Effects of High Pressure on Inactivation Kinetics and Events Related to Proton Efflux in *Lactobacillus plantarum*

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Knowledge of the mechanism of pressure-induced inactivation of microorganisms could be helpful in defining an effective, relatively mild pressure treatment as a means of decontamination, especially in combination with other physical treatments or antimicrobial agents. We have studied the effect of high pressure on *Lactobacillus plantarum* grown at pH 5.0 and 7.0. The classical inactivation kinetics were compared with a number of events related to the acid-base physiology of the cell, i.e., activity of F_0F_1 ATPase, intracellular pH, acid efflux, and intracellular ATP pool. Cells grown at pH 5.0 were more resistant to pressures of 250 MPa than were cells grown at pH 7.0. This difference in resistance may be explained by a higher F_0F_1 ATPase activity, better ability to maintain a ΔpH, or a higher acid efflux of the cells grown at pH 5.0. After pressure treatment at 250 MPa, the F_0F_1 ATPase activity was decreased, the ability to maintain a ΔpH was reduced, and the acid efflux was impaired. The ATP pool increased initially after mild pressure treatment and finally decreased after prolonged treatment. The observations on acid efflux and the ATP pool suggest that the glycolysis is affected by high pressure later than is the F_0F_1 ATPase activity. Although functions related to the membrane-bound ATPase activity were impaired, no morphological changes of the membrane could be observed.

High hydrostatic pressure offers an attractive alternative to heat pasteurization as a means of producing preservative-free, microbiologically safe and stable foods. Yeasts, molds, and vegetative cells of bacteria can be inactivated by pressures in the range of 200 to 700 MPa, and the organoleptic quality of food products is retained at these pressures (1, 8, 22). A better understanding of the effects of pressure on the cell is essential for formulation of effective pressure processes, especially when combination treatments are applied, i.e., with other physical treatments or natural antimicrobial agents. Hence, elucidation of the mechanistic aspects of high-pressure inactivation of microorganisms will help in food preservation.

Enzymes are more susceptible to pressure at low and high temperatures. The optimum temperature at which the majority of enzymes are most resistant to pressure lies between 20 and 40°C (6). Since a similar pattern has been found for pressure resistance in microorganisms (11, 24), we speculate that a target enzyme may play a role in pressure-induced inactivation of microorganisms. Furthermore, there is evidence that membranes are damaged by pressure treatment (12). The similarity between pressure-induced protein denaturation and pressure-induced inactivation of microorganisms and the observation of membrane damage at high pressure directed our attention to membrane-bound enzymes (ATPases) as a target for pressure-induced inactivation of microorganisms. The majority of ATPases have been categorized as F type (F_0F_1 ATPase), V type (vacuolar ATPase), P type (or E_1E_2 ATPase), and ABC transporters (ATP binding type ATPases). It had been shown that pressure affects the activity of ATPases in eukaryotic tissues (2, 3, 15, 19), but most of these studies addressed the P-type ATPases such as Na^+/K^+ ATPases. In lactic acid bacteria, which do not possess an electron transport chain, a pH gradient is maintained via a proton-translocating

ATPase, i.e., the F_0F_1 ATPase (7, 17, 18). Marquis and Bender (16) studied the effects of relatively low hydrostatic pressure (50 MPa) on the F_0F_1 ATPase of isolated membranes of *Enterococcus hirae* (formerly *Streptococcus faecalis*). They concluded from their experimental data that under these conditions, the proton-translocating step, and not the ATP hydrolytic step, is inhibited by pressure.

In the present study, we investigated the effects of high pressure on *Lactobacillus plantarum*, a bacterium which plays a role in food fermentation but is also a well-recognized spoilage microorganism of acidic products, such as processed tomatoes and dressings for salads. Regulation of the cytoplasmic or intracellular pH (pH_{in}) is a prerequisite for the survival and viability of lactic acid bacteria in low-pH environments such as in the food products mentioned above (20). Since the ability to generate a proton gradient is closely related to the activity of F_0F_1 ATPase, we investigated the effect of high pressure on the activity of F_0F_1 ATPase in situ before and after pressure treatment and on the ability to generate a proton gradient. Because of the relationship between proton efflux and proton gradient, we also studied the effect of pressure on acid efflux. Furthermore, the intracellular ATP amount before and after pressure treatment was measured. Transmission electron microscopy was performed to determine whether morphological changes of the membrane, e.g., separation of the cell membrane from the cell wall, occurred. The above-mentioned events were compared with CFU production, the classic indicator of the viability of the cell.

MATERIALS AND METHODS

Bacteria, growth conditions, and media. *L. plantarum* LA 10-11, identified by the American Type Culture Collection, no ATCC number, is a spoilage microorganism which was isolated from onion ketchup. From our experience, we considered it necessary to use identical batches of cells in all experiments for an accurate comparison of the various pressure treatments. Therefore, cells of *L. plantarum* LA 10-11 (8 liters) were grown in fermentors with McFeeters chemically defined medium, modified by the addition of 0.5% (vol/vol) acetate and 1.0% (wt/vol) glucose at 30°C (13). The pH was controlled at 5.0 or 7.0 with 4 N KOH, and anaerobiosis was maintained with a 90% N_2–10% CO_2 mixture. Exponentially growing cells were harvested at an optical density at 660 nm

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(OD_{660}) of 0.5, washed twice, and resuspended in 50 mM sodium piperazine-N,N'-bis(2-ethanesulfonate) (PIPES; pH 7.0) to a final OD_{660} of approximately 100, which was equivalent to 10^{10} CFU/ml. The concentrated cell suspension was rapidly frozen in liquid nitrogen and stored at $-80°C$ until use.

High-pressure treatment. Before pressure treatment, the cells were thawed at room temperature, placed on ice, and subjected to pressure treatment within 30 min. The cells were treated in an isostatic high-pressure vessel (National Force, St. Niklaas, Belgium) at 250 MPa for different periods varying from 2 to 240 min and at 350 MPa for 10 min; all treatments were performed at room temperature ($24 \pm 2°C$). Complete compression and decompression took approximately 2 min. The number of viable cells (CFU per milliliter) was determined before and after the pressure treatments by pour plate counts in MRS agar (Merck, Darmstadt, Germany), and the plates were incubated aerobically for 5 days at 30°C.

Measurement of cytoplasmic volume and determination of ΔpH. The cytoplasmic volume was determined from the distribution of $^{3}H_2O$ (0.5 MBq/mmol) and [^{14}C]inulin (1.4 GBq/mmol) by the silicone oil centrifugation technique (21). From the data, a specific internal volume of 1.68 ± 0.37 μl/mg and an extracellular volume (adhering water after silicone oil centrifugation) of 1.69 ± 0.05 μl/mg could be calculated for the culture grown at pH 5.0. For the culture grown at pH 7.0, a specific internal volume of 1.26 ± 0.17 μl/mg and an extracellular volume of 1.24 ± 0.17 μl/mg were calculated. The ΔpH with respect to the external medium was determined from the distribution of [^{14}C]benzoic acid (0.67 GBq/mmol) after silicone oil centrifugation. A control assay was performed, with the addition of 1 μM valinomycin (K^+ ionophore, dissipates the membrane potential, $\Delta\Psi$) and 1 μM nigericin (facilitates the exchange of K^+ for H^+, dissipates the ΔpH), in which pH_{in} should be equal to pH_{out}. The intracellular pH was also determined after the addition of 1 mM DCCD (N,N'-dicyclohexylcarbodiimide; Sigma no. D3128) to the buffer.

Preparation of membrane vesicles. Inside-out membrane vesicles were prepared as follows: 1 ml of a concentrated cell suspension ($OD_{660} = 100$) was diluted with 1 ml of 50 mM PIPES (pH 7.0) buffer containing 2.5 mM $MgCl_2$, and the cells were disrupted by sonication (Soniprep 150; Beun de Ronde B. V., Abcoude, The Netherlands) at an amplitude of 26 μm for 20 min (cycle of 15 s of treatment and 45 s of cooling). The suspension was diluted by the addition of 50 ml of 50 mM PIPES (pH 7.0) containing 2.5 mM $MgCl_2$. The cell debris was removed by low-speed centrifugation ($7700 \times g$). The upper two-thirds of the supernatant was collected and centrifuged at high speed ($543,000 \times g$). The pellets were resuspended in 500 μl of 50 mM PIPES (pH 7.0) containing 2.5 mM $MgCl_2$. Inside-out membrane vesicles were rapidly frozen and stored in liquid nitrogen until use. The protein concentration of the membrane vesicles was determined by the method of Lowry et al. (10).

ATPase activity measurements. ATP hydrolysis by the membrane-bound ATPase was estimated from the amount of inorganic phosphate released as described by Driessen and Konings (4). Measurements were performed at 30°C in a 25 mM morpholineethanesulfonic acid (MES)–HEPES buffer (pH 6.0) containing 50 mM KCl, 5 mM $MgSO_4$, 1 mM dithiothreitol (Aldrich Chemie no. 15,046-0), and 10 μM carbonyl cyanide m-chlorophenylhydrazone (Aldrich Chemie no. 85,781-5). ATP (Sigma no. A 2383), at a final concentration of 4 mM, was used as the substrate. Samples of 100 μl were taken after 1, 2, and 3 min of incubation at 30°C and added to 1.6 ml of malachite green molybdate reagent (MGM reagent) with 0.1% (vol/vol) Triton X-100. After exactly 5 min of incubation at room temperature, 200 μl of 34% citric acid was added, and after a further 40 min of incubation at room temperature, the absorbance of the solution was measured at 660 nm. A calibration curve was made with a phosphorus standard solution (Sigma no. 661-9) containing 645 nmol of inorganic phosphate per ml. To confirm that F_0F_1 ATPase activity was measured, control experiments were performed in the presence of specific ATPase inhibitors. Vanadate (0.5 mM) was used as a P-type ATPase inhibitor, KNO_3 (50 mM) was used as a V-type inhibitor, and DCCD (1 mM) was used as an F-type inhibitor. The F_0F_1 ATPase activity of membrane vesicles isolated from *L. plantarum* LA 10-11 cells was measured before and after various high-pressure treatments after the addition of 1 mM DCCD or 0.2% Triton X-100. All measurements were carried out in duplicate.

Determination of the acidification of the external medium by *L. plantarum* cells. The cells were washed once with MES buffer (pH 6.5) containing 50 mM KCl, resuspended in the same buffer to a final OD_{660} of approximately 20, and subjected to high pressure as described above. After the treatment, the acidification test was performed at 30°C with 1 ml of the cell suspension in 18 ml of 5 mM MES buffer (pH 6.5) containing 50 mM KCl. Where necessary, glucose was added to a final concentration of 0.5% to energize the cells, and the pH was monitored continuously during incubation for 1 h, until the pH reached 6. If a pH of 6 was not reached within 1 h, the experiment was not continued. The acidification rate (nanomoles of H^+ per minute per milligram of protein) was calculated from the pH drop in time compared with a calibration curve (pH versus μmol of H^+, added as HCl).

Determination of the amount of intracellular ATP after energization. The amount of intracellular ATP, before and after pressure treatment of *L. plantarum* LA 10-11, was determined in a Lumac no. M2500 biocounter (Perstorp Analytical, Oud-Beijerland, The Netherlands) by the luciferin-luciferase reaction. The experiments were performed as specified by the manufacturer. The cells were preincubated for 10 min at 30°C in 50 mM PIPES (pH 7.0), and after incubation for 0, 5, 10, 15, 30, or 45 min at 30°C in 50 mM PIPES (pH 7.0)
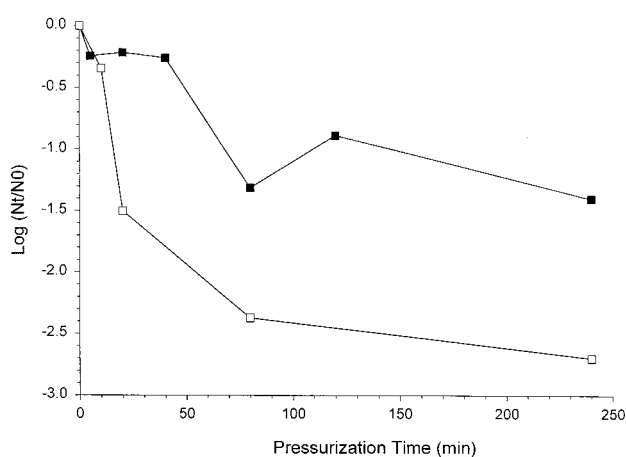

FIG. 1. Inactivation kinetics of *L. plantarum* LA 10-11 cells grown at pH 5.0 (■) and pH 7.0 (□) after various pressure treatments at 250 MPa.

containing glucose (0.2% [vol/vol]), samples were analyzed for intracellular ATP. ATP concentrations were determined from a calibration curve of relative light units plotted against ATP concentration. A value for the total intracellular [ATP] was obtained by subtracting the extracellular [ATP] from the total [ATP].

Transmission electron microscopy. For electron microscopy, cells were grown in McFeeters medium (13) at 30°C and harvested in the exponential growth phase at an OD_{660} of 0.5. The cells were washed twice in 50 mM PIPES (pH 7.0) and resuspended in the same buffer to an OD_{660} of 1.3. This concentrated cell suspension was used for high-pressure treatments of 10 min at 150, 250, or 350 MPa. After the high-pressure treatment, the cells were immediately mixed with a few drops of 2% gelatin and fixed for 4 h at 4°C in 0.1 M PIPES buffer containing 2% glutaraldehyde (pH 7.0). The cells were postfixed for 1 h at 4°C in 2% aqueous osmium tetroxide and embedded in LX112. Prior to dehydration, all samples were treated with 1% tannic acid for 1 h at room temperature, washed with 1% sodium sulfate for 5 min, and stained with 1% uranyl acetate for 2 h at room temperature. The fixed cells were then dehydrated by transfer through a series of 50, 70, 80, 90, and 100% acetone solutions. Finally, the cells were filtered and embedded in LX112. Ultrathin sections (50 nm) were cut with a Reichert-Jung Ultracut-E ultramicrotome. The sections were poststained with uranyl acetate and lead citrate and were examined in a Philips CM12 transmission electron microscope operated at 80 kV.

RESULTS

Effect of freezing on the viability and intracellular pH of *L. plantarum*. The viability of untreated and pressure-treated *L. plantarum* cells was determined before and after freezing as described in Materials and Methods. The same cells were also checked for their ability to maintain a ΔpH. The viable counts and the ability to maintain a ΔpH were not changed after freezing.

Effect of high pressure on the viability of *L. plantarum* LA 10-11 cells. The inactivation kinetics of *L. plantarum* LA 10-11 after high-pressure treatment are shown in Fig. 1. The results show that cells grown at pH 5.0 are more resistant to pressure than are cells grown at pH 7.0.

pH-activity profiles of F_0F_1 ATPase in membrane vesicles. The pH-activity profiles of the ATPase in membrane vesicles from *L. plantarum* LA 10-11 were determined at various pH values of the standard reaction mixture (Fig. 2). The pH optimum of F_0F_1 ATPase activity was between 6.5 and 7.5. The ATPase activity of membrane vesicles from cells grown at pH 7.0 is lower in the pH range 4.5 to 6.0 than is the ATPase activity of cells grown at pH 5.0. Inhibitor studies were carried out to confirm that only F_0F_1 ATPase activity was measured and not another type of ATPase (results not shown). Hardly any inhibition of ATPase was observed in the presence of vanadate, which is a P-type ATPase inhibitor. KNO_3, a V-type ATPase inhibitor, did not inhibit the measured ATPase activ-
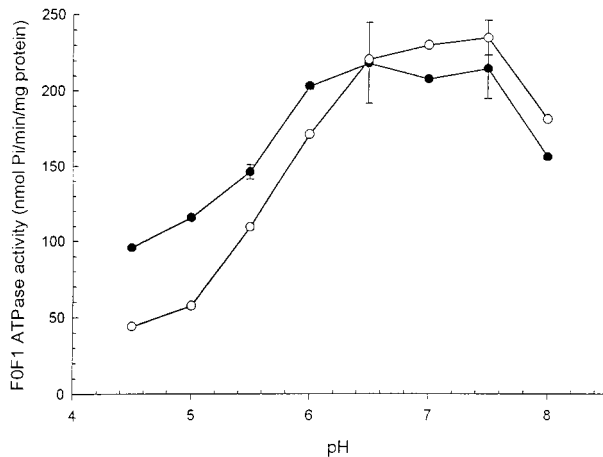
FIG. 2. pH-activity profiles for the F_0F_1 ATPase activity of membrane vesicles obtained from *L. plantarum* LA 10-11 cells grown at pH 5.0 (●) and 7.0 (○). Pi, inorganic phosphate.

ity. The addition of DCCD, a specific, partial inhibitor of F_0F_1 ATPase of gram-positive bacteria, showed the highest reduction in ATPase activity, 49% for pH 7.0-grown cells and 69% for pH 5.0-grown cells.

ATPase activity of membrane vesicles before and after high-pressure treatment. The effect of high-pressure treatment on the F_0F_1 ATPase activity of membrane vesicles isolated from different batches of cells grown at pH 5.0 and 7.0 is shown in Fig. 3. Contrary to our expectation, a residence time of 80 min of pressure treatment resulted in an approximately 1.2-fold increase in the ATPase activity of membrane vesicles from cells cultured at pH 5.0 (Fig. 3A) and a 1.7-fold increase for cells cultured at pH 7.0 (Fig. 3B), in comparison to untreated cells. However, after a longer residence time at the high pressure (240 min), the ATPase activity was decreased by 0.08- and 0.17-fold when the cells were grown at pH 5.0 and 7.0, respectively. A pressure treatment of 10 min at 350 MPa resulted in a reduction of 80% in the membrane ATPase activity of cells grown at pH 7.0. The addition of 1 mM DCCD to the reaction mixture containing membrane vesicles obtained from untreated cells, resulted in a decrease in ATPase activity, confirming that F_0F_1 ATPase activity was measured (Fig. 3). The addition of 0.2% Triton X-100 to the reaction mixture caused an increase of 2.5-fold in the F_0F_1 ATPase activity of membrane vesicles obtained from untreated cells grown at pH 5.0 and in an increase of 1.9-fold for cells grown at pH 7.0 (Fig. 3). Interestingly, the activity of the ATPase in membrane vesicles obtained from untreated cells at pH 7.0 treated with Triton X-100 was approximately the same as the activity observed for the membrane vesicles from cells which were pressure treated for only 10 and 80 min at 250 MPa (Fig. 3B). The addition of 0.2% Triton X-100 to the membrane vesicles from the cells that were pressure treated for 10 and 80 min resulted in only a very minor change in activity.

Effect of high pressure on the intracellular pH. To establish whether F_0F_1 ATPase is important in maintaining a proton gradient, the intracellular pH was measured in the presence of DCCD. Indeed, the internal pH values were lower after the addition of DCCD (Fig. 4), indicating that F_0F_1 ATPase is important in generating a ΔpH. It was shown that *L. plantarum* LA 10-11 cells grown at pH 5.0 (Fig. 4A) maintained a greater ΔpH at low pH values than did cells grown at pH 7.0 (Fig. 4B). High-pressure treatment resulted in a drop in the intracellular

pH of the cells (Fig. 4), which indicates that the regulation of the internal pH is impaired in these cells.

Effect of pressure on the acidification rate by *L. plantarum* LA 10-11 cells. The relative acidification rate of cells grown at pH 7.0 was considerably lower than that of cells grown at pH 5.0. Furthermore, for both culture conditions, the acidification rate decreased after pressure treatment (Fig. 5). The efflux of protons both before and after high-pressure treatment was largely inhibited by DCCD (Fig. 5), suggesting that at least a part of the F_0F_1 ATPase was still active after high-pressure treatment, in accordance with the F_0F_1 ATPase activity measurements described above.

Amount of intracellular ATP after high-pressure treatment. The results of the intracellular ATP measurements after energization with 0.2% glucose after different high-pressure treatments are shown in Fig. 6. After mild pressure treatments, an increase in the intracellular ATP pools was observed. This increase was higher in cells grown at pH 5.0 (Fig. 6A) than in cells grown at pH 7.0 (Fig. 6B). However, after more severe pressure treatments of cells grown at pH 5.0, this increase was
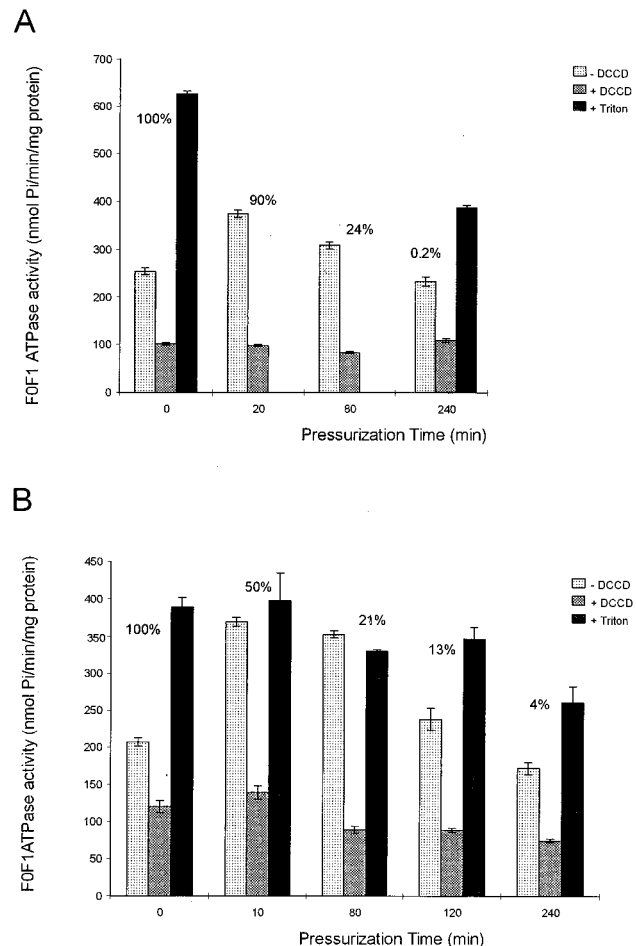

FIG. 3. F_0F_1 ATPase activity of membrane vesicles obtained from *L. plantarum* LA 10-11 cells grown at pH 5.0 (A) and pH 7.0 (B) after pressure treatment at 250 MPa for 0, 10, 20, 80, 120, or 240 min, with or without the addition of 1 mM DCCD and 0.2% Triton X-100. The measurements were performed in 25 mM MES–HEPES buffer (pH 6.0). The results are the mean of two independent experiments, and the standard deviation is indicated with error bars. The percent survival after pressure treatment is indicated. Pi, inorganic phosphate.
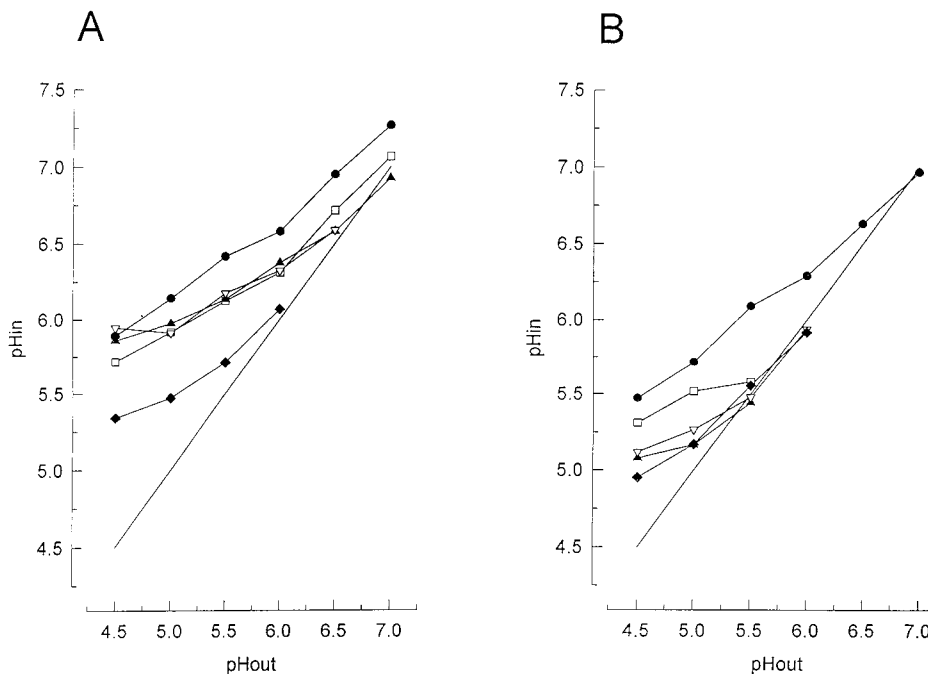
FIG. 4. Ability of *L. plantarum* LA 10-11 cells grown at pH 5.0 (A) and pH 7.0 (B) to maintain a ΔpH at various external pH values after different durations of pressure treatment at 250 MPa. Symbols: ●, 0 min; □, 0 min with the addition of 1 mM DCCD; ▲, 10 min, survival 99% (A) and 3.6% (B); ▽, 20 min, survival 41% (A) and 0.11% (B); ◆, 80 min, survival 6.8% (A) and 0.0032% (B); —, line to indicate $pH_{in} = pH_{out}$. The percent survival is calculated by [N (number of cells after pressure)/N (number of cells before pressure)] × 100.

no longer observed and hardly any ATP could be detected at the longest incubation times.

Membrane morphology determination. No obvious changes were observed in the morphology of the membranes from pressure-treated cells in comparison to control cells at magnifications of up to 750,000 (data not shown). Interestingly, clustering of DNA was observed when the cells were given a pressure treatment for 10 min at 250 MPa. However, almost all the cells (86%) survived this treatment, suggesting that clus-

FIG. 5. Acid efflux, in a 5 mM MES–50 mM KCl buffer (pH 6.5) after energization with glucose, of the *L. plantarum* LA 10-11 cells grown at pH 5.0 without and with DCCD added to the buffer and of the pH 7.0-grown cells without and with DCCD added to the buffer. The experiments were performed after high-pressure treatment at 250 MPa for 0, 10, or 80 min. The percent survival was 100, 111, and 10% (pH 5.0) and 100, 65, and 1% (pH 7.0). The percent survival is calculated by [N (number of cells after pressure)/N (number of cells before pressure)] × 100.

tering of DNA by pressure can be reversible. Clustering of DNA was also observed when cells were given a pressure treatment of 10 min at 350 MPa, with 0.05% survival of the cells. Mackey et al. (14) observed similar effects of high pressure on *Salmonella* and *Listeria*.

DISCUSSION

It can be assumed that the effects of freezing of the cells prior to measurements are negligible, since the viable counts and the intracellular pH of the cells were not affected by freezing.

The kinetics of pressure inactivation show that cells grown at pH 5.0 are more resistant to high pressure than are cells grown at pH 7.0.

As shown in Fig. 3, the measured ATPase activity in vesicles from cells grown at pH 5.0 did not differ significantly from the ATPase activity of cells grown at pH 7.0. When the vesicles were treated with Triton X-100, the apparent ATPase activity increased considerably, especially in the vesicles which were made from cells grown at pH 5.0. This increase might be explained by better exposure of occluded ATPase to ATP after treatment with Triton X-100, since the presence of right-side-out membrane vesicles (ATPase on the inside) cannot be excluded. The F_0F_1 ATPase activity of membrane vesicles isolated from mildly pressurized cells from both culture conditions increased significantly compared to that for untreated cells. Access of ATP to the enzyme might be enhanced by pressure. This is in line with the observation that Triton X-100 treatment did not cause a further increase in the ATPase activity of mild pressurized cells. The ATPase activity decreased gradually after longer pressure treatments.

The observations on intracellular pH seem consistent with the findings mentioned above. The ability of the cells to main-
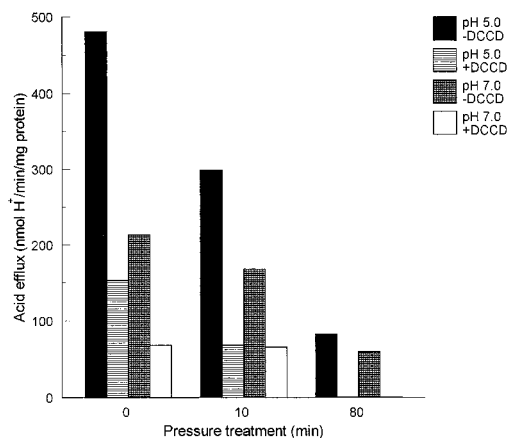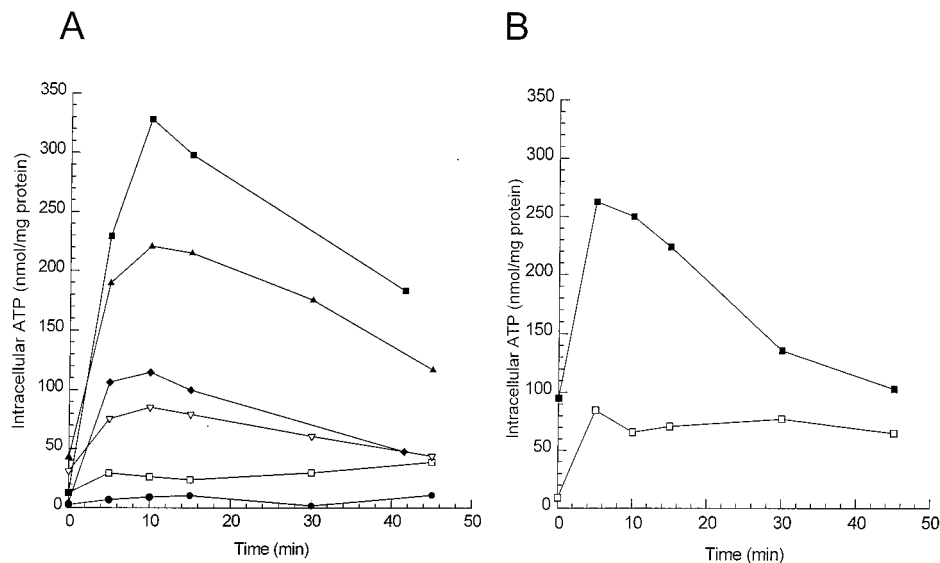
A

B

FIG. 6. Amount of intracellular ATP in *L. plantarum* LA 10-11 cells grown at pH 5.0 (A) and pH 7.0 (B) after different durations of pressure treatment at 250 MPa. Symbols: □, 0 min; ◆, 5 min, survival 57% (A); ▲, 10 min, survival 99% (A); ■, 20 min, survival 41% (A) and 56% (B); ▽, 40 min, survival 55% (A); ●, 80 min, survival 0.4% (A). The percent survival is calculated by [*N* (number of cells after pressure)/*N* (number of cells before pressure)] × 100.

tain a ΔpH decreased with increasing pressure treatment as the cells lost viability (Fig. 4). However, the intracellular pH had already decreased before the number of CFU was reduced (10 min at 250 MPa for pH 5.0 cultured cells). Furthermore, the number of viable cells was smaller after 20 min of pressure treatment than after 10 min of treatment while the ability to maintain a ΔpH remained almost the same. Moreover, Smelt et al. (23) have demonstrated that such pressure treatments extend the lag time of the same microorganism. Both observations indicate that there is an impaired physiological state of *L. plantarum* after high-pressure treatment and that the cells are better able to recover from a mild pressure treatment (10 min) than a severe pressure treatment. At low external pH values, the cells grown at pH 5.0 were able to maintain a greater ΔpH than were the cells grown at pH 7.0. This difference may be explained by the lower ATPase activities found for pH 7.0-grown cells in the range from pH 4.5 to 6.0 (Fig. 2). However, when the external pH was 6.5, the ΔpH was also greater for cells grown at pH 5.0 than for cells grown at pH 7.0, but, as can be seen from Fig. 2, both type of cells have a similar F_0F_1 ATPase activity at this pH. This may be explained by the fact that the ΔpH is measured in cells and the ATPase activity measured in membrane vesicles.

Glucose-induced acid efflux has previously been shown to be a reflection of F_0F_1 ATPase activity in streptococci (5). Indeed, the acid efflux could be inhibited by DCCD. *L. plantarum* cells grown at pH 5.0 were able to expel more protons than were cells grown at pH 7.0. These results are in agreement with earlier findings of Kobayashi et al. on *Enterococcus hirae* (formerly *Streptococcus faecalis*) (9). The relative rate of acidification was decreased after high-pressure treatment.

Addition of an energy source resulted in a dramatic increase in the intracellular ATP concentration after mild high-pressure treatment compared to that of untreated cells. The ATP in the intracellular pool is the net of ATP production minus ATP utilization. It is conceivable that after pressure treatment the F_0F_1 ATPase can no longer utilize ATP while the ATP-generating system is still intact. The findings suggest that the ATPase may be more sensitive than glycolysis to pressure treatment. In addition, the cells were not able to increase their ATP pool

after severe pressure treatments. This might indicate that severe pressure treatment affects glycolysis.

We present evidence which suggests that the membrane-bound F_0F_1 ATPase is involved in high-pressure inactivation of *L. plantarum*. Although F_0F_1 ATPase is membrane bound, no direct morphological changes of the membrane could be observed.

REFERENCES

1. **Cheftel, J.-C.** 1992. Effects of hydrostatic pressure on food constituents: an overview. Colloq. INSERM **224:**195–209.
2. **Chong, P. L., P. A. Fortes, and D. M. Jameson.** 1985. Mechanisms of inhibition of (Na,K) ATPase by hydrostatic pressure studied with fluorescent probes. J. Biol. Chem. **260:**14484–14490.
3. **De Smedt, H., R. Borghgraef, F. Ceuterick, and K. Heremans.** 1979. Pressure effects on lipid-protein interactions in (Na$^+$ + K$^+$)-ATPase. Biochim. Biophys. Acta **556:**479–489.
4. **Driessen, A. J. P., and W. M. Konings.** 1992. Assay of phosphate in lipids. Methods Cell Biol. **34:**147–165.
5. **Harold, F. M., E. Pavlasova, and J. R. Baarda.** 1970. A transmembrane pH gradient in *Streptococcus faecalis*: origin, and dissipation by proton conductors and N,N′-dicyclohexylcarbodiimide. Biochim. Biophys. Acta **196:**235–244.
6. **Hawley, S. A.** 1971. Reversible pressure-temperature denaturation of chymotrypsinogen. Biochemistry **10:**2436–2442.
7. **Kashket, E. R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46:**233–244.
8. **Knorr, D.** 1995. Hydrostatic pressure treatment of food microbiology, p. 159–175. *In* G. W. Gould (ed.), New methods of food preservation. Blackie, Glasgow, Scotland.
9. **Kobayashi, H., T. Suzuki, and T. Unemoto.** 1986. Streptococcal cytoplasmic pH is regulated by changes in amount and activity of a proton-translocating ATPase. J. Biol. Chem. **261:**627–630.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein

measurements with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.

11. **Ludwig, H., C. Bieler, K. Hallbauer, and W. Scigalla.** 1992. Inactivation of microorganisms by hydrostatic pressure. Colloq. INSERM **224:**25–32.

12. **Macdonald, A. G.** 1992. Effects of high hydrostatic pressure on natural and artificial membranes. Colloq. INSERM **224:**67–75.

13. **MacFeeters, R. F., and K.-H. Chen.** 1986. Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Compounds which serve as electron acceptors. Food Microbiol. **3:**73–81.

14. **Mackey, B. M., K. Forestière, N. S. Isaacs, R. Stenning, and B. Brooker.** 1994. The effect of high hydrostatic pressure on *Salmonella thompson* and *Listeria monocytogenes* examined by electron microscopy. Lett. Appl. Microbiol. **19:**429–432.

15. **MacNaughtan, W., and A. G. Macdonald.** 1982. Effects of pressure and pressure antagonists on the growth and membrane-bound ATP-ase of *Acholeplasma laidlawii B*. Comp. Biochem. Physiol. A **72:**405–414.

16. **Marquis, R. E., and G. R. Bender.** 1987. Barophysiology of prokaryotes and protontranslocating ATPases, p. 65–73. *In* H. W. Jannasch, R. E. Marquis, and A. M. Zimmerman (ed.), Current perspectives in high pressure biology. Academic Press Ltd., London, United Kingdom.

17. **Nannen, N. L., and R. W. Hutkins.** 1991. Intracellular pH effects in lactic acid bacteria. J. Dairy Sci. **74:**741–746.

18. **Nannen, N. L., and R. W. Hutkins.** 1991. Proton-translocating adenosine triphosphatase activity in lactic acid bacteria. J. Dairy Sci. **74:**747–751.

19. **Péqueux, A., and R. Gilles.** 1978. Effects of high hydrostatic pressures on the activity of the membrane ATPases of some organs implicated in hydromineral regulation. Comp. Biochem. Physiol. B **59:**207–212.

20. **Poolman, B.** 1993. Energy transduction in lactic acid bacteria. FEMS Microbiol. Rev. **12:**125–147.

21. **Rottenberg, J.** 1979. The measurement of membrane potential and ΔpH in cells, organelles and vesicles. Methods Enzymol. **55:**547–569.

22. **Sale, J. H., G. W. Gould, and W. A. Hamilton.** 1970. Inactivation of spores by hydrostatic pressure. J. Gen. Microbiol. **69:**323–334.

23. **Smelt, J. P. P. M., W. Courier, H. G. A. Cuppers, P. C. Wouters, and A. G. F. Rijke.** 1996. Inactivation kinetics of microorganisms by hydrostatic pressure, p. 273–276. *In* Proceedings of the XXXIVth Meeting of High Pressure Bio-Science and Biotechnology.

24. **Sonoike, K., T. Setoyama, Y. Kuma, and S. Kobayashi.** 1992. Effect of pressure and temperature on the death rate of *Lactobacillus casei* and *Escherichia coli*. Colloque INSERM **224:**297–301.