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A Low-pH-Inducible, Stationary-Phase Acid Tolerance Response in *Lactobacillus acidophilus* CRL 639

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Abstract. Acidity is an important environmental condition encountered by lactobacilli during food fermentation. In this report we show that triggering the stationary-phase acid tolerance response (ATR) in *L. acidophilus* CRL 639 depends on the final growth pH. In free-pH fermentation runs (final pH = 4.5), the cells were completely resistant to acid stress, whereas cells from cultures under controlled pH (pH = 6.0) were very sensitive. The relationship between the final pH and the development of cross-resistance to different kinds of environmental stress was also evaluated. The study of protein profiles showed the overexpression of 16 proteins from 6.5 to 70.9 kDa in stationary phase cells. Seven of these proteins (26.3, 41.4, 48.7, 49.3, 54.5, 56.1, and 70.9 kDa) were expressed as result of the stationary phase itself, while nine proteins (14.1, 18.6, 21.5, 26.9, 29.3, 41.9, 42.6, 49.6, and 56.2 kDa) were exclusively induced as a result of the drop in culture pH during free fermentation runs. These results strongly suggest the involvement of these proteins in cell adaptation to environmental changes.

Lactic acid is produced by lactic acid bacteria (LAB) during sugar fermentation, which implies a frequent confrontation of the cells with acid stress. Being a weak organic acid, lactic acid can easily pass the cell membrane in the protonated form at low pH, reducing the internal pH (pH_i) and determining the beginning of the stationary phase [10]. As in other microorganisms, the physiological mechanism of pH homeostasis in LAB includes the use of an H^+ antiport system to maintain the pH_i relatively constant over a wide range of environmental pH [2].

Modifications in the expression of proteins during the stationary phase at low pH values or during a pH shift have also been reported for *Enterobacteriaceae* [9], *Bacillus subtilis* [17], and *Streptococcus mutans* [7], but little is known in LAB. In *Salmonella typhimurium*, three overlapping inducible systems dependent on protein synthesis have been described that protect the cells from acid death [16]. These inducible survival mechanisms were referred to as the Acid Tolerance Response (ATR).

Within the LAB group, *Lactobacillus acidophilus* is commonly used in dairy fermentations owing to its probiotics properties. The ability of *L. acidophilus* CRL 639 to survive low pH conditions depends on the growth phase: stationary phase cells are naturally acid resistant, whereas exponential phase cells need an adaptation step to induce acid tolerance, which is known as the classical log-phase ATR [14].

In this report, it is shown that triggering the stationary-phase ATR in *L. acidophilus* CRL 639 depends on the final growth pH. In free pH fermentation runs (final pH = 4.5), the cells were completely resistant to acid stress, whereas cells from controlled pH (pH = 6.0) batch cultures remained very sensitive. Changes in the protein profile might be involved in the resistance of *L. acidophilus* to environmental stress.

Materials and Methods

Bacterial strain and growth conditions. The strain *L. acidophilus* CRL 639 used in this study was obtained from the Culture Collection of CERELA and was previously isolated from fermented dairy products. Batch culture experiments were performed in a fermenter (BIOFLO C22, New Brunswick Scientific Co. Inc, Edison, NJ, USA) with or without pH control, having a working volume of 1.5 L. The temperature was kept at 37°C, with agitation speed of 200 rev min^{-1} and

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60 ml min^{-1} pure nitrogen overlay to provide anaerobic conditions. Cultures were carried out in MRS [4] broth at an initial pH of 6.5. Glucose (0.5% [wt/vol] final concentration) was separately sterilized and added to the broth culture (MRS_5). Preliminary studies with different concentrations of glucose have led to the choice of 0.5% glucose to ensure that the exhaustion of sugar triggers transition to stationary phase. When needed, 1 M ammonium hydroxide (Merck, Germany) was used to adjust the culture pH.

Challenge conditions and viability determinations. For challenge, cells were harvested by centrifugation (5000 *g*, 10 min) and resuspended in fresh MRS_5 at pH 3.0 (adjusted with lactic acid) during 60 min at 37°C. For cross-resistance experiments, each cell suspension received one of the following treatments: i) Freezing (−20°C) for 24 h; ii) 20% ethanol (vol/vol) during 30 min; iii) pH 3.0 (adjusted with concentrated lactic acid) for 60 min; iv) 3 M NaCl for 24 h; v) 10 mM H_2O_2 for 60 min; and vi) heating to 60°C for 60 min. All treatments except freezing and heating were performed at 37°C. For lyophilization stress, cells from 16-h-old cultures in MRS broth were harvested, suspended in 0.85% (wt/vol) NaCl solution to the same volume, frozen overnight at −20°C, and dried under vacuum (6.67 Pa) for 8 h in a chamber-type freeze-dryer (Lyovac GT2, Leybold, Köln, Germany). The freeze-dried samples were rehydrated to the original fluid volume with 0.85% NaCl. Serial dilutions of each sample were plated in mass in MRS agar (MRS broth plus 1.5% agar) by the plate dilution method, and plates were incubated at 37°C for 72 h. Results were expressed as CFU/ml, and the survival rate was determined as N/N_0 where N is the CFU/ml after a given incubation time and N_0 is the CFU/ml at zero time (without acid shift).

Preparation of cell extracts and SDS-PAGE. Harvested cells were washed twice with 0.1 M potassium phosphate buffer (pH 7.0) and disrupted by grinding with glass beads. Protein concentration was determined according to Bradford [3], and aliquots of 30 μg protein were used per line. The cell extract was used for protein analysis by sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) according to Laemmli [12], modified by using a 10–15% acrylamide gel gradient (BioRad) and molecular weight markers in a range of 6.5–205 kDa (Sigma Chemical Co., St. Louis, MO, USA). Polyacrylamide gels were silver stained for total protein detection [15]. Quantitative measurements were based on peak areas of the densitograms obtained by using an Ultroscan XL densitometer (Pharmacia LKB, Uppsala, Sweden) with the Gel Scan XL 2.1 software (Pharmacia LKB).

Reproducibility. All results presented in this paper are the average of two independent assays. The differences were less than 5%.

Results

Stationary-phase and acid tolerance response. Cultures of *L. acidophilus* CRL 639 grown without pH control showed a very different acid resistance according to the growth phase; the maximal sensitivity was found at OD_{560} = 0.25 when the pH dropped from 6.5 to 5.6 (Fig. 1). The cells became more tolerant to acid shock as the pH of the culture went down, and the maximum ATR was obtained after 10 h of fermentation (early stationary phase) at a final pH (pH_f) of 4.5. These results suggest that the physiological age of the culture, the pH attained, or both factors might be involved in the survival of stationary-phase cells.

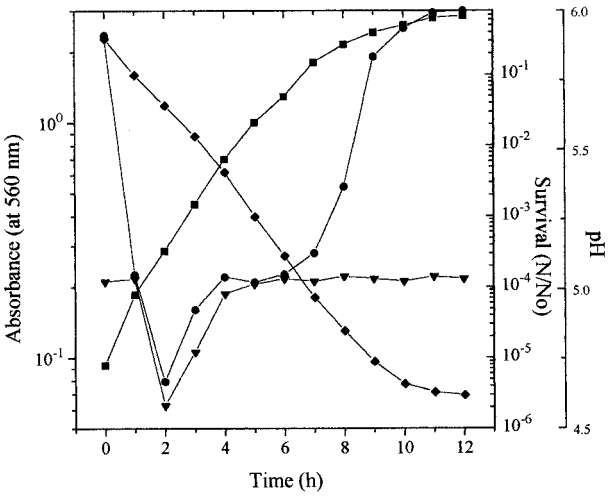

Fig. 1. Effect of the growth phase on the acid tolerance of *L. acidophilus* CRL 639 undergoing free fermentation (●) or in controlled pH batch cultures at pH 6.0 (▼). Survival rates (N/N_0) were determined after cell exposure to pH 3.0 for 60 min. OD_{560} (■) or free fermentations changes in pH were measured at each stage of growth (◆).

To determine the effect of acid environment, *L. acidophilus* CRL 639 was grown in batch cultures at controlled pH 6.0. Figure 1 shows that cells remained very acid sensitive at all growth stages although a 2-log cycle decrease in cell viability was observed in exponentially growing cells with respect to stationary-phase cells. These results put in evidence the close relationship existing between the pH attained by the cultures and the development of ATR.

Thus, we consider the pH_f reached by the cultures as being the main factor involved in the resistance of stationary-phase cells to acid shock. To demonstrate this hypothesis, cells were cultured in MRS broth supplemented with different concentrations of glucose (0.05–0.7%) in order to reach a pH_f in the range of 5.7–4.1. Once glucose was consumed and cultures entered stationary phase, the cells were subjected to acid challenge (pH 3.0). As expected, cultures with a pH_f = 5.7 were very sensitive to acid stress, whereas those with a pH_f lower than 5.0 were very resistant (Fig. 2).

Acid tolerance and cross-resistance stress. Stationary-phase cells obtained from free (pH_f = 4.5) and controlled pH (6.0) fermentation runs were subjected to different stress conditions: heat, ethanol, sodium chloride, hydrogen peroxide, freezing, and lyophilization, and the survival rate compared with that obtained after acid shock. Results obtained are shown in Fig. 3. Again, cells from cultures at pH 6.0 were very sensitive to all challenge conditions, while those from free fermentation runs showed 1 to 3 log-cycle increase in survival to ethanol,

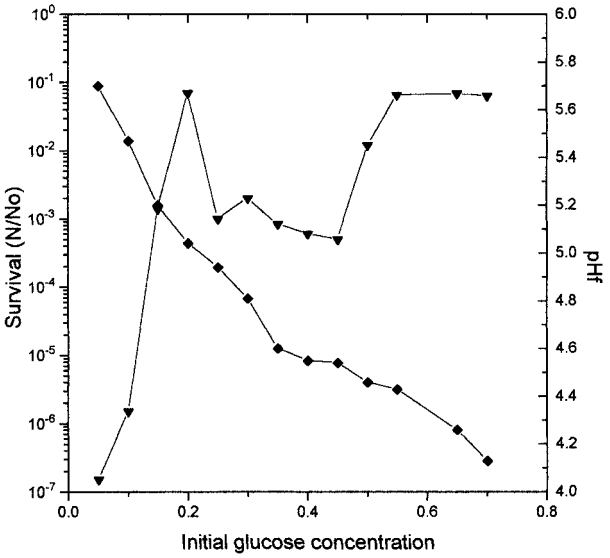

Fig. 2. Effect of final pH on the stationary-phase ATR of *L. acidophilus* CRL 639. Survival rates (N/N_0) were determined after cell exposure to pH 3.0 for 60 min (▼). Final broth pH was measured after glucose exhaustion (◆).

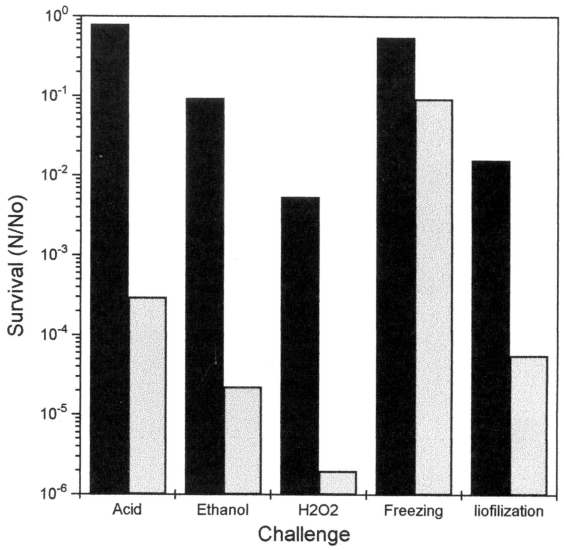

Fig. 3. Stress resistance of *L. acidophilus* CRL 639 undergoing free fermentation (■) or in controlled pH batch cultures at pH 6.0 (▨).

hydrogen peroxide, freezing, and lyophilization. However, cells remained sensitive to heat and osmotic stress.

To confirm the key role that pH plays in the tolerance of *L. acidophilus* CRL 639 to environmental stress, stationary-phase cultures were shifted from pH 4.5 to 6.0 for 30 min. Exposure of *L. acidophilus* CRL 639 to the latter pH resulted in a rapid loss of both the ATR and the cross-resistance of the cells, indicating that a drop in pH during fermentation without pH control would be the main factor that triggers the ATR in stationary-phase cells.

Protein profile. Scanning one dimensional electrophoresis gels made it possible to evaluate the protein profile from cells grown with (pH 6.0) and without (pH_f = 4.5) controlled pH. Table 1 shows the proteins that were significantly overexpressed as defined by integrated intensity ratios greater than 2.0. No significant differences were found between exponentially growing cells at both culture pHs, while changes in the expression of at least 16 proteins from 6.5 to 70.9 kDa were found within stationary-phase cells (Fig. 4). Seven of these proteins (26.3, 41.4, 48.7, 49.3, 54.5, 56.1, and 70.9 kDa) were expressed as a result of the stationary phase itself. The remaining nine proteins (14.1, 18.6, 21.5, 26.9, 29.3, 41.9, 42.6, 49.6, and 56.2 kDa) were exclusively induced as a result of the drop in culture pH during free fermentation runs (Table 1). This effect was also observed in stationary phase cells grown at different glucose concentration and pH_f of 5.7–4.1 (data not shown).

Discussion

Acid tolerance is perceived to be an important property of probiotic LAB, enabling the cells to survive gastric acidity and volatile fatty acids produced as a result of fermentation in the intestine [5]. The ability to resist acid stress is also believed to be necessary for colonizing and establishing a commensal relationship with mammalian hosts. Besides, the production of organic acids (mainly lactate) by LAB during growth implies a frequent challenge of the cells to acid environments.

In this study, it was determined that the low pH_f attained by the cultures rather than the stationary phase by itself is the main inductor of the ATR in *L. acidophilus* CRL 639.

In *Lactococcus lactis*, stationary-phase cells and those starved at pH 6.8 developed strong cross-protection against heat, ethanol, acid, osmotic and oxidative challenges [8]. In contrast, the global stress protection in *L. acidophilus* CRL 639 is induced only at low pH, but the cells were not able to develop tolerance to heat or osmotic stress. A different response was obtained for cold-adapted cells of *L. acidophilus* CRL 639 against heat and osmotic challenge [13].

Results obtained show the close relationship existing between the drop in pH and the induction of specific proteins. From this standpoint, it is assumed that some of the nine proteins expressed at pH_f 4.5 (Table 1) would be involved in the ATR of *L. acidophilus* CRL 639 rather than proteins expressed as a result of the stationary phase itself. The constitutive basal level of the former proteins

Table 1. Overexpression of proteins by *L. acidophilus* CRL 639 during stationary phase, cultured with (pH 6.0) and without (pH_f = 4.5) pH control

Free pH (pH_f = 4.5)			Control pH (pH 6.0)		
kDa	Absolute area[a]	$I.I._{4.5}/I.I._6$[b]	kDa	Absolute area[a]	$I.I._6/I.I._{4.5}$[b]
14.1	2.68	3.3	6.6	8.80	2.0
18.6	0.96	2.9	37.9	0.30	7.5
21.5	3.34	2.8	40.7	1.25	3.8
26.9	2.40	2.1	48.5	0.82	9.4
29.3	1.72	2.0	51.6	0.10	3.1
41.9	0.98	2.4			
42.6	0.77	2.1			
49.6	0.29	5.1			
56.2	0.76	8.1			

[a] Determined as mm · h of the peak areas.
[b] I.I., integrated intensity; 4.5, stationary cells grown without pH control (pH_f = 4.5); 6, stationary cells grown with pH control (pH = 6.0).

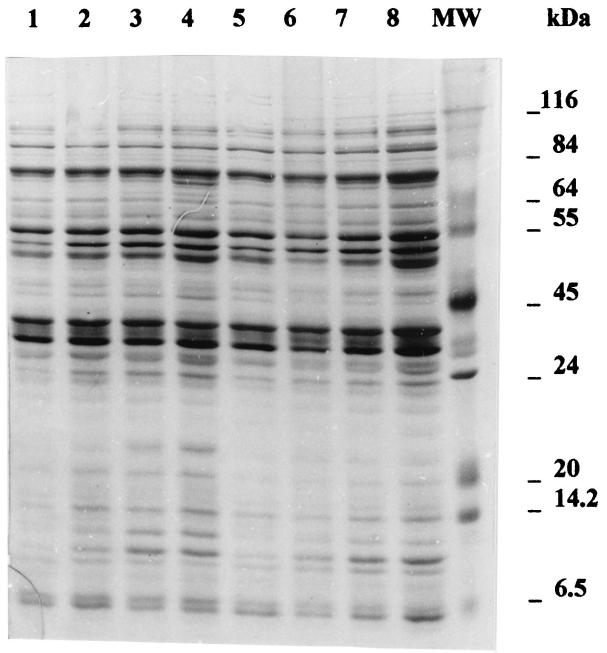

Fig. 4. Protein profile during different stages of growth in cells undergoing free fermentation (1–4) or growing at pH 6.0 (5–8): cells from 3 h (1, 5), 6 h (2, 6), 9 h (3, 7), and 12 h (4, 8). MW: molecular weight marker (Sigma, wide range).

during exponential and stationary phase would indicate that they are also involved in the normal cellular physiology as well, besides the role they play in acid adaptation.

Currently, the identity of various proteins up-regulated under various stress conditions is not known, but the nature of the stress does point to a number of possible proteins associated with physiological responses. For example, the synthesis of stress proteins [6] or the ΔpHi can induce a homeostatic response (mainly ATPases) in *Streptococcus mutants* and *L. casei* [1, 11].

From the results obtained, i. e., induction of ATR by low pH, the validity of growing starter cultures near neutrality under controlled to ensure maximum biomass and active cells is doubtful. It would rather be advisable to grow cells for probiotic purposes at lower pH (about 5.0) in order to improve the resistance of the cells to acid stress during the gastrointestinal passage as well as assure a better survival of starter cultures during the industrial processes to which these bacteria are subjected.

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