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Growth Characteristics, Bile Sensitivity, and Freeze Damage in Colonial Variants of *Lactobacillus acidophilus*†

T. R. KLAENHAMMER* AND E. G. KLEEMAN

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27650

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Rough (R) and smooth (S) colonial variants were isolated from a heterogeneous culture of *Lactobacillus acidophilus* RL8K. R and S types were stable upon repeated transfer on agar, but revertant colonies did appear after broth transfers. When propagated in commercial MRS broth, R and S cultures showed similar growth characteristics, and both cell types were insensitive to freezing and frozen storage at −20°C. Alternatively, during growth in scratch MRS broth, R cultures shifted to a reduced rate of growth during the late logarithmic phase. R cells grown under these conditions were susceptible to death by freezing and injury at −20°C. Microscopically, R cells were observed as long gram-positive rods with small nonstainable blebs protruding from the cell wall. In bile sensitivity studies of R and S cells plated on MRS agar plus oxgall, the S culture was resistant to 1% bile, whereas the R culture was sensitive to 0.6% bile. Differences in the bile resistance and freeze damage of R and S cells suggest that colonial and cellular morphologies are important considerations for the selection of *Lactobacillus* strains as dietary adjuncts and for the development of growth conditions for preparing frozen concentrated cultures from either cell type.

The lactic acid bacteria are composed of a diverse group of bacteria that are responsible for the characteristic end product of most food fermentations, lactic acid. Numerous benefits have been realized due to the fermentative abilities of these food-bioprocessing bacteria. The wide variety of fermented foods is the direct result of the different growth characteristics, metabolic activities, and end products of the lactic acid bacteria. Within this group, the lactobacilli participate in numerous milk, meat, and vegetable fermentations and are critical to the successful completion of many thermophilic food fermentations. Beyond the fermentative abilities of the lactobacilli, an additional role has been attributed to those species that reside in the intestinal tracts of humans and animals. It has long been suggested that *Lactobacillus acidophilus* may enhance resistance to common intestinal disorders through stabilization of normal intestinal microflora (5, 19, 23). More recently, Goldin et al. (9) reported that viable *L. acidophilus* cells favorably altered the formation of fecal bacterial enzymes that contribute to carcinogen formation in the bowel lumen. Unfortunately, studies identifying the beneficial effects of *Lactobacillus* sp. in the intestinal tract are limited and often subject to conflicting reports (25).

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The lactobacilli have been subject to extensive investigations concerning their taxonomy, growth, nutritional requirements, and metabolic activities to clarify their roles in the intestine or enhance their fermentative activities. These characteristics are highly variable among the lactobacilli, and the differences contribute to the difficulty in defining optimum conditions for the growth and activity of different species. Phenotypically, the lactobacilli respond to altered growth conditions by morphological changes apparent microscopically or colonially on solid media (1). These morphological transitions appear characteristic of the lactobacilli (1) but in recent years have been neglected during the selection and propagation of strains for fermentative starters or dietary adjuncts.

Early descriptions of *L. acidophilus* colonial variation identified characteristic "X" and "Y" types. On solid media, the X type appeared as a fuzzy, rough, flat, and irregular colony, whereas the Y type was smooth, regular, and convex in appearance (17, 22). Strains were typically isolated from feces as rough (R) colonies and readily dissociated to smooth (S) colonies upon laboratory transfer (16). S types rarely dissociated back to R types, and the R → S transition was favored upon repeated laboratory propagation (16, 22). Kopeloff (16) also described an intermediate colony type that approached either the

R or S types but would not stabilize at either extreme. Subsequent studies on numerous species of *Lactobacillus*, including *L. acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus casei*, concluded that morphological alterations in colony types are not the result of genetic variability but rather represent differences in the phenotypic expression of a population in different growth environments (18, 21). Growth conditions that have been found to affect the colony morphology of the lactobacilli include the presence or absence of oxygen (18), temperature of incubation (2), pH (18), and components of the growth medium, such as acetate (18), salt (1), fatty acid esters (21), and antibiotics with surfactant properties (1). Microscopically, R strains produce chains of long or filamentous cells, and S strains are more compact bacillary structures in short chains (10). Nutritional deficiencies in deoxyribonucleosides, vitamins, and divalent cations have also been reported to induce filamentous growth in the lactobacilli (4, 12-14, 26). Dramatic alterations in colonial or cellular morphology of entire *Lactobacillus* populations under limiting or variable growth conditions, however, do not address the heterogeneity that occurs from pure isolates on a single complex medium under stable environmental conditions. We have observed that a pure culture of *L. acidophilus* RL8K exhibits heterogeneous colony types on MRS agar. This report describes the isolation of stable R and S types, their growth characteristics in MRS broth, and the response of each type to bile salts and frozen storage, conditions that are important in the selection of *Lactobacillus* strains for use in frozen concentrated cultures and dietary adjuncts.

MATERIALS AND METHODS

Culture propagation, selection, and storage. *L. acidophilus* RL8K was obtained from the dried culture collection maintained by the Department of Food Science at North Carolina State University. The culture was propagated in commercial MRS broth (Difco Laboratories, Detroit, Mich.) at 37°C and held at 4°C between transfers. Unless otherwise noted, Difco MRS broth was used to prepare all liquid and solid propagation media. Incubation conditions were 37°C for 48 h under a flowing CO_2 atmosphere (0.4 liter/min).

R and S colonial variants of *L. acidophilus* RL8K (designated $RL8K^R$ and $RL8K^S$) were isolated from the surfaces of MRS agar plates (1.5% agar; BBL Microbiology Systems, Cockeysville, Md.). A dissecting microscope was used to define colony types and assist in the selection of R and S colonies through repeated isolation until stable types were obtained. Working stock cultures were prepared by picking single colony isolates into MRS broth, transferring once through MRS broth, and then mixing 1 ml of culture with 2.5 ml of autoclaved 11% Matrix milk (Galloway-West Company, Fond du Lac, Wis.) and freezing at −76°C. Stock cultures were thawed a maximum of three times for subculture.

Identification of the cultures as *L. acidophilus* was confirmed by Gram stain, catalase test, growth at 15 and 45°C, and carbohydrate fermentation patterns (8, 20).

Growth studies. From frozen stock cultures, *L. acidophilus* $RL8K^R$ and $RL8K^S$ cells were streaked onto MRS agar and incubated for 48 h at 37°C. Typical R and S colonies were picked into MRS broth and incubated for 24 h at 37°C. The cells were harvested by low-speed centrifugation (3,000 × *g* at 4°C for 10 min) and resuspended in 2 to 3 ml of fresh MRS broth. These cells were used to inoculate 10 ml of MRS broth prepared from the commercial dehydrated medium or broth prepared with the following components as per the Difco MRS broth formula: protease peptone no. 3 (Difco), 10.0 g; beef extract (Difco), 10.0 g; yeast extract (BBL), 5.0 g; dextrose, 20.0 g; Tween 80, 1.0 g; ammonium citrate, 2.0 g; sodium acetate, 5.0 g; magnesium sulfate, 0.1 g; manganese sulfate, 0.05 g; disodium phosphate, 2.0 g; distilled H_2O, 1 liter; pH 6.7. This medium was designated scratch MRS broth. Growth at 37°C was followed optically at 650 nm on a Bausch & Lomb Spectronic 70. Colony types from each culture were confirmed on MRS agar at the start and completion of the growth experiments.

Bile sensitivity. Single colonies of R and S types were isolated, propagated once through 10 ml of MRS broth, and concentrated to a small volume as described above. Concentrated cell suspensions were used to adjust 10 ml of MRS broth to an initial optical density at 650 nm of 0.62 to 0.64. The cultures were immediately plated in MRS agar containing various concentrations (0 to 1%) of oxgall (BBL). Dilutions were prepared in 0.1% peptone (Difco) and 0.01% Antifoam B (Sigma Chemical Company, St. Louis, Mo.).

Pour plates and dilutions were prepared by standard methods (7). The plates were incubated under a flowing CO_2 atmosphere for 72 h at 37°C. Colony types were confirmed by preparing a streak plate on MRS agar from the adjusted cultures.

Freeze studies. Concentrated cells of *L. acidophilus* $RL8K^R$ and $RL8K^S$ were prepared as described above and used to adjust 10 ml of MRS broth to an initial optical density at 650 nm of 1.3. A 1-ml amount of this suspension was used to inoculate 100 ml of tempered (37°C) MRS broth. The culture was incubated for 12 h at 37°C. Colony morphology was confirmed at the beginning and end of this 12-h incubation. Cells were then harvested by centrifugation at 8,000 × *g* at 4°C for 10 min and suspended in 100 ml of cold (4°C) 11% Matrix milk. This suspension was blended (20 to 30 s at high speed in a Waring blender) and dispensed into 2.0-ml Cryule vials (Wheaton Scientific, Millville, N.J.) The vials were frozen at −20°C and stored for 35 days. The *Lactobacillus* population was determined on MRS agar and MRS agar containing 0.15% oxgall (MRSO) before freezing and after 18 and 35 days of frozen storage. Dilutions were prepared as described above, and the pour plates were incubated for 72 h at 37°C under a CO_2 atmosphere before counting.

RESULTS

Colony selection. Original plating of *L. acidophilus* RL8K revealed heterogeneous colony types on the surfaces of MRS agar plates. Isolation of R and S colony types was accomplished by repeatedly picking the roughest and smoothest colonies until stable R and S types were obtained. As a surface colony, the R type was large, irregular, and flat to umbonate, with a matte surface and mottled opacity (Fig. 1). In contrast, the S colony was smaller, circular, with a smooth edge, and convex with a glistening translucent appearance. Removal of the colonies from the agar surface with an inoculating needle revealed that the S colony was moist and creamy, and the R colony was dry and granular. Gram stains showed that R colonies were composed of a mixture of long and short rods in long chains and filaments, often observed in tangled masses of cells. Alternatively, homogeneous and individual short rods were observed in Gram stains of S colonies.

The R and S colonies isolated from *L. acidophilus* RL8K were homogeneous and stable, although colony characteristics varied slightly between experiments. This variation was probably caused by the sensitive response of the lactobacilli to slight differences in culture and incubation conditions, such as dryness of agar surfaces or total CO_2 atmosphere (1, 18). The R and S types were gram-positive rods, were catalase negative, did not grow at 15°C, and showed variable growth at 45°C. Fermentation patterns, characterized with the Minitek system (BBL Microbiology Systems) (8), were identical for both types and consistent with typical fermentation reactions of *L. acidophilus*.

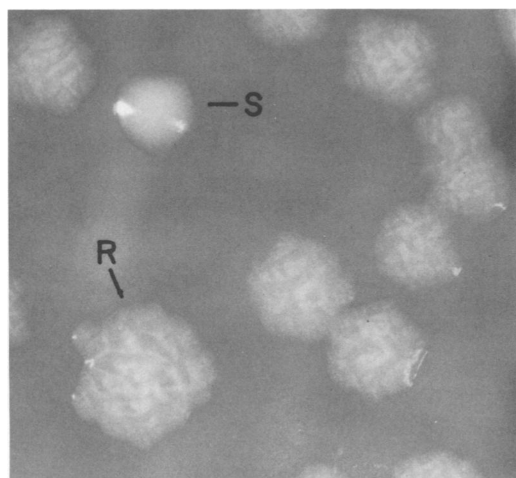

FIG. 1. *Appearance of L. acidophilus RL8K^R and RL8K^S colonies on the surface of MRS agar. Objective magnification, ×3.2, reflected light.*

Growth studies. In commercial MRS broth (Difco), both RL8K^S and RL8K^R cells showed excellent growth when examined turbidometrically (Fig. 2A). However, a slight reduction in the growth rate during the later stages of logarithmic growth was consistently observed for the RL8K^R culture but was not detected in the RL8K^S culture. Gram stains of the RL8K^S culture revealed short rods found individually or in short chains, whereas the RL8K^R culture grew as longer rods found in short or long chains mixed with filamentous cells (data not shown).

The late log reduction in the growth rate for RL8K^R cultures in MRS broth was enhanced by propagation in scratch MRS broth prepared from individual components by the Difco recipe (Fig. 2B). After 4 h of growth in the scratch broth, the R culture showed a temporary cessation of growth, with subsequent growth proceeding at a reduced rate. The *L. acidophilus* RL8K^S culture showed identical growth in either the commercial or scratch MRS broths. Microscopic analysis of cells from the RL8K^S and RL8K^R cultures in the scratch MRS broth revealed a dramatic difference in cell morphology beyond the typical short versus long rods. Blebs protruding from numerous points about the cell wall were detected for RL8K^R cultures examined during the reduced growth phase (Fig. 3). The blebs did not retain crystal violet stain and were observed as red protrusions from the gram-positive cell wall. Bleb formation was common in the RL8K^R cells propagated in the scratch broth, occasionally observed in RL8K^R cells from commercial broth, and rare in RL8K^S cells from either broth formulation.

Bile sensitivity. The bile sensitivity of R and

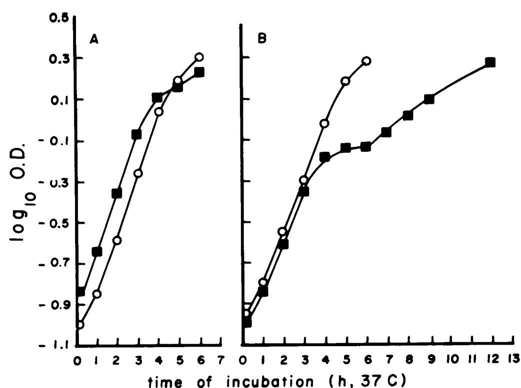

FIG. 2. *Growth of R and S cells in commercial (A) and scratch (B) MRS broths. Symbols: ■, RL8K^R cells; ○, RL8K^S cells. O.D., Optical density.*

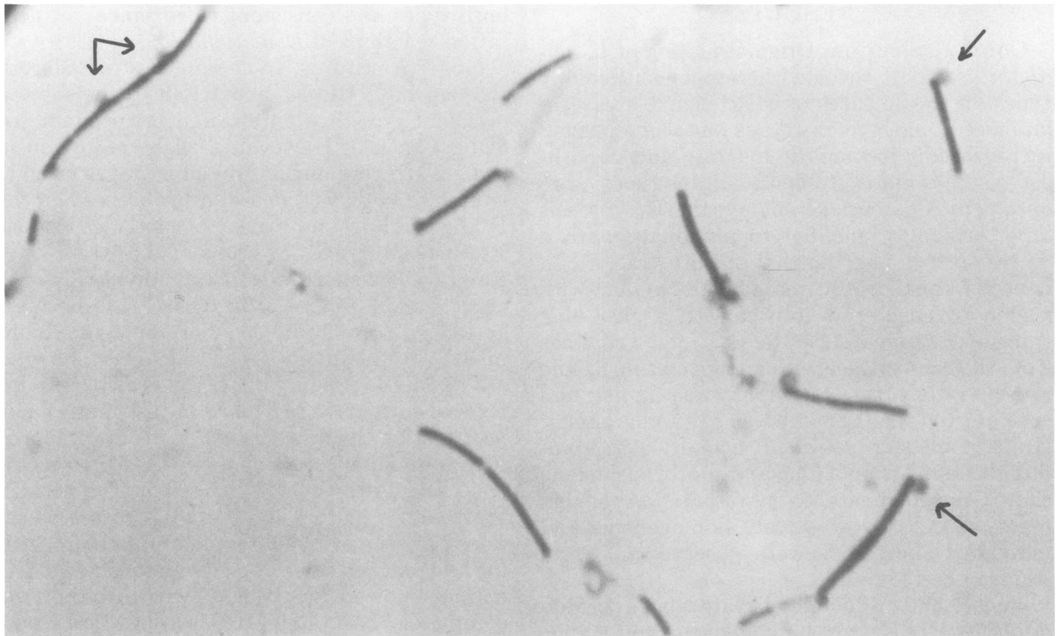

FIG. 3. *Photomicrograph of R cells with blebs (indicated by arrows) after 5 h of growth in scratch MRS broth at 37°C. Cells were Gram stained. Objective magnification, ×100.*

S cells was tested by pour plating broth cultures on MRS agar containing increasing concentrations of bile (Fig. 4). RL8K^S cells were resistant to 1% bile and maintained the original population level through all tested concentrations. Alternatively, RL8K^R cells were more sensitive to bile, and a significant reduction in colony-forming ability was observed as the bile concentration was increased from 0.6 to 1.0%.

In MRS pour plates, the differences between R and S colonies were apparent but were less striking than forms observed on the agar surface (Fig. 5). However, in MRSO, R and S colonial morphologies were dramatically different when compared with each other and with their respective morphologies in MRS agar without oxgall. In MRSO, the S colony was disk to round in shape and much smaller than that in MRS agar (Fig. 5A and B). No difference in colony size was observed between the R type in MRS agar and that in MRSO. However, in the presence of 0.15% oxgall, the R type formed a very rhizoid colony (Fig. 5C and D). The results demonstrate a significant difference between R and S types of *L. acidophilus* RL8K in the level of bile resistance and the types of colonies formed in the presence of 0.15% bile.

Freeze damage. In early literature, reports suggested that exposure of the lactobacilli to stress favored the development of the S colony type, generally considered more resistant to en-

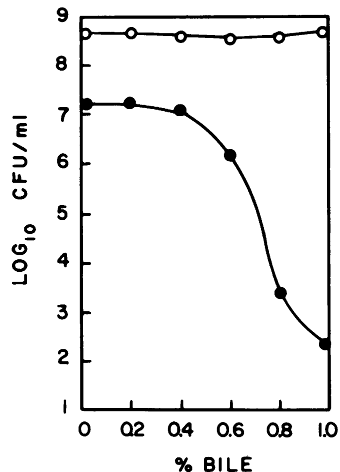

FIG. 4. *Sensitivity of RL8K^S* (○) *and RL8K^R* (●) *cells to increasing concentrations of bile. CFU, Colony-forming units.*

vironmental stress (2, 15). *Lactobacillus* strains are commonly incorporated into frozen concentrated cultures, and therefore, it was of interest to determine the stability of R and S types of *L. acidophilus* RL8K under frozen storage conditions. As shown in Fig. 6, both R and S cells cultured in commercial MRS broth were relatively stable during freezing and frozen storage at −20°C. Over the 35-day storage period, little

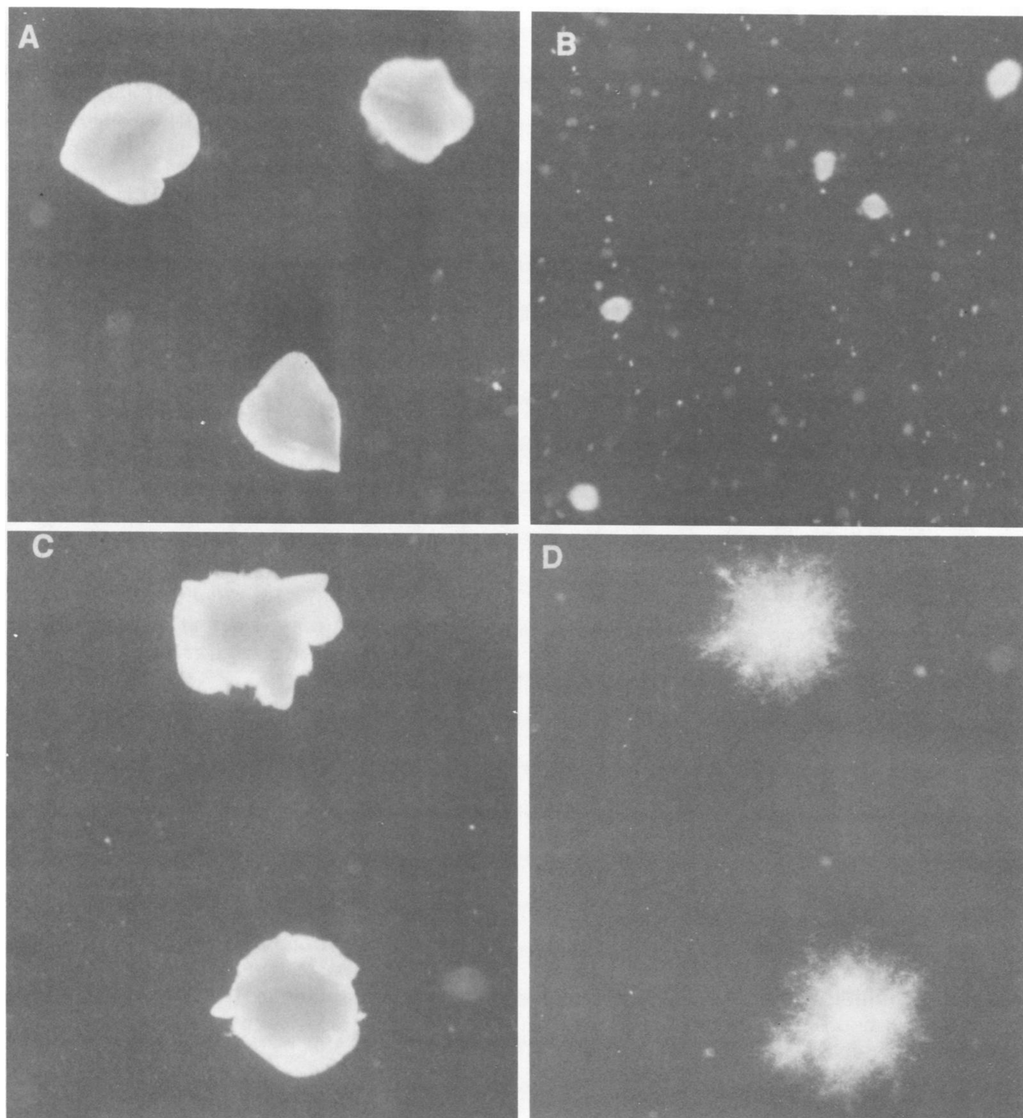

FIG. 5. *Appearance of RL8K^S and RL8K^R colonies in MRS and MRSO plates. (A) S colony in MRS agar; (B) S colony in MRSO; (C) R colony in MRS agar; (D) R colony in MRSO. Magnification, ×3.2, transmitted light. Precipitated bile appeared as small specks in the background of MRSO plates.*

death or sublethal injury was detected. However, R cells prepared from a scratch MRS broth culture were highly susceptible to freeze damage. A 90% reduction in viable cells was observed after 35 days of frozen storage and, of the remaining cells, approximately 60% were freeze injured and unable to form colonies on MRSO plates. S cells prepared in the scratch broth were stable at −20°C. The data indicated that R and S cells were not significantly different in their resistance to freezing unless the scratch broth was used to propagate the cells.

DISCUSSION

Bergey's Manual of Determinative Bacteriology (20) describes homofermentative *Lactobacillus* colonies as normally R, becoming S upon laboratory transfers in the presence of Tween 80 or sodium oleate. Use of various compounds in the growth medium can induce the transition of an entire *Lactobacillus* population from one type of colonial morphology to another (21). However, it must also be recognized that heterogeneity can occur within a single popula-

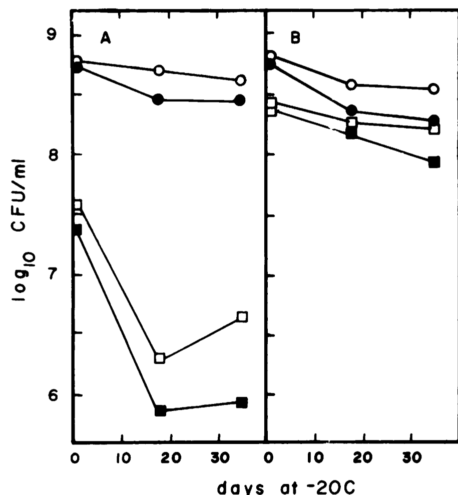

FIG. 6. *Effect of the growth medium on the freeze stability of L. acidophilus RL8K*^{R} *(squares) and RL8K*^{S} *(circles). (A) R and S cultures were grown in and plated on scratch MRS (□ and ○) and MRSO (■ and ●). (B) R and S cultures were grown in and plated on commercial MRS (□ and ○) and MRSO (■ and ●). MRSO was used to evaluate the degree of sublethal injury of surviving cells. CFU, Colony-forming units.*

tion under a single set of environmental conditions. In this study, it was observed that purified R and S colonies of *L. acidophilus* RL8K were stable on MRS agar, but dissociation would periodically occur at low levels. It is not known whether this dissociation results from phenotypic variability or represents the beginning of a gradual population transition caused by environmental stimuli. In either case, *L. acidophilus* strains may be heterogeneous during growth in MRS-based media. As a result of this heterogeneity, strains, media, and incubation conditions should be evaluated to assure homogeneous cell suspensions when studying the characteristics and function of this genus.

During this study, differences were apparent upon comparison of the level of bile resistance and freeze damage exhibited by R and S cultures. RL8K^R cells were more sensitive than RL8K^S cells to increasing concentrations of bile. The elongated cells may expose more sites susceptible to agents with the surfactant and detergent properties of bile salts. Additionally, differences in the susceptibility of RL8K^R and RL8K^S cells to freezing were manifested only after growth in scratch MRS broth. In this medium, RL8K^R cells displayed increased bleb formation and an altered rate of growth during the logarithmic phase. The reason for the differences effected by the two MRS broth media is not known. However, it is apparent that minor changes in the preparation of the growth medium can dramatically alter the multiplication and resulting characteristics of R cells.

Morphology differences in the cells of R and S *Lactobacillus* cultures included filamentous and short bacillary rods (2). Close examination of the RL8K^R cells revealed bleb-like structures protruding from the bacterial cell wall. In R cells, these structures occurred at regular intervals along the cell and could represent protrusions of the cytoplasmic membrane through the bacterial cell wall. Bleb formation in the outer membranes of *Escherichia coli* and *Salmonella typhimurium* has been reported in septation-division mutants (6, 24). These protrusions occur at the suspected sites of cell division during growth of elongated cells. In *L. acidophilus* Higgins et al. (11) observed that during autolysis of strain 63AM Gasser, gaps in the cell wall occurred at major polar and septal sites, allowing the extrusion of membrane and cytoplasm in small evaginations. By analogy, bleb formation in RL8K^R cells may occur at sites of defective cross wall formation during growth of these long, rod-shaped cells.

The freeze damage and bile sensitivity of bacterial membranes has been well established. In RL8K^R cells, bile sensitivity and freeze damage suggests that the bleb-like protrusions from the bacterial cell wall are, in fact, evaginations of the cyotplasmic membrane. This defect in the cellular structure may be inherent to R cells due to the growth of elongated cells that is characteristic of this colony type. Bleb formation was rarely observed in RL8K^S cells. Previous reports have addressed the more resistant state of S *Lactobacillus* colonies to environmental stress (2). Our results substantiate the resistant state of bacillary or S cells, but only on the basis that S cells are less sensitive to alterations in the growth environment that may selectively affect R cells and render them susceptible to stress.

The roles of *L. acidophilus* in the intestinal tracts of humans and animals are not well defined. In the selection of strains for use in defining these roles, it is important that the bacterium survive and establish itself under the conditions encountered in the intestinal environment. Successful entrance of *L. acidopilus* into the intestinal tract would be dependent on a physiological state of the organism that is compatible with the environment. Curran et al. (3) found that 73% of known intestinal isolates of *Lactobacillus* exhibit the X or R colonial morphology and suggested that the R colony is characteristic of these strains. Whether R colonies represent the morphological state of the lactobacilli found within the intestinal environment is unknown.

However, all the available evidence suggests that in vitro filamentous growth of the lactobacilli results from growth conditions that are not supportive of proper cell multiplication (4, 12–14). Bleb formation, bile sensitivity, and freeze damage in $RL8K^R$ cells support this conclusion. On this basis, it appears that the media and environmental conditions employed during the isolation and propagation of intestinal lactobacilli may be somewhat inappropriate. Heterogeneity of *Lactobacillus* populations resulting in R, S, or intermediate dissociants may reflect adaptive tendencies of the cells either to the laboratory environment ($R \rightarrow S$) or to another physiological state compatible with the intestinal environment ($S \rightarrow ?$). For this latter tendency, however, the environment established for the growth of the bacterium may be inadequate, resulting in the appearance of R colonies under the standard laboratory conditions. Therefore, although the S type is more stable in vitro, it is unclear whether this type represents the in vivo form of the intestinal lactobacilli or a form that is incompatible with the intestinal environment that has adapted to laboratory conditions.

ACKNOWLEDGMENT

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