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Calcium-Induced Alteration of Cellular Morphology Affecting the Resistance of *Lactobacillus acidophilus* to Freezing†

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Examination of factors affecting the resistance of *Lactobacillus acidophilus* NCFM culture concentrates to freeze injury induced during frozen storage at −20°C revealed that calcium supplementation of the growth medium contributed to the storage stability of cells prepared in static culture. Culture concentrates of *L. acidophilus* NCFM were prepared from cells propagated in MRS broth or MRS broth supplemented with 0.1% calcium carbonate, calcium chloride, or calcium phosphate. After 28 days of frozen storage at −20°C, concentrated cells (3.2×10^9 colony-forming units per ml) prepared from MRS broth cultures showed an 84% reduction in viable cells. Of the remaining viable cells, 88% were sublethally injured and unable to form colonies on MRS agar supplemented with 0.15% bile. Cells prepared in calcium-supplemented MRS broths demonstrated more resistance to frozen storage. Viability and injury losses in the frozen concentrates were limited to 10 to 39% and 3 to 23%, respectively. It was observed that calcium supplementation of MRS medium resulted in a morphological transition of *L. acidophilus* NCFM from filamentous to bacilloid rods, and the bacilloid cells were more resistant to freezing and storage at conventional freezer temperatures. The results suggest that the morphology of the *L. acidophilus* cell may be an important consideration in the preparation of freeze-stable culture concentrates.

Concentrated starter cultures are composed of single or mixed strains of lactic acid bacteria that have been prepared under batch fermentor conditions, concentrated to a smaller volume, and then placed in frozen or dried storage (7). These concentrated bacterial cell suspensions are used to inoculate milk vats directly or to prepare bulk cultures for milk fermentations. Culture concentrates have found wide acceptance within the dairy industry because starter culture maintenance and "buildup" for vat inoculation within individual dairy plants can be eliminated, and centralized concentrate preparation can be monitored carefully to produce cultures that are highly active, contaminant free, and of uniform quality (2). An additional application has been the use of concentrated cell suspensions of *Lactobacillus acidophilus* to inoculate low-fat milk with high populations of this bacterium (26). In the preparation of "sweet acidophilus" milk, growth of the lactobacilli is not required after inoculation because the milk serves only to provide a suspending menstruum for the organism.

The development and use of concentrated cultures has resulted in a major advancement of fermentation technology. However, numerous problems and limitations in concentrated culture preparation and distribution remain to be resolved. Few concentrates prepared from the group N streptococci, leuconostocs, or lactobacilli respond well to lyophilization, spray drying, or conventional frozen storage. Sharpe (22) has stated that typical viability losses during freeze or spray drying approach 40 to 70% for the mesophilic streptococci (27), 50 to 80% for the lactobacilli, and 20 to 50% for the thermophilic streptococci. Use of cryoprotective agents and selection of insensitive strains allows, in some cases, satisfactory frozen storage at conventional temperatures of −20°C to −40°C (16). To minimize cell death and sublethal injury of the lactic acid bacteria during freezing, use of liquid nitrogen has been recommended as the preferred method for maintaining the highest level of culture viability and activity (7, 19). Unfortunately, the cost of maintaining active cultures under these conditions is extremely high and has, therefore, limited their widespread use.

In the preparation of culture concentrates, production methods and conditions must be tailored to the requirements of the specific organism to be concentrated (7). Ideally, growth medium formulations and batch fermentor conditions should prepare high cell populations adapted to the enzymatic and physical states

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required for cell harvest and concentration, stability during freezing and drying, and, most importantly, proper fermentative or biological activity (2). Conditions of batch culture that have been found to affect achievable cell mass, resultant cellular activity, and storage stability include the composition of the growth medium, temperature and time of fermentation, the pH and type of neutralizer, and the method of harvesting the cells (2, 15, 17, 19, 22). Selection of strains for incorporation into concentrated cultures is often dependent on intrinsic properties of the bacterium that dictate the suitability of that strain to commercial fermentation, concentration, and storage conditions. Unfortunately, active bacterial strains frequently are unable to meet these requirements.

Lactobacillus culture concentrates are of commercial importance in the production of both fermentative starters and dietary adjuncts. Prolonged frozen storage of *L. acidophilus* concentrates used in the preparation of sweet acidophilus milk leads to freeze injury (N. Austin, M.S. Thesis, North Carolina State University, Raleigh, 1977). Freeze-injured cells are incapable of growth in the presence of bile salts and, therefore, are no longer suitable for use as dietary adjuncts. Furthermore, frozen storage of *Lactobacillus* culture concentrates used for food bioconversions often results in the loss of fermentative activity, rendering them unsuitable for industrial use. Therefore, the growth, preparation, and frozen storage of *Lactobacillus* concentrates must facilitate the production of viable and uninjured cells.

Production of freeze-stable *L. acidophilus* culture concentrates has been reported in two instances where a milk or whey-based medium was used to propagate the cells. By using the whey-based medium, Duggan et al. (5) prepared cells that were stable to freezing at −10 to −60°C for 6 months. Later, Austin (N. Austin, M.S. Thesis, North Carolina State University, Raleigh, 1977) described a milk-based fermentor medium that was used to prepare cells stable to frozen storage at −76 or −196°C, but sensitive to −20°C. Retrieval of *L. acidophilus* cells from the milk medium required casein solubilization with sodium citrate before centrifugation (27). The increased stability observed for *L. acidophilus* cell crops propagated in milk or whey-based media suggested to us that calcium may participate in predisposing the bacterial cells to a physiological state less susceptible to freeze-induced death and injury. This study attempted to prepare freeze-stable cell crops of *L. acidophilus* NCFM in a nutrient broth medium, therefore eliminating problems commonly encountered with retrieval of cells from a milk-based medium. It was observed that calcium supplementation of the growth medium resulted in a morphological transition of *L. acidophilus* NCFM, and these cells were insensitive to freezing and storage at conventional freezer temperatures.

(This work was taken from a thesis submitted by C.T.W. to the Graduate School of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. acidophilus* NCFM and RL8K were obtained from the stock culture collection at North Carolina State University. The characteristics of *L. acidophilus* NCFM, including sugar fermentations, percent guanine plus cytosine, and growth at 15 and 45°C, have been described previously (N. Austin, M.S. Thesis, North Carolina State University, Raleigh, 1977). Stock cultures of *L. acidophilus* NCFM were characterized and found to harbor all characteristics previously reported, with the exception of growth at 45°C (S. Sutherland, M.S. Thesis, North Carolina State University, Raleigh, 1980). Growth at 45°C appears to be a variable characteristic in *L. acidophilus* (unpublished data), and *L. acidophilus* NCFM has not maintained this growth ability.

L. acidophilus strains were propagated in MRS broth (Difco Laboratories, Detroit, Mich.), using a 1% inoculum and incubation at 37°C for 12 h. Frozen stock cultures were prepared from MRS broth cultures, mixed with an equal volume of autoclaved 11% nonfat dry milk solids (NFDMS), and maintained at −76°C. Before experimental use, the frozen stock culture was thawed and propagated twice through MRS broth at 37°C, using a 1% inoculum.

Growth media and preparation of culture concentrates. MRS broth prepared from the standard formula (4) was used in the preparation of cell crops for culture concentrates. In some studies, MRS broth was supplemented with the following calcium derivatives: $CaCl_2 \cdot 2H_2O$, $Ca(OH)_2$, $CaCO_3$ (Fisher Chemical Company, Raleigh, N.C.), and $CaHPO_4$ (Allied Chemical Corp., Morristown, N.J.). For preparation of calcium-supplemented MRS broth, derivatives were added either before or after autoclaving (see below). MRS broth supplemented with calcium derivatives before autoclaving was adjusted to pH 6.8. For addition to sterile MRS broth (pH 6.5), $CaCO_3$, $Ca(OH)_2$, and $CaHPO_4$ were weighed, individually packaged, and sterilized in the desired quantities for 1 liter of broth supplementation. $CaCl_2 \cdot 2H_2O$ was prepared and dispensed as a stock solution to sterile MRS broth. After autoclaving, 10 or 20 ml of $CaCl_2 \cdot 2H_2O$ stock solution (10% [wt/vol] $CaCl_2 \cdot 2H_2O$ in distilled water) was added to 1 liter of sterile MRS broth to yield a final concentration of 1 or 2 g of $CaCl_2 \cdot 2H_2O$ per liter. These media were adjusted to pH 6.5 with sterile 1 N HCl.

L. acidophilus NCFM cell crops were propagated statically in MRS or supplemented MRS growth media. After 12 h of incubation at 37°C, cells were har-

vested by centrifugation at 8,000 × *g* for 10 min. Concentrated cell suspensions were prepared by suspending 4 g of the resulting cell pellet in 10 ml of 10% NFDMS. Cell concentrates were placed in 2-ml Cryovac vials (Wheaton Scientific, Millville, N.J.) and used immediately or frozen and stored at −20°C.

Determination of bacterial populations. Cell populations of culture concentrates before and after frozen storage were determined by standard plate count on MRS (Difco Laboratories) or Lactobacillus Selection (BBL Microbiology Systems, Cockeysville, Md.) agars. Frozen concentrates were thawed under tap water for 3 min, and serial dilutions were prepared in 0.1% NFDMS and 0.01% Antifoam B emulsion (Sigma Chemical Co., St. Louis, Mo.). Duplicate plates of each dilution were incubated for 72 h at 37°C in a flowing CO_2 atmosphere (0.4 liter/min). Colonies appearing on the plates were enumerated with the aid of a Quebec colony counter.

To assess sublethal cellular injury, concentrates were also plated on MRS or LBS agars containing 0.15% Oxgall (BBL Microbiology Systems, Cockeysville, Md.) as previously described (8). Plating media containing Oxgall were designated as MRSO or LBSO. Injury within the bacterial population was calculated by using the following formula: % injury = 1 − [(count on selective medium)/(count on nonselective medium)] × 100.

Bacterial growth and acid production. Growth of *L. acidophilus* NCFM in MRS broth or MRS broth supplemented with calcium was analyzed spectrophotometrically at 650 nm with a Bausch & Lomb Spectronic 20. Overnight cultures in 20 ml of MRS broth were centrifuged, and the pelleted cells were suspended in 1 ml of fresh MRS broth. Sterile cuvettes containing 10 ml of MRS broth or calcium-supplemented MRS broth were adjusted to an initial optical density of 0.1 by dropwise addition of the suspended culture. The cultures were then incubated at 37°C, and the optical density was read at 1-h intervals.

Acid production in MRS broth cultures was monitored at 37°C with a Beckman model 900 pH analyzer. All electrodes were calibrated to pH 7.0 with standard buffer, sterilized with 95% ethanol, and placed in 40-ml centrifuge tubes containing 25 ml of MRS broth or MRS broth supplemented with calcium derivatives. The broths were adjusted to pH 6.4 to 6.6 with sterile 1 N HCl and seeded from overnight cultures in MRS broth at a 1% inoculum. The pH was monitored for 12 h during growth.

RESULTS

Freeze stability of *L. acidophilus* cell crops propagated in Ca^{2+}-supplemented growth media. The survival of frozen concentrates of *L. acidophilus* NCFM propagated in MRS broth and MRS broth containing various calcium derivatives is shown in Fig. 1. After 28 days of storage at −20°C, the cell concentrate prepared from an MRS broth culture showed an 84% decrease in viability. Addition of $CaCO_3$, $CaHPO_4$, or $CaCl_2$ to MRS broth resulted in bacterial cell crops more resistant to freezing

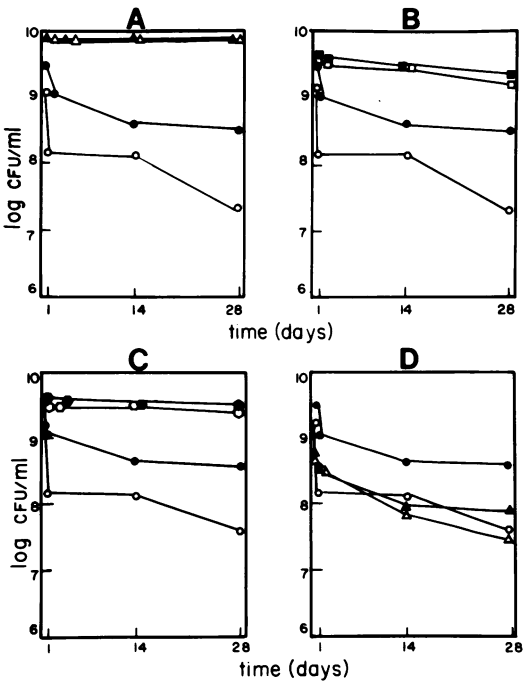

FIG. 1. *Frozen storage of L. acidophilus NCFM at −20°C. Cell concentrates were prepared from 12-h cultures in MRS or MRS calcium-supplemented broth. Total and uninjured cell populations were determined by enumeration on MRS (closed symbols) and MRSO (open symbols) agars. Each graph contains MRS-cultured cells (●, ○) for internal comparison. A, $CaCO_3$ (▲, △); B, $CaCl_2$ (■, □); C, $CaHPO_4$ (● ○); D, $Ca(OH)_2$ (▲, △). CFU, Colony-forming units.*

death and frozen storage as evidenced by viability losses of 18, 10, and 39%, respectively, over the 28-day storage period. Cell populations from MRS-$CaCl_2$, MRS-$CaCO_3$, and MRS-$CaHPO_4$ broths remained stable at −20°C, whereas cells grown in MRS-$Ca(OH)_2$ broth showed no resistance to frozen storage as compared with the control cells propagated in the absence of calcium. Cells propagated in MRS broth containing calcium also showed a reduction in the level of injury induced during frozen storage (Fig. 1). Although the control cells displayed 89% injury after 4 weeks of frozen storage at −20°C, concentrates prepared from broth supplemented with $CaCO_3$, $CaCl_2$, and $CaHPO_4$ showed injury levels of 3, 20, and 23%, respectively.

Heat sterilization of MRS broth containing calcium derivatives in the above experiment resulted in precipitate formation. Furthermore, $Ca(OH)_2$, $CaCO_3$, and $CaHPO_4$ were only slightly soluble in MRS broth before autoclaving at the

1-g/liter concentration used in the above stability experiment. Therefore, the enhancement of a cellular state that was resistant to freezing and frozen storage may have resulted from calcium precipitation of some unknown medium component. To examine this possibility, an additional stability experiment was conducted with cells propagated in MRS broth supplemented with $CaCl_2$ that remained soluble in the medium. Calcium chloride was prepared as a solution, sterilized separately, and then added to sterile MRS broth. Upon addition to MRS broth, no precipitate formed at the 1- or 2-g/liter levels. *L. acidophilus* NCFM cells propagated in the $CaCl_2$-supplemented medium again showed increased resistance to frozen storage in comparison with cells prepared in MRS broth (Fig. 2). Survival was assessed via enumeration on the more selective LBS and LBSO agars to further detect injury and death during the evaluation of calcium effectiveness. Little difference in cellular resistance to freezing death or injury was observed between the 1- or 2-g/liter $CaCl_2$-propagated cells, indicating that higher concentrations of $CaCl_2$ did not exhibit any further extension of freezing protection. Although cellular death during freezing was significantly reduced in $CaCl_2$-propagated cells, freeze injury was present and detectable on LBSO agar for cells propagated with or without calcium supplementation. Therefore, calcium supplementation of MRS broth for the growth of *L. acidophilus* NCFM resulted in cells more resistant to freezing and frozen storage. However, these cells were not completely insensitive to storage at −20°C, and cellular injury and death were detected with highly selective plating media.

Roles of calcium: cryoprotection and buffering capacity. Additional experiments were conducted to determine whether or not calcium derivatives provided stability to *L. acidophilus* concentrates during frozen storage by establishing a cryoprotective effect. The possible cryoprotective effectiveness of calcium has been suggested previously. Rudnick (21) observed that calcium carbonate supplementation of cottage cheese starters provided increased resistance to frozen storage over unsupplemented cultures. It has also been recommended that milk medium supplemented with calcium carbonate be added to lactic acid bacterial cultures to enhance survival during refrigerated storage at 4°C (23). Precipitate formation during heating of calcium-supplemented media or precipitation of calcium complexes during growth of *L. acidophilus* in MRS broth (19) could result in substantial recovery of calcium derivatives in cell pellets harvested from spent broth. Carryover of these derivatives into the freezing menstrum could then elicit a cryoprotective effect. To examine this possibility, 11% NFDMS was supplemented with 1 g of either $CaCl_2 \cdot 2H_2O$, $CaCO_3$, $Ca(OH)_2$, or $CaHPO_4$ per liter. These calcium-supplemented milk preparations were then used as a freezing menstrum for *L. acidophilus* culture concentrates prepared from cells propagated in a standard formula MRS broth. Table 1 shows that no cryoprotective effect was afforded to the culture concentrate when suspended in a freezing menstrum of 11% NFDMS containing calcium. Extremely high levels of injury were observed in all cultures frozen in this manner, and cellular death during storage was comparable for all cultures.

Calcium derivatives used in this study have component anions (CO_3^{2-} and PO_4^{3-}) which may facilitate neutralization of lactic acid produced during the growth of *L. acidophilus* in static culture. Prevention of acid damage to the bacterial cells before harvest and concentration

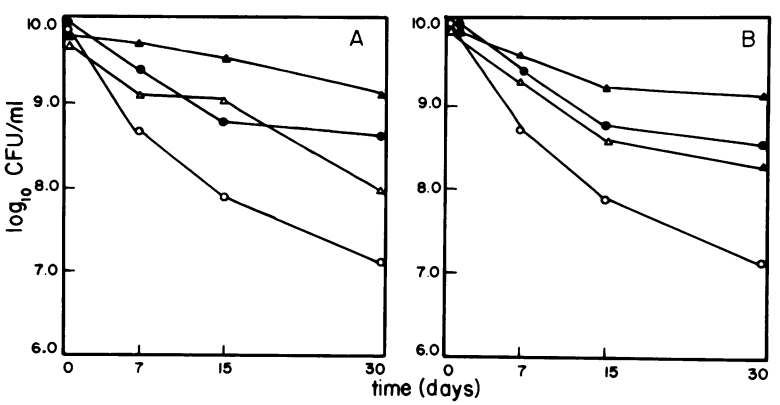

FIG. 2. *Storage stability of L. acidophilus NCFM at −20°C as determined by enumeration on LBS (closed symbols) and LBSO (open symbols) agars. A. Cells propagated in MRS broth (●, ○) or in MRS broth supplemented with 0.1% $CaCl_2 \cdot 2H_2O$ (▲, △). B. Cells propagated in MRS broth (●, ○) or in MRS broth supplemented with 0.2% $CaCl_2 \cdot 2H_2O$ (▲, △). CFU, Colony-forming units.*

may, therefore, contribute to the stability of these cells observed during frozen storage. To test for acid production and possible buffering capacity of calcium derivatives, frozen concentrates of *L. acidophilus* NCFM were inoculated into MRS broth containing $CaCl_2$, $CaCO_3$, $CaHPO_4$, and $Ca(OH)_2$. Cultures were incubated at 37°C, and acid production was monitored over a 12-h period. Although no difference in acid production was observed for organisms grown in the presence of MRS, MRS-$CaCl_2$, MRS-$Ca(OH)_2$, and MRS-$CaHPO_4$ broths, a slight buffering effect was noted for cells grown in the presence of MRS-$CaCO_3$ (Fig. 3). This buffering effect may have contributed to the cellular stability observed previously for *L. acidophilus* NCFM during storage at −20°C. Interestingly, cell crops prepared in MRS-$CaCO_3$ broth resulted in the highest levels of viability and bile resistance after frozen storage for 1 month at −20°C. However, where calcium supplementation to MRS broth did not exert a buffering effect, substantial levels of freeze resistance were still observed in *L. acidophilus* cells. Therefore, the resistance of *L. acidophilus* cells prepared in calcium-supplemented MRS broth to frozen storage at −20°C was not attributable to the cryoprotective effects of calcium or solely to the buffering capacity of the growth medium.

TABLE 1. *Effectiveness of calcium as a cryoprotective agent in 11% NFDMS*

Calcium supplement[a]	% Death[b]	% Injury[b]
None	56	>99
$CaCl_2$	48	>99
$CaCO_3$	53	>99
$CaHPO_4$	31	>99
$Ca(OH)_2$	65	>99

[a] Calcium derivatives were added to 11% NFDMS at a level of 0.1%.

[b] Cells were suspended in the freezing menstruum at levels greater than 10^9 colony-forming units per ml and frozen and stored at −20°C. After 2 weeks of storage, populations were enumerated on MRS and MRSO agars.

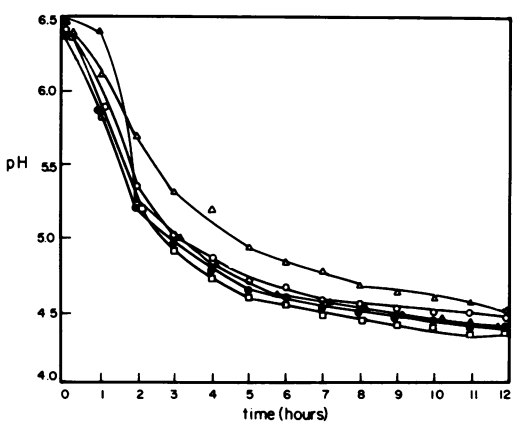

FIG. 3. *Effect of supplementation of MRS broth with calcium derivatives upon acid production by L. acidophilus NCFM. All calcium derivatives were added to sterile MRS broth at a level of 0.1%. Symbols: ●, MRS; □, MRS-$CaCl_2 \cdot 2H_2O$; △, MRS-$CaCO_3$; ○, MRS-$CaHPO_4$; ▲, MRS-$Ca(OH)_2$.*

Cellular morphology. Bacterial growth curves showed that growth rates were identical for MRS broth and MRS broth supplemented with calcium when examined turbidimetrically or via agar plate counts (data not shown). Thus, cells used in this investigation were in identical growth phases, and the stability observed for calcium-grown cells could not be attributed to these cells being in a more resistant growth phase. Microscopic examination of *L. acidophilus* NCFM cultures after growth for 12 h in MRS broth revealed that two predominant cell types were present (Fig. 4A). Long, thick, filamentous rods constituted the major morphological type present in the microscopic smear. Smaller, more bacilloid rods were also present. After growth in MRS broth in the presence of $CaCl_2$, *L. acidophilus* NCFM showed more short bacilloid rods present individually or in small chains (Fig. 4B). The extended cell chains and filamentous cells observed for MRS broth cultures were less pronounced when Ca^{2+} was supplemented to the growth medium. When extended chain lengths were observed in Ca^{2+}-MRS broth cultures, cross wall formation was more apparent and suggested that calcium was enhancing the divisional process.

The transition from filamentous to bacilloid cells during growth in $CaCl_2$-supplemented MRS broth suggested that the stability of *L. acidophilus* NCFM to frozen storage was dependent, in part, on the morphological state of the cell. Barber and Frazier (1) described rough and smooth colonial variants of *L. acidophilus*. Microscopically, long filamentous rods were observed from smears of the rough colonies, whereas smooth colonies were composed of short bacilloid cells. Rough and smooth variants of *L. acidophilus* RL8K (RL8K^r and RL8K^s, respectively) have been isolated in our laboratory (T. R. Klaenhammer and E. Kleeman, manuscript in preparation), and *L. acidophilus* RL8K^r and RL8K^s were examined to determine the effect of cellular morphology on frozen storage stability at −20°C. RL8K^r and RL8K^s were propagated in MRS broth and in MRS broth supplemented with $CaCl_2$ for 12 h at 37°C, and culture concentrates were prepared. After growth in MRS broth, RL8K^r showed long, thick, filamentous rods (Fig. 5A) similar to those

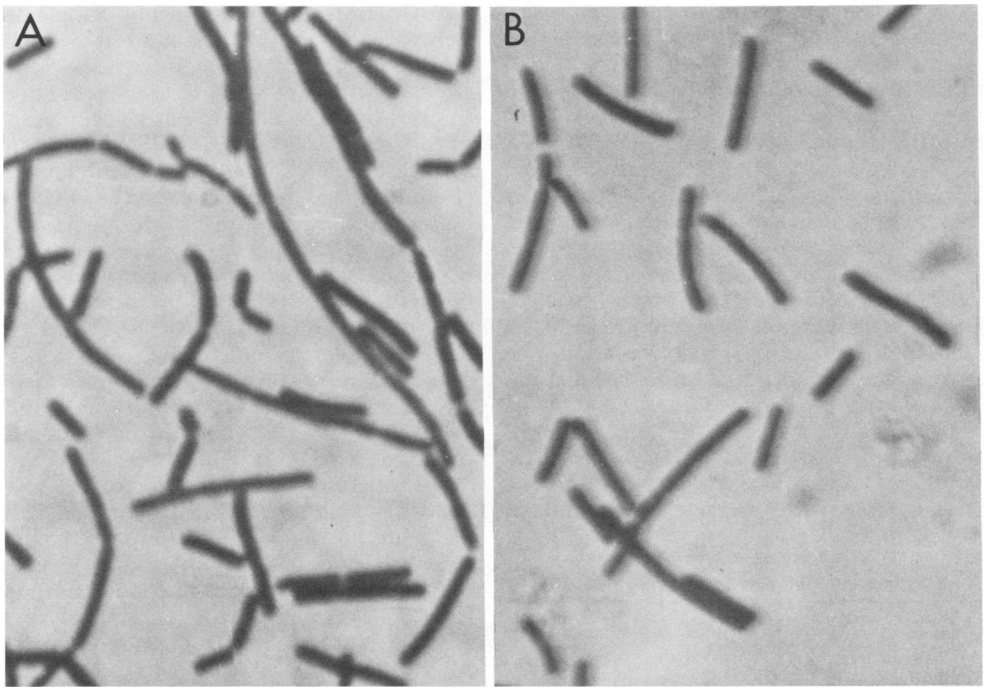

FIG. 4. *Photomicrographs of concentrated cultures of L. acidophilus NCFM prepared from cells propagated in MRS broth (A) or MRS broth supplemented with 0.1% $CaCl_2 \cdot 2H_2O$ (B). Magnification, ×100.*

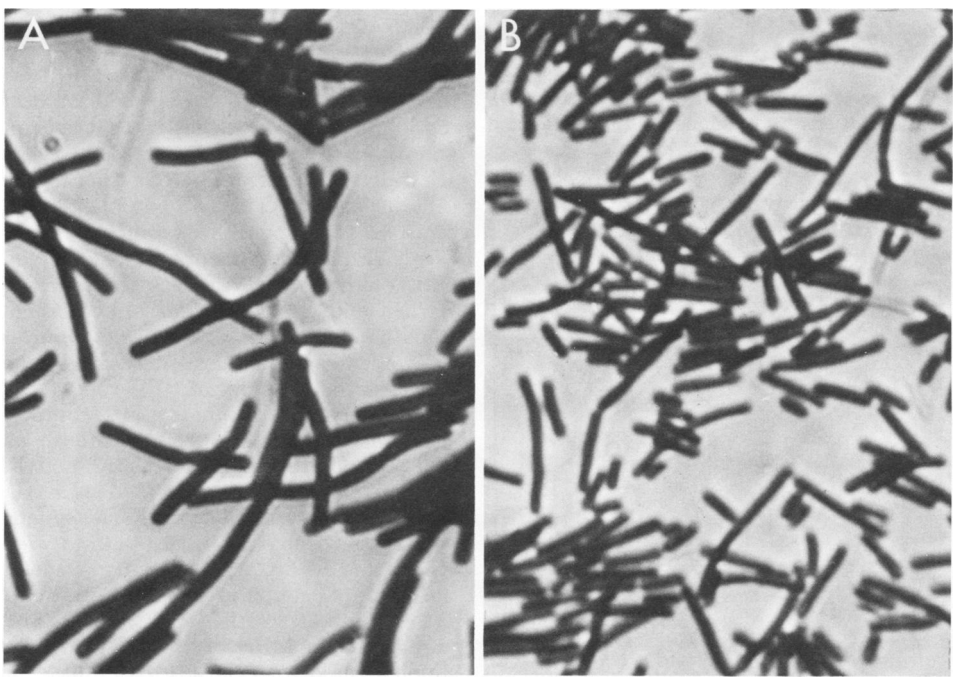

FIG. 5. *Photomicrographs of concentrated cultures of L. acidophilus RL8K. A, RL8K^r; B, RL8K^s. Magnification, ×100.*

reported previously by Barber and Frazier (1). *L. acidophilus* RL8K^s strains were observed as being short bacilloid rods (Fig. 5B). Growth in MRS broth supplemented with $CaCl_2$ did not result in an alteration of either morphological type (data not shown). This suggests that calcium-induced transitions in morphology are highly strain specific and dependent on the environmental conditions employed to propagate any particular type.

Dramatic differences in the survival of *L. acidophilus* RL8K^r and RL8K^s were observed during storage of culture concentrates at −20°C. *L. acidophilus* RL8K^r showed a marked reduction in the viable count, whereas RL8K^s was insensitive to freezing death during 30 days of storage at −20°C (Fig. 6). Calcium supplementation of the growth medium did not confer any resistance to the cells stored at −20°C. Therefore, under these growth conditions, RL8K^r cells were far more sensitive to frozen storage. Calcium did not alter the morphology or enhance the freeze resistance of this cell type.

DISCUSSION

The use of liquid nitrogen freezing and storage for the preservation of viable and active culture concentrates of the lactic acid bacteria has been highly advocated (14, 19, 27). Unfortunately, the high cost associated with liquid nitrogen storage prohibits the widespread use of frozen culture concentrates maintained and distributed under these conditions. These economic considerations have prompted development of alternative methods of frozen or dried storage for culture concentrates. Frozen storage at conventional freezer temperatures (−20 to −40°C), lyophilization, and spray drying of cultures are economically practical and convenient methods to maintain culture concentrates. However, under these conditions, few members of the lactic acid bacteria retain their viability or activity during prolonged storage. Past efforts to improve the viability and activity of culture concentrates have focused primarily on propagation, concentration, and storage techniques to minimize cellular death and sublethal injury. Few reports have addressed intrinsic bacterial resistance to frozen or dried storage in the lactic acid bacteria and selective parameters that could be used to identify insensitive strains.

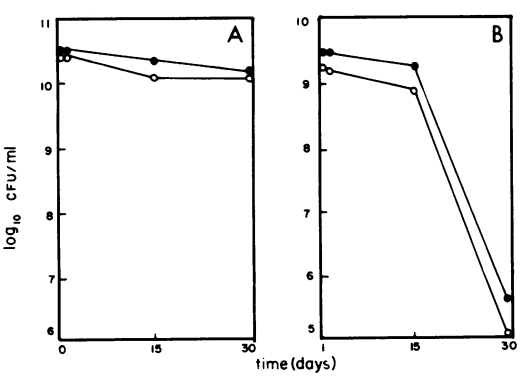

FIG. 6. *Survival of L. acidophilus RL8K^s (A) and RL8K^r (B) during frozen storage at −20°C. Cell crops were propagated for 12 h in MRS broth. Population levels were determined on MRS (●) and MRSO (○) agars.*

Susceptibility of the lactobacilli to freezing and drying varies considerably among species and different strains of the same species. Upon examination of three *Lactobacillus bulgaricus* strains subjected to liquid nitrogen freezing, Smittle et al. (24) observed cellular death at levels of 95, 54, and 0%. Recently, Porubcan and Sellars (19) noted that different strains of *L. bulgaricus*, *L. acidophilus*, and *Lactobacillus helveticus* exhibit marked differences in their sensitivity to spray drying. Reports in the literature concerning the levels of death resulting from frozen or dried storage of the lactobacilli are variable and could be attributed to the different strains employed during these studies. It is apparent that development of highly active and storage-stable culture concentrates must consider the intrinsic resistance or susceptibility of the lactobacilli to stress encountered during freezing and frozen storage. Results from this investigation suggest that the physiological state of the lactobacilli that is maintained or adapted through batch culture may be an important parameter when evaluating culture resistance to freezing and frozen storage.

Concerning the effects of frozen storage on bacterial cells, it is generally accepted that: (i) the rate of storage death increases with the duration of storage; (ii) the lower the storage temperature and fluctuation, the lower the rate of storage death; (iii) cryoprotective compounds usually provide good protection against freezing damage; (iv) higher concentrations of cells enhance protection against freezing damage; and (v) nonmetabolizing or stationary-phase cells show increased resistance over younger, more active cultures (20). Stability of *L. acidophilus* NCFM cell crops, prepared in calcium-supplemented MRS broth, could not be attributed solely to cryoprotection, cell concentration, or culture age. Culture mass and growth rates also were found to be identical between supplemented and unsupplemented MRS broth cultures of *L. acidophilus* NCFM. The effectiveness of calcium used in these studies was attributable, in part, to the minor buffering capacity

of calcium carbonate. *L. acidophilus* NCFM grown in static culture in the presence of this derivative displayed the highest viability levels after frozen storage and the lowest incidence of freeze injury. The pK_a of carbonic acid is 6.3, and since buffering agents are most effective at the pH of their pK_a, this would account for the buffering capacity of calcium carbonate in MRS broth at pH 6.5. Alternatively, $Ca(OH)_2$ was completely ineffective in producing freeze-stable cells during static culture. With a pK_a of 2.4, $Ca(OH)_2$ would be completely undissociated, and, therefore, calcium ions would not be available to the cells during growth.

Filament formation of gram-positive bacteria has historically been associated with nutritional deficiencies in the growth medium. For the lactobacilli, it has been observed that growth medium deficiencies in deoxyribonucleic acid, vitamin B_{12}, and vitamin B_6 result in filament formation in *L. acidophilus*, *Lactobacillus leichmanii*, and *Lactobacillus arabinosus*, respectively (3, 9, 10). *Lactobacillus brevis* isolates from tomato juice initially displayed a filamentous morphology, but upon propagation in a complex nutrient broth medium containing tomato juice, bacilloid structures were observed (28). *Lactobacillus bifidus* is also observed in two morphological states: a bifid form displaying extensive branching, and a bacilloid form (11). Cultivation in a calcium-free medium results in the exclusive appearance of the bifid structure, whereas supplementation of the medium with calcium generates bacilloid structures (12). The calcium content of the bifid form was found to be significantly less (82%) than that of the bacilloid cells, indicating incorporation of the cation into the cellular structure. Fontana et al. (6) and Sokatch (25) proposed that rod-shaped bacteria bear two sites for peptidoglycan assembly: one responsible for lateral wall elongation, and the other responsible for septum formation. Strains defective in the site for septum formation would form filaments, whereas those defective in the site for lateral wall elongation would grow as cocci. Fontana et al. (6) suggested that the septum is a portion of peptidoglycan which grows at a faster rate and that divalent cations may accumulate specifically in this portion to form linkages and increase the rigidity of the polymer. Extensive cross-division is observed in *L. acidophilus* NCFM after the growth of these cells in the presence of calcium, and perhaps the accumulation of divalent cations enhances this divisional process.

Filamentous growth in cation-deficient media has been found to be a general characteristic of many gram-positive bacilli (29). It is interesting to note that MRS broth, which contains added Mn^{2+} and Mg^{2+} components, required added calcium to support growth of bacilloid type cells of *L. acidophilus* NCFM. Furthermore, calcium supplementation of MRS broth did not alter the filamentous growth characteristics of *L. acidophilus* RL8K^r. Therefore, either RL8K^r is phenotypically subject to filamentous cellular growth, or MRS broth is additionally deficient in some other unknown component necessary for bacilloid growth of this strain.

Upon consideration of the physiology of gram-positive cells, Matthews et al. (18) and Sokatch (25) suggested that the walls of gram-positive bacteria are natural cation exchangers because of the numerous phosphate and carboxylate ions of peptidoglycan. Fontana et al. (6) observed that the accumulation of divalent cations appeared to be necessary for the peptidoglycan of *Klebsiella pneumoniae* to acquire sufficient rigidity for shape determination and cell protection. These cells grown in the presence of ethylenediaminetetraacetic acid were susceptible to autolysis and were rapidly killed by freezing and thawing. A routine practice in the dairy industry involves the addition of sodium citrate to bacterial cells grown in media containing NFDMS. Injury previously observed in *L. acidophilus* NCFM cell crops prepared in milk-based media could to be attributed to the destabilization of cellular structure by sodium citrate used to facilitate bacterial cell harvest from this medium. Sodium citrate destabilizes the casein micelle by removal of calcium from this complex. Although calcium is removed from the casein micelle by sodium citrate, it could also be removed from the bacterial cell, assuming it was incorporated into the cellular structure during growth, thus resulting in the onset of freeze injury upon frozen storage of *L. acidophilus* NCFM concentrates. If calcium does afford stability to some bacterial strains during prolonged frozen storage, the practice of citration of milk media should be seriously reconsidered.

Barber and Frazier's (1) description of rough and smooth colonies of *Lactobacillus* species, composed of filamentous and bacilloid cells, respectively, indicated that exposure of the lactobacilli to stress favored the development of the smooth colony type. These stresses included ultraviolet and X-ray irradiation and heat, and suggested that the smooth colony, or bacilloid structure, was more resistant to environmental stress. Earlier, Kopeloff et al. (13) found that rough strains lost viability more rapidly at 4°C than did the smooth colony type, further supporting the more resistant state of bacilloid *Lactobacillus* cells. These early reports suggest that bacilloid cells of the lactobacilli would also show increased resistance to freezing and frozen stor-

age. However, this relationship has not been examined previously. Results from our investigation show that filamentous and bacilloid cells display dramatic differences in sensitivity to death and injury during freezing and frozen storage. Because bacilloid cells appear desirable from a freeze stability standpoint, production methods facilitating conversion of filamentous forms or prior selection of strains harboring the bacilloid phenotype may serve to enhance the freeze stability of *Lactobacillus* culture concentrates. It appears that calcium as well as other divalent cations are inductive of these morphological changes in some strains, and inclusion of these compounds in fermentor broth formulations may be beneficial in the production of freeze-stable cell crops.

In addition to freeze stability, selection of strains for incorporation into concentrated cultures must also evaluate fermentative or biological activity of fermentor cell crops. Whether or not morphological transitions and divalent cation accumulation affect desirable fermentative or biological activities in the lactobacilli has not, as yet, been investigated. It is apparent, however, that selection of lactobacilli for use as fermentative or biological cultures via frozen concentrates must consider both the physiological and genetic characteristics of the bacterial strains under consideration.

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