Inc.) equipped with a flame ionization detector. A stainless-steel column packed with EGSSX (10%) on Chromosorb Q (100/120 mesh, Applied Science Co., State College, Pa.) was employed. The oven temperature was held at 140 C for 3 min after sample injection and then increased linearly at 4 C per min until the final temperature of 190 C was reached. The injector and detector temperatures were 230 and 205 C, respectively. Nitrogen served as the carrier gas with a flow rate of 20 ml/min at 22 C.

The fatty acid methyl esters were tentatively identified by comparing their retention times with known standards. The peak areas were determined by triangulation, and the total area was used to determine the relative percent of each fatty acid present. Gas chromatographic detector response was linear over the range studied.

Statistical analysis. The regression of percent death on percent fatty acid content was determined by using methods outlined by Snedecor and Cochran (26).

RESULTS

Relationship of sodium oleate to storage stability. Previous work in this laboratory (25) demonstrated that cells of *L. bulgaricus* grown in broth containing Tween 80 were more resistant to freezing in liquid nitrogen than cells grown in broth without Tween 80. When most of the free oleic acid associated with Tween 80 was removed by silicic acid column chromatography, the detergent lost part of the ability to impart freezing resistance to cells grown in its presence. Sodium oleate added to a broth composed of Tryptone, yeast extract, and lactose was toxic to the growth of *L. bulgaricus*. However, Tween 20 (polyoxyethylene sorbitan monolaurate) was added to the medium to detoxify the fatty acid (15, 30). Cells grown in the control broth without sodium oleate exhibited susceptibility to freezing similar to that observed in a previous report (25). An improvement in the storage stability was noted for each strain when grown in the presence of sodium oleate (Table 1). The cultures grown without sodium oleate

varied in their resistance to freezing. Strain NCS4 was most resistant to freezing; strain NCS1 was most sensitive and exhibited the greatest response to sodium oleate. Although the level of sodium oleate utilized in these experiments improved the stability of strains NCS2 and NCS3, it did not impart to the cells sufficient resistance to survive the freezing completely. Previous studies in our laboratories (25) indicated that strains of *L. bulgaricus* varied with respect to the optimum level of Tween 80 required to produce cells that were resistant to freezing. Presumably a similar situation exists with respect to the optimum level of sodium oleate.

Incorporation of [^{14}C]sodium oleate. Radioactive tracer studies demonstrated that sodium oleate was incorporated into the lipid portion of *L. bulgaricus* NCS1 (Table 2). Analysis of whole cells revealed that 100.8% of the radioactivity associated with the cells was incorporated as lipid material of which 28.5% was bound lipid and 72.3% was free lipid. The free lipid was further separated by silicic acid col-

umn chromatography into neutral and polar lipid fractions. There was approximately 2.5 times more ^{14}C associated with the polar lipids than with the neutral lipids. Gravimetric analysis of the lipids revealed that the percentage by weight of each fraction was similar to the percent distribution of radioactivity in each fraction. There was little quantitative difference in lipid content between cells grown with and without sodium oleate. The total amount of lipid for cells grown with and without sodium oleate was 4.2 and 4.9%, respectively.

Fatty acid analysis of lipids from hydrolyzed whole cells. Lipids from *L. bulgaricus* NCS1, NCS2, NCS3, and NCS4 grown with and without sodium oleate were evaluated for fatty acid composition. Chromatograms of fatty acid methyl esters prepared from lipids extracted from hydrolyzed cells of strain NCS1 grown with and without sodium oleate are presented in Fig. 1. Similar chromatograms were obtained for strains NCS2, NCS3, and NCS4. Quantitative evaluations comparing percentage compositions based on peak areas for all four strains are presented in Table 3. The primary fatty acids in lipids from hydrolyzed whole cells grown in sodium oleate were tetradecanoic, hexadecanoic, hexadecenoic, octadecenoic, and C_{19} cyclopropane fatty acids. The predominant fatty acids of strains NCS1, NCS2, and NCS3 grown without sodium oleate were dodecanoic, tetradecanoic, hexadecanoic, hexadecenoic, and octadecenoic acids. Strain NCS4 grown without sodium oleate contained considerably more C_{19} cyclopropane fatty acid than the other three strains. Cells grown in sodium oleate in all cases contained larger percentages of octadecenoic and C_{19} cyclopropane fatty acids than cells grown without sodium oleate. Cells

TABLE 1. Sodium oleate supplementation of the growth medium and its effect on stability of *L. bulgaricus* to freezing in liquid nitrogen^a

Culture	Growth medium	Death (%)
NCS1	Control	99
	Sodium oleate ^b	9
NCS2	Control	67
	Sodium oleate	48
NCS3	Control	69
	Sodium oleate	39
NCS4	Control	15
	Sodium oleate	0

^a Cells suspended in 10% nonfat milk solids were frozen 24 h in liquid nitrogen.

^b Control broth plus 100 μg of sodium oleate per ml.

TABLE 2. Distribution of lipids in *L. bulgaricus* NCS1

Lipid fraction	Gravimetric analysis (% of total lipid)		Radioisotope measurement (% of total incorporated ^{14}C)
	Growth medium		
	Control	Sodium oleate ^a	
Free	73.6	73.9	72.3
Neutral	22.8	25.6	19.0
Polar	52.2	55.2	47.1
Bound	26.4*	26.3	28.5

^a Control broth plus 100 μg of sodium oleate per ml.

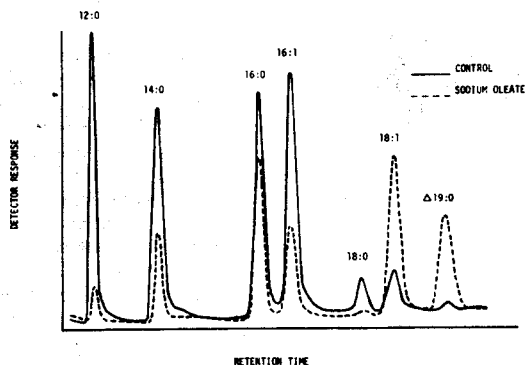


FIG. 1. Chromatograms of fatty acid methyl esters from lipids extracted from hydrolyzed whole cells of *L. bulgaricus* NCS1 grown with and without sodium oleate.

grown without sodium oleate contained a larger percentage of the saturated fatty acids.

Fatty acid composition of lipid fractions from *L. bulgaricus* NCS1. Results from gas chromatographic analyses of lipid fractions from cells of strains NCS1 grown with and without sodium oleate are presented in Table 4. The same fatty acids were observed in all fractions. The major differences appeared to be the presence of greater amounts of octadecenoic and C₁₉ cyclopropane fatty acids along with lesser amounts of the saturated fatty acids in cells grown in sodium oleate than in cells grown in the control medium. More C₁₉ cyclopropane fatty acid was present in the polar fractions than in the neutral fraction. The cells grown in sodium oleate had more octadecenoic acid in the neutral than in the polar fraction; the reverse was true for cells grown in the control broth.

Relationship of fatty acid composition to death resulting from freezing. The amount of death that resulted from freezing was closely associated with the cellular content of C₁₉ cyclopropane fatty acid. Figure 2 shows the percent death plotted against the cellular content of C₁₉ cyclopropane fatty acid for the *Lactobacillus* cultures. The line had a negative regression that was significant at $P < 0.001$. Similar comparisons involving dodecanoic and tetradecanoic acids revealed positive regression lines that were significant at $P < 0.001$ and $P < 0.005$, respectively. Octadecanoic acid also had a positive regression line that was significant at $P < 0.025$. C₁₉ cyclopropane fatty acid content exhibited the smallest standard deviation from the regression line of all the individual fatty acids. The regression coefficient of percent death on percent total saturated fatty acids was significant at $P < 0.001$.

TABLE 3. Fatty acid composition of lipids from different strains of *L. bulgaricus*

Fatty acids ^a	Relative percentages in							
	NCS1		NCS2		NCS3		NCS4	
	Control ^b	Sodium oleate ^c	Control	Sodium oleate	Control	Sodium oleate	Control	Sodium oleate
12:0	18.8 ^d	2.9	8.6	3.2	11.7	3.3	9.1	3.0
14:0	21.4	10.4	8.9	5.0	13.8	5.6	6.9	4.5
16:0	25.6	23.0	34.4	37.4	26.2	22.8	25.1	25.6
16:1	24.1	13.8	27.9	10.1	28.2	21.7	26.1	13.6
18:0	3.9	0.6	2.8	2.7	5.8	2.3	2.7	2.9
18:1	5.8	27.1	14.3	29.2	13.8	36.8	11.7	18.9
Δ19:0	0.4	22.2	3.1	12.2	1.3	7.2	12.0	26.6

^a Fatty acid methyl esters are designated by the number of carbon atoms to the left of the colon, and the number of double bonds to the right. Δ19:0 denotes a 19 carbon (C₁₉) cyclopropane fatty acid.

^b Control broth.

^c Control broth plus 100 μg of sodium oleate per ml.

^d Relative percentages.

TABLE 4. Fatty acid composition of *L. bulgaricus* NCS1 grown with and without sodium oleate

Lipid fraction	Relative percentages in													
	12:0 ^a		14:0		16:0		16:1		18:0		18:1		Δ19:0	
	Control broth	Sodium oleate broth	Control broth	Sodium oleate broth	Control broth	Sodium oleate broth	Control broth	Sodium oleate broth	Control broth	Sodium oleate broth	Control broth	Sodium oleate broth	Control broth	Sodium oleate broth
Free	18.8 ^b	4.8	21.4	12.1	26.7	22.2	28.8	14.0	3.8	1.6	5.2	27.2	0.2	19.4
Neutral	18.8	7.3	24.8	11.6	29.2	19.6	14.4	7.5	6.5	3.2	6.4	40.9	Trace	8.0
Polar	17.8	4.0	16.6	11.5	22.4	23.9	26.1	15.4	4.1	3.1	11.0	20.8	1.8	21.2
Bound	16.4	1.9	15.1	8.7	27.1	22.7	29.3	16.1	3.3	0.1	7.5	25.9	1.0	24.7

^a Fatty acid methyl esters are designated by the number of carbon atoms to the left of the colon, and number of double bonds to the right. Δ19:0 denotes a 19 carbon (C₁₉) cyclopropane fatty acid.

^b Relative percentages.

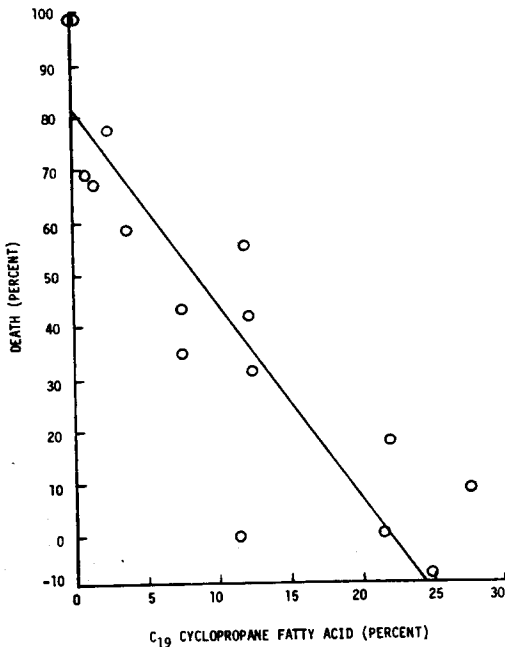


FIG. 2. Regression of percent death on percent C_{19} cyclopropane fatty acid content of *L. bulgaricus*.

DISCUSSION

Results from this and a previous study (25) suggested that sodium oleate was the active portion of Tween 80 responsible for producing cells that were stable to freezing in liquid nitrogen. The incorporation of [^{14}C]sodium oleate into the lipid fraction of cells of certain lactobacilli has been reported (17, 21). The amount of labeled sodium oleate incorporated into the lipid fractions of *L. bulgaricus* NCS1 was similar to the percent of each fraction on a weight basis, which suggested that the incorporation was random and not selective for a specific lipid fraction. The total amount of cellular lipids and the ratio of polar to neutral lipid were similar to those of other lactobacilli (12, 13). Since cyclopropane fatty acids are usually associated with phospholipids of bacteria (5, 11) it was not surprising that the polar lipids of *L. bulgaricus* NCS1 contained more C_{19} cyclopropane fatty acid than the neutral lipids. The phospholipid fraction thus appears to be important in protecting *L. bulgaricus* during freezing. The data indicate that the oleate was incorporated into the lipids intact or was converted to C_{19} cyclopropane fatty acid. Such a conversion of octadecenoic or *cis*-vaccenic acid to C_{19} cyclopropane fatty acid has been demonstrated in other lactobacilli (17, 21; J. A. Croom and J. J. McNeill, *Bacteriol. Proc.*, p. 170, 1961).

Generally, the major fatty acids of all strains were similar to those reported by Veerkamp (27) for *L. bulgaricus*. Alteration of the growth medium resulted in changes in the relative percentages of the individual fatty acids present in the cells. Similar effects of growth medium composition on the fatty acid content have been reported for other bacterial cells (14, 27). The increases in octadecenoic or C_{19} cyclopropane fatty acid, accompanied by decreases in saturated fatty acids that were observed when *L. bulgaricus* cells were grown in the presence of sodium oleate, can be explained by metabolic control mechanisms involving these fatty acids (1, 9, 28).

The lipids of gram-positive microorganisms, which are found predominately in the cell membrane (13), are important in maintaining membrane structure. The primary site of damage to certain cells during freezing is the cell membrane (19). Thus, the fatty acid composition might be involved in maintaining cell membrane integrity during freezing. Kodicek (16) suggested that cyclopropane fatty acids prevent close packing of lipids in cell membranes, making them more elastic and flexible during exposure to adverse environmental conditions. Jungkind and Wood (*Abstr. Annu. Meet. Amer. Soc. Microbiol.*, p. 143, 1972) reported that strains of *Streptococcus faecalis* deficient in cyclopropane fatty acids were more sensitive to deoxycholate, NaCl, sodium lactate at pH 4.0, and incubation at 47 C than the parent strain that contained more of these acids. However, work on liposomes prepared from structural lipids of *Escherichia coli* suggested that the formation of cyclopropane acids from unsaturated fatty acids does not alter their physicochemical properties (8). The results from the present study to support the flexible membrane theory proposed by Kodicek (16) and the work of Jungkind and Wood (*Abstr. Annu. Meet. Amer. Soc. Microbiol.*, p. 143, 1972). A relationship has been shown between the amount of unsaturated and saturated fatty acids and fragility to the cell membranes of *Mycoplasma laidlawii* (23, 24). Gier et al. (7) demonstrated that an increase in double bonds in the fatty acids of liposomes increased their permeability. Plant mitochondria that were sensitive to chilling had a higher content of saturated fatty acids than chill-resistant plant mitochondria (18). Chill-sensitive mitochondria were apparently injured due to an inflexibility of the membrane at low temperatures. A similar phenomenon may exist with regard to *L. bulgaricus*, because cell death appears to be related to a decrease in saturated fatty acid content.

Both unsaturated and cyclopropane fatty

acids are believed to have important roles in cell membrane structure (5). However, in the case of *L. bulgaricus* C₁₉ cyclopropane fatty acid appears to be the one most closely related to the resistance of cells to freezing. The exact mechanism of protection is not known, but death is closely related to both the amount of saturated and cyclopropane fatty acids. Perhaps the cause of increased protection is due to a favorable balance of C₁₉ cyclopropane and saturated fatty acids.

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