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Acid tolerance mediated by membrane ATPases in *Lactobacillus acidophilus*

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Abstract

The acid tolerance response in *Lactobacillus acidophilus* CRL 639, induced at pH 4.2 for 15 min, is mediated by the cell membrane F_1-F_0 ATPase. The specific activity of the enzyme was induced 1.6-fold after acid adaptation compared to a non-adapted control. The ATPase was optimal at pH 6 with a $K_m = 0.8$ mM and a $V_{\max} = 100$ mM.

Introduction

Many studies have suggested beneficial effects of lactic acid bacteria in human nutrition and health (De Simone *et al.* 1991). Within this group of bacteria, *Lactobacillus acidophilus* is widely used because of its potential beneficial properties (Dunne *et al.* 1999).

We have previously reported that *L. acidophilus* CRL 639 develops crytolerance and an acid tolerance response (ATR) after growing the cells at low temperature or by cell adaptation at low pH (Lorca *et al.* 1998, Lorca & de Valdez 1998, 1999). Maximal ATR was achieved after adaptation at pH 4.2 and pH 5, protein synthesis being required only in the latter case. For several organisms that inhabit the gastrointestinal tract, the F_1F_0-ATPase is an important element in the tolerance to low pH.

This report concerns the characterisation of a membrane associated F_1F_0-ATPase in *L. acidophilus* CRL 639 which plays a role in the homeostatic response induced at pH 4.2 for 15 min.

Material and methods

Microorganism and culture conditions

L. acidophilus CRL 639, of dairy origin, was grown at 37 °C in MRS broth (De Man *et al.* 1960) containing 0.5% (w/v) glucose (MRS_5) (initial pH = 6.5). Bacterial growth was measured turbidimetrically at 560 nm and calibrated to the cell dry weight. One absorbancy unit corresponded to 0.25 g dry wt per litre.

Adaptation and challenge conditions

The early exponential phase cells (0.06 g l^{-1}) were adapted (A) in MRS_5 broth at pH 4.2 (adjusted with lactic acid) for 15 min. Non-adapted (NA) cells were used as control. When needed, chloramphenicol (50 μg ml^{-1}, final concentration) or *N*,*N*′-dicyclohexylcarbodiimide (DCCD, 250 μg ml^{-1}, final concentration) were added during adaptation. Control and adapted cells were harvested by centrifugation and challenged by resuspension in fresh MRS_5 at pH 3 for 60 min. Serial dilutions of each sample were plated in mass in MRS agar, and plates were incubated at 37 °C for 72 h. Results were expressed as c.f.u. ml^{-1}; the survival rate was determined as N/N_0 were N is the number of c.f.u. ml^{-1} after a given incubation

time and N_0 is the number of c.f.u. ml^{-1} at zero time (without acid shift).

Membrane preparation and enzyme assays

L. acidophilus CRL 639 (control and adapted cells) before acid stress were washed twice in 100 mM Tris/HCl, pH 6.5 with 2 mM Mg_2Cl, and disrupted by grinding with 0.1 mm glass beads. The suspension was centrifuged to pellet unbroken cells and debris (10 000 *g*, 10 min, 4 °C). The supernatant was centrifuged (48 000 *g*, 60 min, 4 °C); the wall/membrane fraction obtained was washed once with the same buffer and resuspended in a small volume of the same solution.

The F_1F_0-ATPase activity was determined by measuring the inorganic phosphate (Pi) liberated from ATP by the method of Fiske & Subbarow (1925). The enzyme was assayed in a standard reaction mixture consisting of 50 mM Tris/HCl pH 6, 3 mM $MgCl_2$ and 2.5 mM Na-ATP in a final volume of 3 ml. The reaction was started by addition of 100 μl of membrane extract and further incubation at 37 °C for 15 min. The reactions were stopped by addition of 1 ml 12% (w/v) trichloroacetic acid, centrifuged at 8000 *g* for 5 min, and the supernatant fluids were used for determination of inorganic phosphate. Absorbancy was measured against backgrounds consisting in the corresponding reaction mixtures without membrane extracts. One unit of ATPase activity (UE) was defined as the amount of enzyme required to liberate one nmol Pi from ATP per min. The specific activity was expressed UE per mg proteins (UE mg^{-1}).

The optimal pH of the enzyme activity was assayed by using 50 mM Tris/maleate/NaOH buffer at pH values from 5.5 to 7.4 and acetate buffer from 3.6 to 5.5. The K_m value for ATPase was determined by varying ATP concentration from 0.5 to 5 mM and with a constant molar ratio Mg^{2+}:ATP of 1.2. The optimum Mg^{2+}:ATP ratio was determined measuring the enzyme activity at various concentrations of $MgCl_2$ and at constant amount of ATP (2.5 mM).

Measurement of internal pH

The internal pH (pHi) was measured by loading the cells with the conjugated fluorescent probe 5,6-carboxyfluorescein succinimidyl ester (cFDASE) according to Breeuwer *et al.* (1996). Harvested cells were washed and resuspended in 50 mM potassium HEPES buffer pH 8. Subsequently, the cells were incubated at 30 °C for 10 min in the presence of 1 μM cFDASE, washed, and resuspended in 50 mM potassium phosphate buffer, pH 7. To eliminate nonconjugated cFSE, glucose (final concentration, 10 mM) was added and the cells were incubated for an additional 30 min at 30 °C. The cells were then washed twice, resuspended in 50 mM potassium phosphate buffer (pH 7), and placed on ice until required.

Cells containing fluorescent probe were diluted to a concentration of approximately 10^7 cells per ml in a 3 ml glass cuvette and placed in the spectrofluorometer. Fluorescence intensities were measured at excitation wavelength of 490 and 440 nm by rapidly alternating the monochromator between both wavelengths. The emision wavelength was 525 nm, and the excitation and emission slit widths were 5 and 10 nm, respectively. The 490 to 440 nm ratio were corrected for backgroung signal due to buffer.

Calibration curve was determined in 50 mM citric acid K_2HPO_4 buffer at pH values from 3 to 6 and KH_2PO_4 K_2HPO_4 buffer from 6 to 8. The pHi and external pH were equilibrated by addition of valinomycin (1 μM) and nigericin (1 μM), and the ratios were determined as described previously.

Reproducibility

All experiments were performed in triplicate. The means of the data and the standard deviations are presented (mean $\pm$ SD).

Results and discussion

The acid tolerance response (ATR) triggered at pH 4.2 increased the survival of *L. acidophilus* CRL 639 to subsequent acid stress at pH 3 (Figure 1A). This response is not dependent on *de novo* protein synthesis as it was not inhibited by the presence of chloramphenicol (Lorca *et al.* 1998). To evaluate if the homeostatic response was mediated by membrane F_1F_0-ATPase, a specific F_1F_0-ATPase inhibitor DCCD was included during the cell adaptation at pH 4.2. Figure 1A shows that the ATR was almost completely inhibited in presence of DCCD.

The F_1F_0-ATPase specific activity of adaptated cells before acid shock showed a higher activity (1.6 fold) than that from control cells (Figure 1B). The hydrolysis of ATP by membrane extracts of adapted cells was completely inhibited when DCCD was added to the reaction mixture (Figure 1B) indicating that the activity measured in membrane extracts was due solely

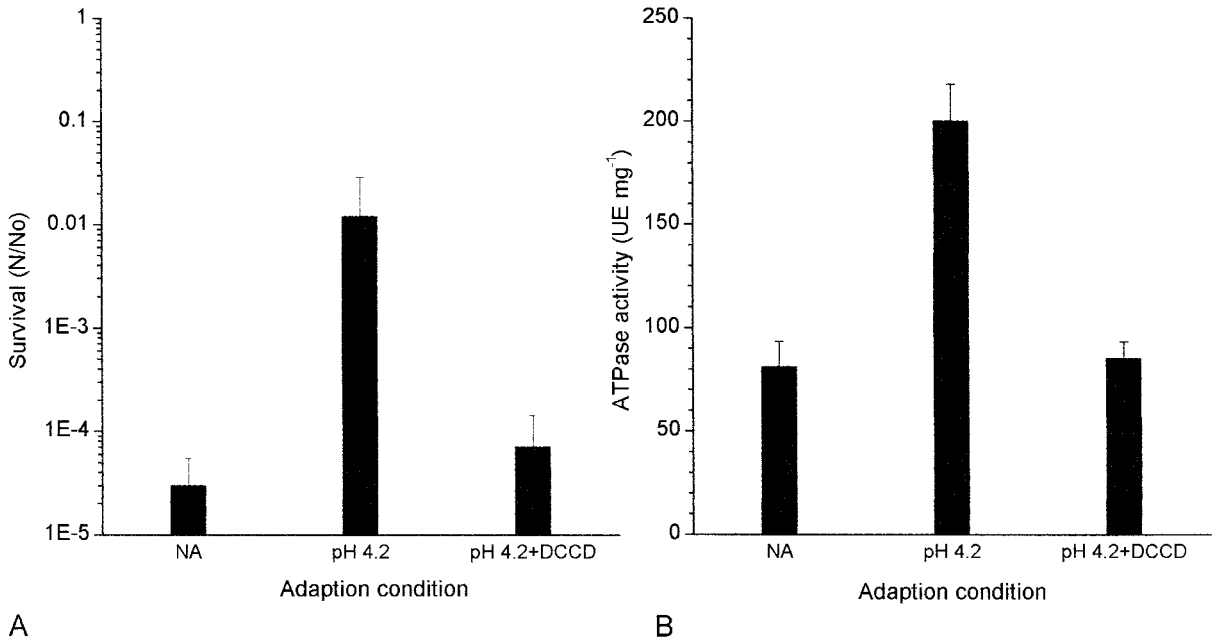

Fig. 1. Effect of N, N'-dicyclohexylcarbodiimide (DCCD, 250 μg ml^{-1}, final concentration) on the acid tolerance response (A) and on the H^+-ATPase activity (B) of *L. acidophilus* CRL 639 adapted to pH 4.2 for 15 min. For acid stress, control and adapted cells were harvested by centrifugation and challenged by resuspension in fresh MRS_5 at pH 3 for 60 min. Survival rate was determined as N/N_0 were N is the number of c.f.u. ml^{-1} after a given incubation time and N_0 is the number of c.f.u. ml^{-1} at zero time (without acid shift). Prior to acid shock, control and adapted cells were harvested, washed and disrupted with glass beads; the membrane fraction was separated by ultracentrifugation (48 000 g, 60 min, 4 °C) and used for ATPase activity determinations. One unit of ATPase activity (UE) was defined as the amount of enzyme required to liberate 1 nmol Pi from ATP per min. The specific activity was expressed as UE per mg proteins (UE mg^{-1}).

to the presence of the F_1F_0-ATPase. No differences in the enzyme activity were obtained after acid shock.

The optimum enzyme pH was 6 and it was not modified after adaptation of the cells at pH 4.2. The F_1F_0-ATPase is a saturable system with a typical kinetics of Michaelis–Menten. According to Lineweaver plot, a K_m value of 0.8 mM and a V_{max} = 100 mM were obtained. Similar values were obtained for the membrane F_1F_0-ATPase of cells adapted at pH 4.2 (data not shown).

The pHi of adapted cells at pH 4.2 was slightly higher (pHi = 7.2 ± 0.11) than non-adapted cells (pHi = 6.8 ± 0.05) while cells treated with DCCD showed a similar pHi as non-adapted cultures (pHi = 6.7 ± 0.12). The elimination of the small increase in pHi after incubation with DCCD abolish the resistance of the cells to acid stress and decrease the ATPase activity (Figure 1B). The slight differences found in the pHi values between adapted and non-adapted cells were statistically significant ($p < 0.05$).

In this report, a direct relationship between ATPase activity and acid tolerance was found for *L. acidophilus* CRL 639. The higher enzyme activity is not related to *de novo* protein synthesis but to an increase in the enzyme activity which could potentially be regulated by the pHi or at the step of enzyme assembly (Arikaido *et al.* 1999). In *Enterococcus hirae*, the cytoplasmic pH is maintained by an increase in the amount and activity of the ATPase (Kobayashi *et al.* 1986) while in *L. acidophilus* NCFM/N2 an increase in the ATPase-specific mRNA has been found after acid shock at pH 3.5 (Kullen & Klaenhamer 1999).

Current knowledge of the physiology of LAB suggests that the F_1F_0-ATPase plays a major role in maintaining the ΔpH across the cell membrane, with an alkaline cytoplasm relative to acid environment (Belli & Marquis 1991). The inducible pH homeostasis system, mediated by a proton-translocating ATPase as a mechanism to increase the pHi, might be an important mechanism in the success of *L. acidophilus* to survive low pH environments.

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