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Changes in the Surface Potential of *Lactobacillus acidophilus* under Freeze–Thawing Stress

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The zeta potential of *Lactobacillus acidophilus* CRL 640, a measure of the net distribution of electrical charges on the bacterial surface, is a function of the glucose concentration in the growing media. With 2% glucose, cells in the stationary phase showed a zeta potential of -45 ± 2 mV. With these cells, the zeta potential after freezing and thawing decreased to -32 ± 2 mV and there was a decrease in viability. The changes in the surface potential correlated with damage to the cell surface as shown by electron microscopy. Freeze–thawed cells incubated in a rich medium recovered a zeta potential of -38 ± 2 mV without cell growth. *L. acidophilus* CRL 640 showed the same value of surface potential as control cells when they were frozen and thawed in 2 M glycerol.

Key Words: Lactobacillus acidophilus; freeze–thawing; surface potential.

Lactobacillus acidophilus is a thermophilic lactic acid bacterium used on a worldwide scale for the elaboration of probiotic fermented milks. The beneficial effects of *L. acidophilus* are related to its ability to adhere to the small intestines of humans and animals (9). These adhesion properties of the bacteria are related to their surface properties, such as hydrophobicity and surface potential, to which specific structures may contribute (1, 11). However, the high sensitivity of this microorganism to very low temperatures, resulting in structural and physiological injury, makes it difficult to preserve (2, 7).

In spite of this, freezing is commonly used for the preservation and storage of lactic acid bacteria for the production of concentrated starter cultures for the food industry (4). The thermal, dehydration, and osmotic stresses involved in the freezing and thawing process can drastically alter the bacterial surface. For these reasons, it would be of interest to determine the state of the bacterial surface after cryogenic stress and the possibility of controlling such changes by the addition of cryoprotectants.

Zeta potential is defined as the difference in electrical potential between the surface of the bacterium and the bulk surrounding medium. It is a measure of the net distribution of electrical charge on the surface of the bacterium. In this paper we have measured the zeta potential of a strain of *L. acidophilus* CRL 640 grown in different concentrations of glucose and the changes that follow freeze–thaw treatment in the presence or the absence of glycerol. To do this we have determined the electrophoretic mobility of *L. acidophilus* CRL 640 bacteria throughout the growth curve and after the freeze–thaw stress. The changes in zeta potential were correlated with the number of surviving or damaged cells at each glycerol concentration.

MATERIALS AND METHODS

Microorganism, Media, and Growth Conditions

L. acidophilus CRL 640 was obtained from the CERELA stock culture collection and was

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originally isolated from fermented milk. The cultures were kept at −20°C in 10% nonfat skim milk (NFSM) supplemented with 0.5% (w/v) yeast extract and 0.5% (w/v) glycerol.

The growth medium was MRS broth (5) containing 0.1 or 2% glucose. The cultures were subcultured at least three times before use. Bacterial growth in MRS broth at 37°C for 24 h was measured by turbidimetry at 560 nm (OD_{560}) with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, NY). Cells were collected by centrifugation at 5000*g* for 20 min, washed twice in Milli Q water, and finally resuspended in 1 mM NaCl solution for the measurement of electrophoretic mobility.

Freezing and Thawing Procedures

Cells grown in MRS (2% glucose) for 16 h at 37°C were harvested by centrifugation at 5000*g* for 20 min at 10°C and resuspended in 0.85% (v/v) NaCl at a cell concentration of 2×10^9 colony-forming units per milliliter (CFU/ml). Aliquots of this cell suspension were added to 0, 0.5, 1.0, 1.5, and 2.0 M glycerol solutions at 20°C and allowed to equilibrate for 10 min prior to freezing. Five-milliliter aliquots of cell suspensions were placed in glass borosilicate tubes (15 × 1.5 cm), frozen at −20°C, and stored at the same temperature for 24 h. The samples were thawed at 37°C for 5 min in a circulating water bath. Unfrozen samples were used as controls.

Determination of Dead and Damaged Cells

The viability of control and freeze–thawed samples was determined by the plate dilution method. Cell suspensions were serially diluted and aliquots were pour-plated in MRS agar and in MRS containing 2% NaCl (MRSNa). The plates were incubated at 37°C for 72 h and the resulting colonies were counted. The difference in colony counts in MRS agar (rich medium) before and after freeze–thaw treatment was used to calculate the number of dead cells (14). The difference between the number of CFU/ml in MRS and the number in MRSNa (selective medium) after freeze–thaw treatment was assumed to measure the number of damaged cells among the survivors (7). The results were expressed in percentages (%).

Nucleic Acid and Protein Determination

Samples were taken before and after the freeze/thaw procedure and filtered through 0.22-μm Bedford Millipore membranes. The clarified supernatant fluid was measured at OD_{260} and OD_{280} to determine the presence of nucleic acid and proteins, respectively (3).

Electrophoretic Mobility and Zeta Potential

Electrophoretic mobilities of bacteria (μ) resuspended in 1 mM NaCl were determined in a capillary H-cell with Ag/AgCl electrodes. The electrodes were connected to a direct current source at 40 V. Measurements were obtained by alternately changing the polarity of the electrodes to avoid polarization. Cells were collected by centrifugation at 5000*g* for 20 min, washed twice in Milli Q water and finally resuspended in 1 mM NaCl solution for the measurement of electrophoretic mobility. The bacterial concentration was around 2×10^7 cell/ml. The electrophoretic mobility (μ) was determined by measuring the rate of migration of the bacteria in the stationary layer when a constant electric field was applied. The effective electrical distance was calculated by using NaCl solutions of known conductivity at 25°C. Conditions were standardized by determining the zeta potential (ξ) of phosphatidylserine liposomes, which is −120 mV in 1mM NaCl at pH 7.4. The rate of migration was determined by microscopic observation of the displacement of individual cells that showed rectilinear and uniform movement along a reticular lattice (length 1 mm). The results reported here are the means and standard deviations of at least 10 determinations in each direction made with different cells. The temperature was maintained at 25°C. The zeta potential was calculated by the equation of Smoluchowski (8),

$$\xi = \eta\mu/\varepsilon.\ \varepsilon_o,$$

where η is the viscosity, ε_o is the permittivity of a vacuum, and ε. is the relative permittivity (dielectric constant) of the solution. The experimental reproducibility of all the zeta potential values reported is ± 2 mV.

Transmission Electron Microscopy (TEM)

Cell suspensions in 0.85% NaCl before and after freeze–thaw treatment were harvested by centrifugation at 5000*g* for 20 min, washed once with 0.1 M sodium phosphate buffer, pH 7.4, and resuspended (1:1 by volume) in the same buffer containing 2.5% (v/v) glutaraldehyde and 1% (w/v) $CaCl_2$. Samples were kept overnight at 4°C and were then embedded in agar. Secondary fixation was with 1% OsO_4 and uranyl acetate (1:1 by volume) for 2 h at room temperature (20°C), followed by dehydration in an ethanol series and embedding in durcupan. Staining was performed according to methods described by Reynolds (13). Thin sections were examined by transmission electron microscopy (EM 109 microscope, Zeiss, Koln, Germany).

RESULTS AND DISCUSSION

Growth and Zeta Potential

The kinetics of growth and the time course of the zeta potential for *L. acidophilus* CRL 640 at 37°C in the presence of 2% glucose are shown in Fig. 1A. In the lag phase the zeta potential is around -27 mV. After 6 h of growth, the zeta potential is -49 mV and falls to -45 mV when cells enter in the stationary phase. The doubling time for cells grown in 2% glucose was 1.3 h (Table 1). In 0.1% glucose (data not shown) the doubling time was 2.2 h and the zeta potential changed from -23 mV in the lag phase to -35 mV in the stationary phase, i.e., when the doubling time decreases by 50 min the surface potential became 20% more negative (Table 1).

Van der Mei *et al.* (16) studied thermophilic dairy streptococci and, working with different types of sugar, reported that the zeta potential reached more negative values at higher rates of growth: the shorter the doubling time, the more negative was the value of the zeta potential in the stationary phase, independent of the way in which the growth rate varied.

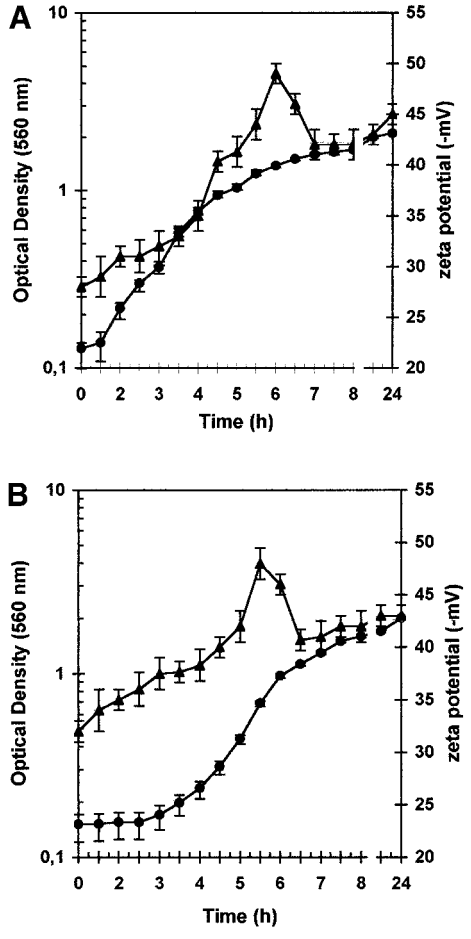

FIG. 1. Growth (●) and zeta potential (▲) of *l. acidophilus* crl 640 grown in mrs 2% glucose at 37°C (A) before and (B) after freezing and thawing.

Effects of Freezing and Thawing

After the freeze–thaw treatment, the bacterial population is composed of cells in three states: uninjured or normal cells; irreversibly injured or dead cells; and reversibly injured cells. According to Johnson *et al.* (7), the last group can be identified by means of selective agents such as NaCl, bile salts, and others; it is assumed that these cells are potentially recoverable under suitable conditions.

TABLE 1
Correlation of the Zeta Potential of *L. acidophilus* CRL 640 with Growth Parameters

	Duplication time (h)	Lag time (h)	Initial zeta potential (mV) in the lag phase	Zeta potential (mV) in the stationary phase
0.1% glucose	2.2	1	−23 ± 2	−35 ± 2
2% glucose	1.3	1	−27 ± 2	−45 ± 2
2% glucose after freeze/thawing	1.56	2.15	−32 ± 2	−43 ± 2

The extent of dead and injured cells in *L. acidophilus* CRL 640 during the freeze–thaw procedure was studied by plating the samples in a rich medium (MRS agar) to obtain total viable counts, and selective medium (MRSNa) to determine the number of injured cells. No differences in colony counts between the plating media were observed before freezing, indicating that unfrozen cells were not sensitive to NaCl. Stationary phase cells grown in 2% glucose to a concentration of 4.1×10^9 CFU/ml showed a zeta potential of −45 mV; when they were frozen and thawed the zeta potential decreased to −32 mV and the number of viable cells fell to 3.1×10^7 CFU/ml. TEM examination of the cell wall of unfrozen *L. acidophilus* CRL 640 (Fig. 2A) showed a typical bilaminar structure with a more electron-dense innermost layer (Fig. 2, II) probably containing peptidoglycan as has been observed in other lactobacilli (10). The other components of the cell wall would be formed by nonpeptidoglycan components such as proteins, polysaccharides, and teichoic acids (10). There was a graded increase in electron density from the inside to the outside, which gave the appearance of a second layer (Fig. 2, I). The absence of stain adjacent to the internal layer of the wall is ascribed to the presence of a cytoplasmic membrane (Fig. 2, III).

As indicated in Fig. 2B, following freezing and thawing, the cell wall showed an irregular and nonuniform structure and a decrease of zeta potential to −32 mV.

The number of colonies counted in selective medium (MRSNa) after the freezing and thawing was much lower than that obtained in MRS agar. The calculation described under Materials and Methods indicates that 98% of the cells were injured when the freeze–thaw process was done without glycerol (Fig. 3B). These cells may be recovered under appropriate conditions and it is of interest to investigate the possibility of following such recovery by observing variations in the zeta potential. To evaluate this, unfrozen samples were resuspended in rich medium (MRS broth) at 37°C. The curves of Figure 1B indicate that the zeta potential was −32 mV immediately after thawing and increased to −38 mV during the lag phase of cell growth (the first 2 h).

The doubling time of the cells after freezing and thawing was 1.56 h, reaching the stationary phase after 8 h (Table 1). During the logarithmic phase the zeta potential changed from −38 to −49 mV; this value is comparable to that observed at the beginning of the stationary phase in nonfrozen cultures (Fig. 1A).

In addition to the difference between the zeta potential in the stationary and the lag phases, there were also differences during the growth phase of control and frozen–thawed cells. The unfrozen culture showed a smooth increase in the zeta potential with time, whereas after freezing and thawing there was a sharp increase from −32 to –38 mV at a constant optical density followed by changes similar to those seen in the unfrozen cultures. This denotes that, after freezing and thawing, the cells reach a critical potential of around −38 mV before starting to grow as the unfrozen cells. The evolution of the zeta potential to more negative values under conditions in

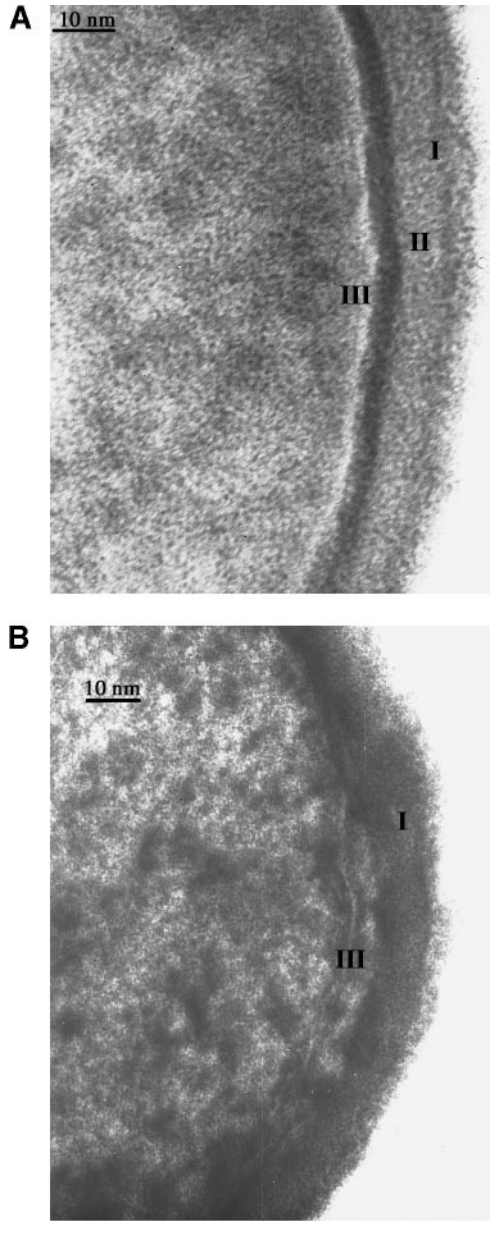

FIG. 2. Transmission electron micrographs of *L. acidophilus* CRL 640 grown in MRS 2% glucose at 37°C (A) before and (B) after freezing and thawing. Bars, 10 nm. (I, II) Bilaminar cell wall; (III) cytoplasmic membrane.

which there is no cell growth could be related to the recovery of damaged cells during the first two hours. Fernández Murga *et al.* (6) showed that the cells partially repair their damage during this period. This might be related to the presence of magnesium and phosphate in the repair medium, since these ions are required for the stability of the cell envelopes in cold-shocked bacteria (12) and for the synthesis of energy-rich compounds (15).

Cryoprotectant Effects

It is known that glycerol is an effective cryoprotectant. This molecule permeates the cell membrane rapidly, allowing equilibration in 2–3 min. *L. acidophilus* CRL 640 cells were resuspended in glycerol for 10 min to allow full equilibration before freezing and thawing. When cells were freeze–thawed in a physiological solution containing glycerol, fewer cells were killed than in controls without glycerol (Fig. 3A). When the concentration of glycerol exceeded 1 M, the number of surviving cells reached a constant value. However, to obtain a zeta potential comparable to that found in unfrozen cells, the bacteria had to be frozen and thawed in 2 M glycerol. These results suggest that, although the number of surviving cells in 1 M glycerol is as high as in 2 M glycerol, the state of the cell surface differs greatly. In other words, the zeta potential reflects other properties that are not revealed by the viability test. The zeta potential values correlate better with the proportion of damaged cells. Figure 3B shows that the percentage of damaged cells decreases as the concentration of glycerol increases. The least damage was observed with 2 M glycerol, with which a zeta potential equal to that of unfrozen cells (−45 mV) was obtained. The lack of effect on the zeta potential of cells frozen and thawed in 2 M glycerol suggests that the cell envelope is undamaged.

It is interesting to observe that over the range of glycerol concentrations in which the cell damage decreases, there is a constant loss of nucleic acid and that of protein showed only a slight change (Fig. 3C). This suggests that freezing and thawing does not involve a major change in permeability. However, the changes

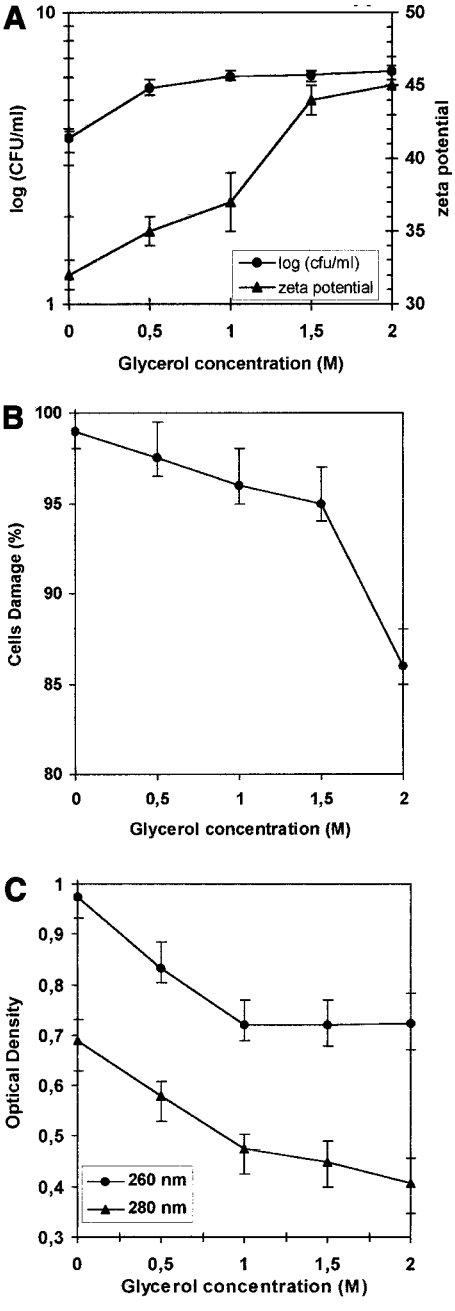

FIG. 3. Effect of glycerol concentration on (A) the number of colony-forming units (CFU/ml) and zeta potential values, (B) the percentage of damaged cells, and (C) the loss of nucleic acid (●) and proteins (▲) after freezing and thawing of *L. acidophilus* CRL 640.

in zeta potential do suggest that the cell wall is damaged and this might affect properties such as adhesion.

We suggest that changes in the zeta potential of bacteria suspended in liquid media could provide markers of cell damage under conditions in which the number of colony-forming units and the leakage of cellular material are unaffected. The correlation of the changes in the zeta potential with the changes observed in the cell surface by electron microscopy suggests that surface potential measurements could be used as a rapid method to detect surface changes in bacteria after freezing and thawing. In addition, zeta potential measurements and the development of more negative values during the growth phase could be an indicator of the recovery of cells that had been damaged by freezing and thawing.

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