APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1983, p. 785–792
0099-2240/83/100785-08$02.00/0
Copyright © 1983, American Society for Microbiology
Vol. 46, No. 4

Influence of Calcium and Manganese on Dechaining of *Lactobacillus bulgaricus*†

CATHERINE T. WRIGHT AND TODD R. KLAENHAMMER*
Department of Food Science, North Carolina State University, Raleigh, North Carolina 27650

Received 10 June 1983/Accepted 29 July 1983

The events responsible for the transition of *Lactobacillus bulgaricus* 1243-F from long filamentous chains to short bacilloid rods were examined in a cation-depleted liquid medium. In the presence of magnesium only, cells grew as long chains of unseparated cells. The addition of 100 μM to 1 mM calcium or manganese to this medium resulted in the dechaining of these cells to short bacilloid rods. Fe^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} failed to induce dechaining. Induction of calcium and manganese dechaining functioned under controlled pH maintained at 5.0 and 6.0 but not at pH 7.0. This was consistent with a previous report showing failure in synthesis of dechaining enzymes by *L. bulgaricus* under pH conditions approaching alkalinity (S. K. Rhee and M. Y. Pack, J. Bacteriol. **144**:865–868, 1980). We conclude that under pH conditions which permit synthesis of dechaining enzymes, calcium and manganese are necessary for dechaining activity.

Lactobacillus bulgaricus is a gram-positive, thermophilic microorganism used in the manufacture of numerous dairy products, including Swiss cheese, mozarella cheese, and yogurt. Within designated strains of *L. bulgaricus*, diverse colonial variants exist (1). Morphological variants of the lactobacilli have been shown to display different susceptibilities to environmental stress. In general, lactobacilli possessing short bacilloid rod morphologies exhibit superior survival during frozen storage over filamentous forms of these organisms (12). In a previous study (19), we isolated two morphological variants from a parent culture of *L. bulgaricus* 1243. The first isolate, designated *L. bulgaricus* 1243-F, was rough and translucent on agar plates. Gram stains of *L. bulgaricus* 1243-F grown in a basal broth medium revealed that this organism grew as rods which existed singly or in pairs. The second isolate, *L. bulgaricus* 1243-O, was opaque and smooth on agar plates. In broth culture, this organism existed in chains, often exceeding 40 cells per chain, which exhibited clumping during growth in broth media. Although morphology did not influence the susceptibility of either variant to death during frozen storage, growth in media supplemented with calcium before freezing prevented death in both isolates. However, the role of calcium in promoting cellular stability was unknown. It was also unclear why these variants displayed such diverse morphologies.

Divalent cations have been reported to mediate cell division and separation during cellular growth (6, 13). Calcium was found to enhance cell division in a species of *Erwinia* and was responsible for the pleomorphic transition of *Lactobacillus bifidus* from branched to bacilloid forms. Autolytic enzymes also function to mediate cell division in bacteria. Under appropriate conditions, these lytic enzymes hydrolyze specific bonds in the cell wall peptidoglycan, permitting cell surface growth and cell division (9). Most bacterial species studied appear to have a variety of specificity in their autolytic enzyme activities. Of these lytic activities, dechaining enzymes were reported to be involved in the separation of *Bacillus subtilis* and *Diplococcus pneumoniae* (4, 7, 10). Purification of the dechaining activity from these organisms revealed that *N*-acetylmuramyl-L-alanine amidases were the lytic enzymes responsible for dechaining. *N*-Acetylmuramyl-L-alanine amidases isolated from *B. subtilis* required calcium, magnesium, and manganese for maximum activity (7).

The present study was conducted to examine the influence of calcium on the growth and cellular morphology of two colonial variants of *L. bulgaricus* 1243. The influence of environmental pH during growth in the presence of calcium was also examined to further understand the influence of this parameter on the cellular morphology of *L. bulgaricus*. Previous evidence (15) has shown that environmental pH

† Paper number 8918 of the journal series of the North Carolina Agricultural Research Service, Raleigh, NC 27650.

dramatically influences the chain length of *L. bulgaricus* NLS-4. We present evidence that defines a requirement for calcium or manganese in dechaining of *L. bulgaricus* 1243 to short bacilloid rods.

MATERIALS AND METHODS

Organism. *L. bulgaricus* 1243 and its homologous phage were obtained from the National Collection of Dairy Organisms, National Institute for Research in Dairying, Shinfield, Reading, England. Morphological variants of this organism were isolated and stored as described previously (19). Both isolates displayed equal sensitivity to lysis by the homologous phage.

Growth medium. Basal broth used in these studies has been described previously (19) and consisted of yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 5.0 g; beef extract (Difco Laboratories, Detroit, Mich.), 10.0 g; Proteose Peptone no. 3 (Difco), 10.0 g; dextrose, 20.0 g; and Tween 80 (Fisher Scientific Corp., Raleigh, N.C.), 1.0 g in 1 liter of distilled water. The pH of the medium was adjusted to 6.5 with 12 N HCl before autoclaving (121°C for 15 min). To remove divalent mineral ions, basal broth was exchanged through Chelex 100 cation-exchange resin (Na^+ form; 200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.) at 25°C in a Pyrex column (66 by 4.5 mm inner diameter) at a flow rate of 10 ml/min. After medium exchange, the resin was regenerated to the Na^+ form in the following sequence: 2 bed volumes of 1 N HCl, 5 bed volumes of distilled water, and 4 bed volumes of 0.5 M sodium acetate buffer (pH 6.25). Exchanged medium was sterilized by autoclaving at 121°C for 15 min. Mineral analysis of the medium was performed by using a Perkin-Elmer Atomic Absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) according to the manufacturer's recommendations for sample treatments. $CuSO_4 \cdot 5H_2O$, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and chlorides of Ca^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} were supplemented to Chelex-exchanged basal broth (CEB) to yield divalent cation concentrations ranging from 1 μM to 100 mM.

Growth studies. Spectrophotometric growth studies were conducted at 650 nm with a Bausch & Lomb Spectronic 70. Cultures had been propagated from frozen stock and transferred twice through basal broth at 37°C before use. Cells from 10-ml cultures were harvested by centrifugation at 10,400 × *g* for 10 min and resuspended in 2 ml of Chelex-exchanged medium. Sterile matched cuvettes containing 10 ml of divalent cation-supplemented basal broth were inoculated to an initial optical density of approximately 0.05 at 650 nm. Cultures were incubated at 37°C, and the optical density at 650 nm was monitored at hourly intervals.

Growth studies under controlled pH. For growth studies under controlled pH, a 500-ml final working volume of Chelex-exchanged medium was placed in a sterile fermentor connected to a New Brunswick automatic pH controller (New Brunswick Scientific Co., Edison, N.J.) maintained at 37°C with continuous agitation. For media at pH 4.0, 5.0, and 6.0, the initial pH was adjusted with sterile 85% lactic acid (Fisher). A neutralizer consisting of 20% Na_2CO_3 and 20% NH_4OH was used to initially adjust media to pH 7.0, and this neutralizer was used to maintain the pH in all fermentor studies. Growth was monitored at hourly intervals by agar plate count on Difco MRS agar. Chains and clumps of cells were disrupted by mild sonication and then plated by using the Spiral Plate Technique (Spiral Systems, Inc., Bethesda, Md.) as described previously (19).

RESULTS

Growth studies. Growth and cellular morphology of *L. bulgaricus* 1243-F and 1243-O were monitored in CEB supplemented with various concentrations of divalent mineral ions. Without mineral supplementation, CEB failed to support the growth of either isolate (Fig. 1 and 2).

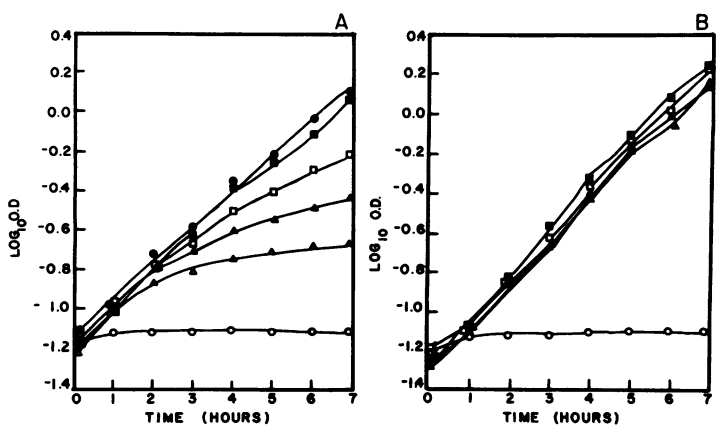

FIG. 1. Growth response of *L. bulgaricus* 1243-F to magnesium and calcium. (A) CEB supplemented with 1 mM Mg^{2+} (△), 5 mM Mg^{2+} (▲), 10 mM Mg^{2+} (□), 20 mM Mg^{2+} (■), and 30 mM Mg^{2+} (●). (B) CEB containing 30 mM Mg^{2+} supplemented with 1 μM Ca^{2+} (△), 10 μM Ca^{2+} (▲), 100 μM Ca^{2+} (□), and 1 mM Ca^{2+} (■). ○, CEB. Mg^{2+} and Ca^{2+} were added as solutions of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$. O.D., Optical density.

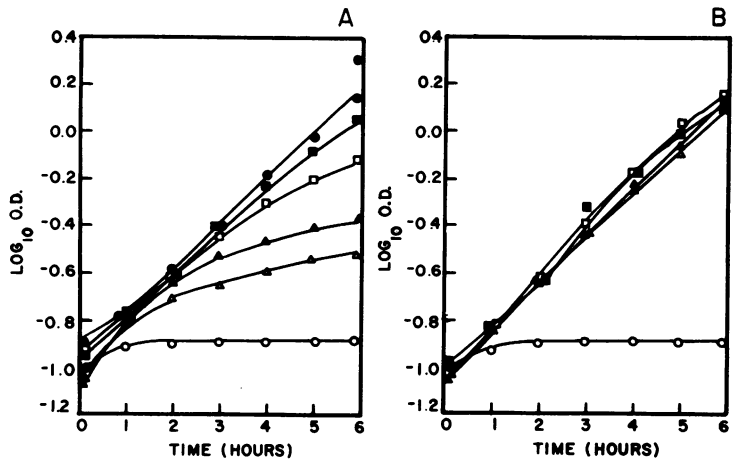

FIG. 2. Growth response of *L. bulgaricus* 1243-O to magnesium and calcium. (A) CEB supplemented with 1 mM Mg^{2+} (△), 5 mM Mg^{2+} (▲), 10 mM Mg^{2+} (□), 20 mM Mg^{2+} (■), and 30 mM Mg^{2+} (●). (B) CEB containing 30 mM Mg^{2+} supplemented with 1 μM Ca^{2+} (△), 10 μM Ca^{2+} (▲), 100 μM Ca^{2+} (□), and 1 mM Ca^{2+} (■). ○, CEB. Mg^{2+} and Ca^{2+} were added as solutions of $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$. O.D., Optical density.

Supplementation of this medium with magnesium resulted in a growth response proportional to the concentration of magnesium used. Levels of 30 mM magnesium were optimal for the growth of these isolates, and both isolates displayed identical growth responses (Fig. 1A and 2A). CEB containing 30 mM magnesium (CEB-Mg^{2+}) was used throughout this study. Morphologically, *L. bulgaricus* 1243-F and 1243-O grown in CEB-Mg^{2+} were indistinguishable and existed in long filamentous chains. Cells grown in CEB-Mg^{2+} exhibited clumping.

Neither morphology nor growth response was affected by the addition of various concentrations (1 μM to 10 mM) of Fe^{2+}, Cu^{2+}, Co^{2+}, or Zn^{2+} to CEB-Mg^{2+} (data not shown). The addition of calcium at levels ranging from 1 μM to 1 mM to CEB-Mg^{2+} did not affect growth rate (Fig. 1B and 2B); however, a morphological response was observed. Increasing the calcium concentration of the growth medium resulted in the transition of *L. bulgaricus* 1243-F from long filamentous chains to short bacilloid rods (Fig. 3). At calcium concentrations exceeding 1 mM, precipitation was observed in culture tubes. Under conditions where calcium remained soluble, 1 mM calcium facilitated maximum dechaining in this organism. Cells which exhibited clumping when grown in CEB-Mg^{2+} displayed even turbidity throughout culture tubes when the medium contained 100 μM to 1 mM calcium. Unlike *L. bulgaricus* 1243-F, *L. bulgaricus* 1243-O failed to respond morphologically to the addition of calcium to CEB-Mg^{2+} and remained in long chains (data not shown).

Manganese was also found to exert a similar morphological effect when added to CEB-Mg^{2+} at concentrations identical to those of calcium. Growth rate remained unaffected, and, as observed with calcium, manganese concentrations

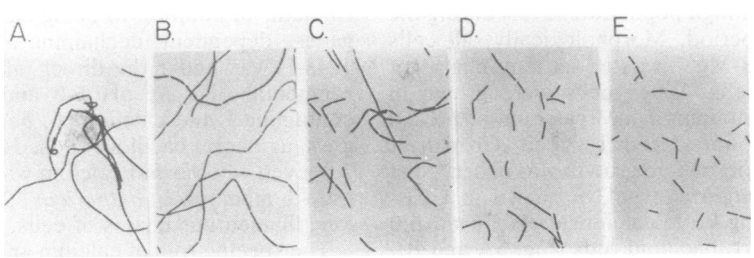

FIG. 3. Morphological response of *L. bulgaricus* 1243-F to calcium. Photomicrographs were prepared from Gram stains of 6-h cultures propagated in CEB supplemented with (A) 30 mM Mg^{2+}, (B) 30 mM Mg^{2+} and 1 μM Ca^{2+}, (C) 30 mM Mg^{2+} and 10 μM Ca^{2+}, (D) 30 mM Mg^{2+} and 100 μM Ca^{2+}, and (E) 30 mM Mg^{2+} and 1 mM Ca^{2+}. ×100.

TABLE 1. Magnesium, manganese, and calcium content of basal broth

Medium	μM of (mean value ± SD):		
	Mg^{2+}	Mn^{2+}	Ca^{2+}
Unexchanged	264.4 ± 24.7[a]	1.5 ± 0.0[b]	93.4 ± 9.4[a]
Exchanged	0.65 ± 0.64[c]	1.2 ± 0.15[d]	4.1 ± 2.7[c]

[a] Five analyses.
[b] Two analyses.
[c] Forty-seven analyses.
[d] Eighteen analyses.

of 100 μM to 1 mM facilitated dechaining of *L. bulgaricus* 1243-F (data not shown). *L. bulgaricus* 1243-O again failed to respond morphologically to the addition of manganese to the growth medium.

Mineral content of basal broth medium. Atomic absorption analysis of basal broth and CEB was performed to determine levels of magnesium, manganese, and calcium in the growth media (Table 1). Magnesium and calcium were present in high concentrations in unexchanged medium, and levels of these ions were substantially reduced by Chelex exchange. Although detectable levels of calcium remained in the exchanged medium, these levels were not sufficient to induce a morphological response in *L. bulgaricus* 1243-F, as complete dechaining did not occur until 100 μM to 1 mM calcium was present in CEB-Mg^{2+}. Manganese, which was present at extremely low concentrations in unexchanged medium, was not further reduced by Chelex exchange.

pH effects. The influence of environmental pH on the growth response and morphology of *L. bulgaricus* 1243-F was examined. Cells were grown at divalent cation concentrations known to inhibit dechaining (magnesium only) and those which facilitated dechaining (1 mM Ca^{2+} or 1 mM Mn^{2+}); environmental pH was varied between 4.0 and 7.0. When *L. bulgaricus* 1243-F was grown in CEB-Mg^{2+}, poor growth was observed at pH 4.0, 5.0, and 7.0 (Fig. 4A, B, and D). At pH 6.0 (Fig. 4C), cells grown in CEB-Mg^{2+} achieved high population levels during the 12-h growth period. Morphologically, all cells grown in CEB-Mg^{2+} existed as long chains of unseparated cells. When cells were grown in CEB-Mg^{2+} containing 1 mM calcium, at pH 5.0 and 6.0, luxuriant growth resulted. Growth at pH 7.0 was poor, and no growth was observed at pH 4.0. *L. bulgaricus* 1243-F grown in CEB-Mg^{2+} containing 1 mM calcium at pH 5.0 and 6.0 existed as short, bacilloid rods (Fig. 5A and B). When cells were grown at pH 7.0, however, even in the presence of 1 mM calcium, long filamentous growth of *L. bulgaricus* 1243-F was observed (Fig. 5C).

In CEB-Mg^{2+} containing 1 mM manganese, growth at pH 5.0, 6.0, and 7.0 approximated the growth response observed with 1 mM calcium (data not shown). However, under controlled pH, manganese was not as efficient in facilitating dechaining of *L. bulgaricus* 1243-F as was calcium. At pH 6.0, in medium containing 1 mM manganese, cells existed in short chains of rods (Fig. 6). Again, as observed with cells grown in medium containing 1 mM calcium at pH 7.0, even in the presence of 1 mM manganese, long filamentous growth of *L. bulgaricus* 1243-F was observed.

Appearance of membrane protrusions. During the growth of *L. bulgaricus* 1243-F in medium containing low levels of calcium or manganese, cells existed as long chains of connected cells. Concomitant with the failure of separation by these cells under reduced concentrations of mineral ions, spherical protrusions were observed at regular, discreet intervals on the cell surface. These evaginations appeared to be portions of the cell membrane, as they existed as nonstainable particles when cells were stained with Gram stain (Fig. 7). When cells were propagated in the presence of increased levels of calcium (1 mM), membrane protrusions could not be observed.

DISCUSSION

In this study, we examined the influence of calcium and other divalent cations on the growth and cellular morphology of two colonial variants of *L. bulgaricus* 1243. The bacilloid rod morphology exhibited by *L. bulgaricus* 1243-F was under the direct influence of the calcium content of the growth medium. When *L. bulgaricus* 1243-F was grown in a divalent cation-depleted liquid medium which contained magnesium only, this organism existed as long chains of unseparated cells. The addition of 1 mM calcium to this medium facilitated dechaining of *L. bulgaricus* 1243-F to short bacilloid rods. Of other divalent cations examined, Fe^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} failed to induce dechaining. In static culture, manganese was found to facilitate dechaining at concentrations identical to those of calcium. In addition to being calcium and manganese dependent, dechaining of *L. bulgaricus* 1243-F was under the direct influence of environmental pH. At pH 5.0 and 6.0 in media containing 1 mM calcium, *L. bulgaricus* 1243-F grew as short, bacilloid rods. However, at pH 7.0, even when 1 mM calcium was present in the growth medium, *L. bulgaricus* 1243-F existed as long filamentous chains of cells.

The specific role of calcium and manganese in promoting dechaining is unclear. Dechaining in bacterial cells is an enzymatic function, and, to function as such, this activity must be highly regulated during cellular growth and division.

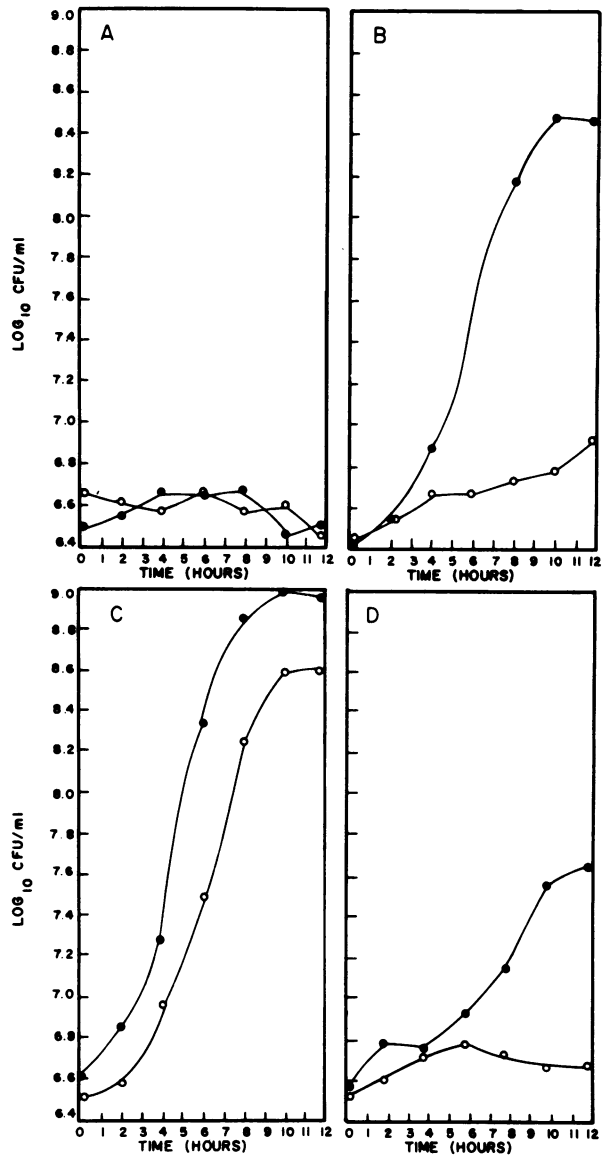

FIG. 4. Influence of environmental pH on the growth response of *L. bulgaricus* 1243-F. (A) pH 4.0; (B) pH 5.0; (C) pH 6.0; (D) pH 7.0. ○, CEB containing 30 mM Mg^{2+}; ●, CEB containing 30 mM Mg^{2+} and 1 mM Ca^{2+}. Counts are expressed as $\log_{10}$ CFU per milliliter on MRS agar.

For dechaining to occur, environmental conditions must be established whereby dechaining enzyme synthesis occurs (15); enzymes must be bound to suitable sites, namely, teichoic acids, in the cell wall (8); divalent cations, specifically manganese and calcium, must be present (7). It is apparent from this study that calcium and manganese are required for dechaining of *L. bulgaricus* 1243-F. However, whether these divalent cations affect the activity or binding of dechaining enzymes was not determined. Mutants which filament due to a failure of cells to separate after cell division have been described in a number of bacterial systems (2, 5, 18). Often these mutants either lack lytic activities responsible for cell separation or are deficient in teichoic acid-binding sites for dechaining enzymes (8, 10). *L. bulgaricus* 1243-O failed to dechain under all growth conditions, including supplementation with calcium or manganese. This suggests that 1243-O was deficient in dechaining enzymes or appropriate binding sites for the enzymes and thus remains unresponsive to cations which may promote dechaining activities.

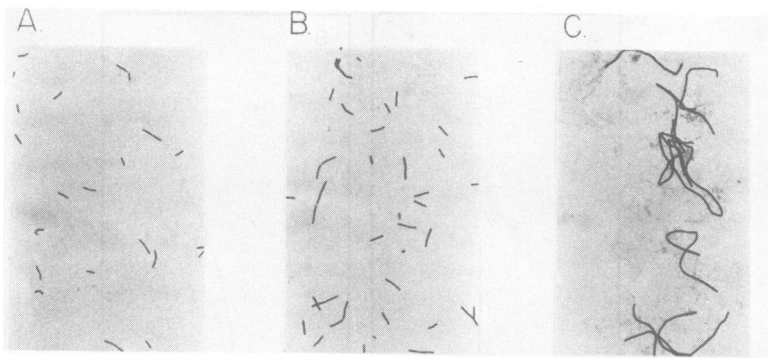

FIG. 5. Morphological response of *L. bulgaricus* 1243-F to environmental pH. Photomicrographs were prepared from Gram stains of 6-h cultures propagated in CEB containing 30 mM Mg^{2+} and 1 mM Ca^{2+}. (A) pH 5.0; (B) pH 6.0; (C) pH 7.0. ×100.

When morphological variants of *L. bulgaricus* 1243 were first isolated (19), *L. bulgaricus* 1243-F existed as short, bacilloid rods when propagated through basal broth medium. Examination of the cation content of this medium indicated that calcium levels would be sufficient to permit dechaining and the consequent morphological effects. It is interesting that basal broth contains little manganese and suggests that in basal broth medium, calcium provided for the main dechaining activity. The fact that these organisms demonstrated dechaining activity in the presence of manganese or calcium may be a function of their ecological niche. Lactobacilli are commonly isolated from milk and fermenting green plant material, which are rich sources of calcium and manganese, respectively.

Previous reports have suggested that the morphological state of the bacterial cell has a profound influence on cell resistance to freezing death and injury (12). Examination of a freeze-sensitive cell type of *Lactobacillus acidophilus* RL8K revealed blebs protruding from the cell wall at regular intervals. It was proposed that these blebs represent membrane evaginations through the cell wall and are thus susceptible targets for structural damage during freezing. Detection of membrane evaginations or blebs have been reported previously for lactobacilli (11). In two reports, appearance of these struc-

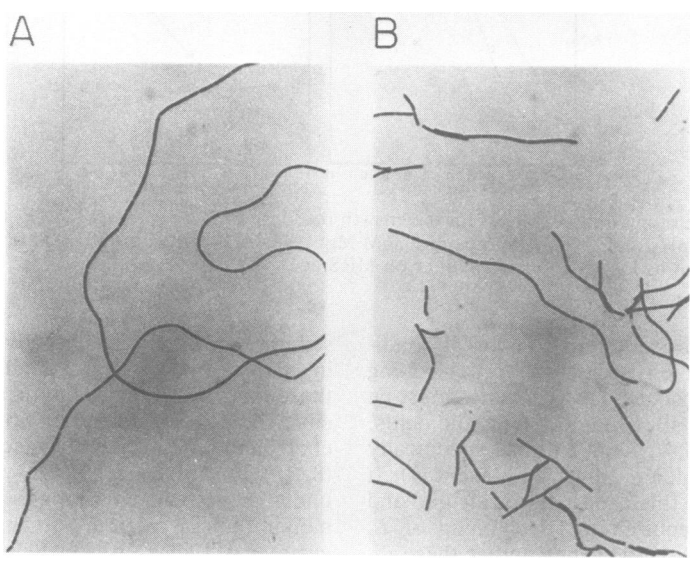

FIG. 6. Morphological response of *L. bulgaricus* 1243-F to manganese. Photomicrographs were prepared from Gram stains of 6-h cultures propagated in CEB (pH 6.0) containing (A) 30 mM Mg^{2+}, (B) 30 mM Mg^{2+} and 1 mM Mn^{2+}. ×100.

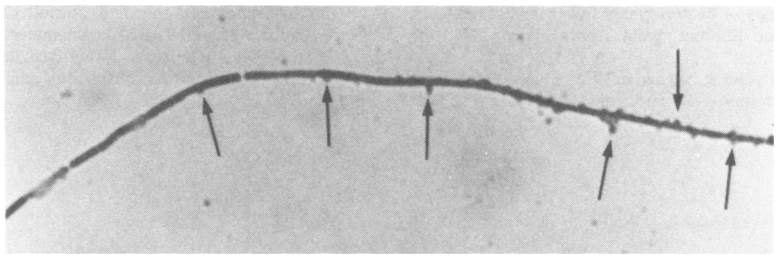

FIG. 7. Photomicrograph of *L. bulgaricus* 1243-F cells with blebs (indicated by arrows) after 5 h of growth at 37°C in CEB containing 30 mM Mg^{2+}. Ten percent sucrose was added to the growth medium to stabilize these structures. Cells were stained with Gram stain. ×100.

tures was attributed to cell wall damage via penicillin disruption of peptidoglycan cross-linking (16) and autolysin activity (9). In the present study, cells propagated in a medium containing low levels of calcium showed bleb-like protrusions from the cell wall. The addition of calcium to the medium eliminated bleb formation. Noting the demonstrated ability of divalent cations to retard autolysin activity (3) and the correlation of autolysis with bleb formation (9), it appears probable that calcium may act to minimize autolysin-induced wall damage in *L. bulgaricus* 1243-F. Furthermore, *L. bulgaricus* 1243 grown in the presence of calcium is more resistant to freezing and freeze-drying than cells propagated in the absence of calcium (19). Apparently, calcium and other divalent cations which play a role in the growth and cell assembly of *L. bulgaricus* 1243-F ultimately influence the freeze stability of this organism.

Although the use of *L. bulgaricus* in the food and dairy industry is widespread, the nutritional requirements of this organism are poorly understood. Previously, no role has been ascribed to calcium for this organism, other than its necessity for bacteriophage adsorption (17). In the preparation of culture concentrates of the lactobacilli, the bacilloid rod morphology is desirable in that those cells are easily centrifuged and concentrated. Cells which exist in long filamentous chains exhibit a clumping phenomenon, similar to the agglutinin phenomenon (14), making their use unsuitable for milk fermentations. The necessity of magnesium, calcium, and manganese for the growth and proper cell assembly of *L. bulgaricus* cannot be overlooked and raises concern over current methods of *L. bulgaricus* culture preparation and propagation, in which phosphated media are employed.

ACKNOWLEDGMENTS

We thank Wayne P. Robarge and Beverly Johnson of the Soil Science Analytical Laboratory, North Carolina State University, for conducting the mineral analysis by atomic absorption.

C.T.W. was supported by a research fellowship grant from the Johnson Wax Fund, Inc., Racine, Wis. This work was also supported in part by the North Carolina Dairy Foundation.

LITERATURE CITED

1. **Barber, F. W., and W. C. Frazier.** 1945. Dissociants of lactobacilli. J. Bacteriol. **50**:637–649.
2. **Chatterjee, A. N., W. Wong, F. E. Young, and R. W. Gilpin.** 1976. Isolation and characterization of a mutant of *Staphylococcus aureus* deficient in autolytic activity. J. Bacteriol. **125**:961–967.
3. **Coyette, J., and G. D. Shockman.** 1973. Some properties of the autolytic *N*-acetylmuramidase of *Lactobacillus acidophilus*. J. Bacteriol. **114**:34–41.
4. **Fan, D. P.** 1970. Autolysin(s) of *Bacillus subtilis* as dechaining enzyme. J. Bacteriol. **103**:494–499.
5. **Forsberg, C. W., and H. J. Rogers.** 1974. Characterization of *Bacillus licheniformis* 6346 mutants which have altered lytic enzyme activities. J. Bacteriol. **118**:358–368.
6. **Grula, E. A., and M. M. Grula.** 1962. Cell division in a species of *Erwinia*. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin, and ultraviolet light. J. Bacteriol. **83**:981–988.
7. **Herbold, D. R., and L. Glaser.** 1975. *Bacillus subtilis* N-acetylmuramic acid L-alanine amidase. J. Biol. Chem. **250**:1676–1682.
8. **Herbold, D. R., and L. Glaser.** 1975. Interaction of N-acetylmuramic acid L-alanine amidase with cell wall polymers. J. Biol. Chem. **250**:7231–7238.
9. **Higgins, M. L., J. Coyette, and G. D. Shockman.** 1973. Sites of cellular autolysis in *Lactobacillus acidophilus*. J. Bacteriol. **116**:1375–1382.
10. **Holtje, J.-V., and A. Tomasz.** 1975. Specific recognition of choline residues in the cell wall teichoic acid by the N-acetylmuramyl-L-alanine amidase of pneumococcus. J. Biol. Chem. **250**:6072–6076.
11. **Hurst, A., and J. M. Stubbs.** 1969. Electron microscopic study of membranes and walls of bacteria and changes occurring during growth initiation. J. Bacteriol. **97**:1466–1479.
12. **Klaenhammer, T. R., and E. G. Kleeman.** 1981. Growth characteristics, bile sensitivity, and freeze damage in colonial variants of *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **41**:1461–1467.
13. **Kojima, M., S. Suda, S. Motta, K. Hamada, and A. Suganuma.** 1970. Necessity of calcium ion for cell division in *Lactobacillus bifidus*. J. Bacteriol. **104**:1010–1013.
14. **Lawrence, R. C., T. D. Thomas, and B. E. Terzaghi.** 1976. Reviews of the progress of dairy science: cheese starters. J. Dairy Res. **43**:141–193.
15. **Rhee, S. K., and M. Y. Pack.** 1980. Effect of environmental pH on chain length of *Lactobacillus bulgaricus*. J. Bacteriol. **144**:865–868.
16. **Rogosa, M.** 1970. Characters used in the classification of lactobacilli. Int. J. Syst. Bacteriol. **20**:519–533.
17. **Sozzi, T., R. Maret, and J. M. Poulin.** 1976. Study of

plating efficiency of bacteriophages of thermophilic lactic acid bacteria on different media. Appl. Environ. Microbiol. **32**:131–137.

18. **Wolf-Watz, M., and S. Normark.** 1976. Evidence for a role of *N*-acetylmuramyl-L-alanine amidase in septum separation in *Escherichia coli*. J. Bacteriol. **128**:580–586.
19. **Wright, C. T., and T. R. Klaenhammer.** 1983. Survival of *Lactobacillus bulgaricus* during freezing and freeze-drying after growth in the presence of calcium. J. Food Sci. **48**:773–777.