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Characterization of the Protein-Synthesis Dependent Adaptive Acid Tolerance Response in *Lactobacillus acidophilus*

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Abstract

Exposure of *L. acidophilus* CRL 639 cells to sublethal adaptive acid conditions (pH 5.0 for 60 min) was found to confer protection against subsequent exposure to lethal pH (pH 3.0). Adaptation, which only occurred in complex media, was dependent on *de novo* protein synthesis and was inhibited by amino acid analogues. There was no modification in the protein synthesis rate during adaptation, but the protein degradation rate decreased. Synthesis of acid stress proteins may increase the stability of pre-existing proteins. By 2D-PAGE, induction of nine acid stress proteins and repression of several housekeeping proteins was observed. Putative heat shock proteins DnaK, DnaJ, GrpE, GroES and GroEL (70, 43, 24, 10 and 55 kDa, respectively) were among the proteins whose synthesis was induced in response to acid adaptation.

Introduction

Lactic acid bacteria (LAB) are used worldwide for the manufacture of cheese, yogurt, and fermented milk products. However, knowledge of physiological adaptions of LAB to dairy processing conditions remains limited. A better understanding of this adaptive response is important since dairy manufacturing processes often expose LAB to adverse environmental conditions such as shifts in pH and temperature.

Survival of *Escherichia coli* under acid stress conditions is often linked to the expression of an adaptive stress response characterized by the transient induction of specific proteins and physiological changes which frequently enhance their ability to withstand harsh environmental conditions (Bearson *et al.*, 1997). Three complex medium-dependent acid resistance (AR) systems have been described for *E. coli* (Lin *et al.*, 1995); two of them are fermentative AR systems which involve the inducible amino acid decarboxylases, arginine decarboxylase and glutamate decarboxylase. These are also present in *Shigella* (Lin *et al.*, 1995) but not in *Salmonella typhimurium*. In summary, the enteric group of microorganism have been shown to be diverse in the ways that they handle acid stress. Relatively little is known about stress responses in dairy starter cultures although heat shock has been studied (Hartke *et al.*, 1997).

Molecular chaperones, which include many well-studied heat shock proteins (HSP), are essential for maintenance of bacterial growth and viability (Hartl *et al.*, 1992). The most abundant and physiologically important chaperones in *E. coli* include DnaK, DnaJ, GrpE, GroEL, and GroES (Georgopoulos *et al.*, 1994), the synthesis of which is under positive control of a minor σ factor (σ^{32}) encoded by the *rpoH* gene. In the gram-positive bacterium *Bacillus subtilis,* chaperones belong to the Class I stress genes that constitute the CIRCE regulon and are negatively controlled by HrcA (Hecker *et al.*, 1996). Several lines of evidence have indicated that the two major chaperone teams, DnaK-DnaJ-GrpE and GroEL-GroES, play distinct but cooperative roles in protein folding, stability, and assembly of individual proteins (Georgopoulos *et al.*, 1994). Heat shock induction of stress proteins has been observed in numerous Gram positive species, including *Bacillus subtilis*, *Enterococcus faecalis*, *Lactococcus lactis* subsp. *lactis, Lactobacillus helveticus*, and *L. sakei* demonstrating the universality of this response (Kim and Batt, 1993; Broadbent *et al.*, 1997). Among LAB, the possible involvement of molecular chaperones in acid stress has only been addressed in *Lactococcus lactis* (Hartke *et al.*, 1997).

In a previous report we observed that stationary-phase cells of *L. acidophilus* CRL 639 are acid sensitive during growth at pH 6.0 but naturally acid resistant when the pH of the culture gradually decreases during free fermentation runs (Lorca and Font de Valdez, 2001). Two acid tolerance responses (ATR) are induced in exponentially growing cells upon exposure to sublethal pH (3.8–6.0) (Lorca *et al.*, 1998): (i) a homeostatic response related to an increase in ATPase activity (independent of *de novo* protein synthesis) induced by adaptation at pH 4.2 for 15 min (Lorca and Font de Valdez, 2001), and (ii) a protein-synthesis dependent ATR induced at pH 5.0 for 60 min. The purpose of this study is to characterize the protein-dependent ATR of *L. acidophilus* CRL 639 and to

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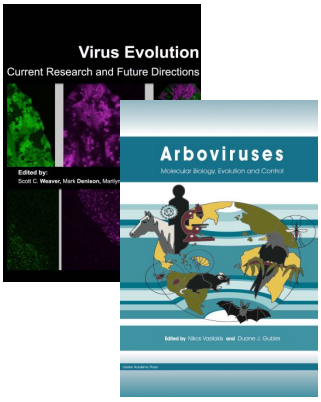

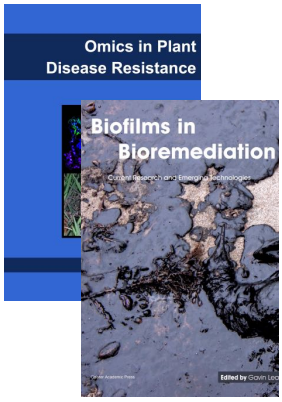

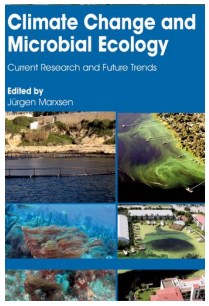

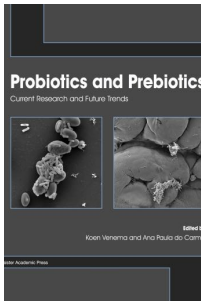

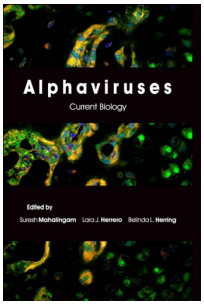

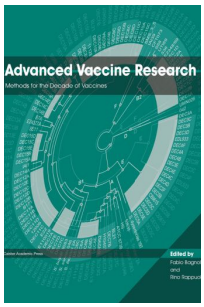

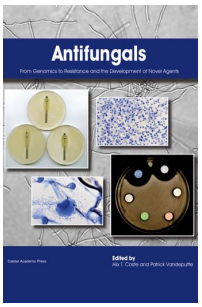

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identify putative HSP's induced in response to acid adaptation.

Results

ATR in Complex and Minimal Media

In *L. acidophilus* CRL 639 acid tolerance is induced by shifting cells to pH 5.0 (during 60 min). There is no cell growth during this adaption. The adapted cells are then challenged at pH 3.0 (Lorca *et al.*, 1998). A synthetic medium (SM) was used to know whether acid survival of *L. acidophilus* in minimal versus complex medium utilizes similar mechanisms, or if distinct systems are involved. To this end, cells were grown in MRS_5 until exponential phase, washed and resuspended in SM adjusted at pH 5.0.

Figure 1 shows that cells adapted in synthetic media (SM) were completely sensitive to acid stress while cells preincubated in complex media (MRS_5 or a modified MRS broth, Mm) developed ATR and displayed an acid tolerant phenotype. Subsequent attempts to determine if the nitrogen component(s) of MRS were responsible for survival at pH 3.0 were made by adding an amino acid pool as peptides (80% casein hydrolysate; SM-C) or as free amino acids (80% casamino acids; SM-Ca) to SM broth. Neither amino acids nor peptides induced the ATR in SM (Figure 1), indicating that a complex pattern of induction is needed.

Rate of Protein Synthesis and Degradation During Adaptation

The induction of protein synthesis during adaptation of the cells in MRS_5, Mm and SM was determined by incorporation of [^{3}H]leucine. The ^{3}H uptake was higher (3000 c.p.m. after 60 min) in SM compared to MRS_5 and Mm (90 c.p.m. after 60 min). However, this low rate of incorporation was completely inhibited by the addition of 50 μg ml^{-1} of chloramphenicol, indicating a specific incorporation of leucine to the proteins. The ^{3}H uptake was further analyzed in MRS_5 since, as described previously, the cells adapted in SM display an acid sensitive phenotype. In MRS_5, adapted (pH 5.0) and non-adapted (pH 6.0) cells showed the same rate of protein synthesis (data not shown).

Amino acid analogues were added to pH 5.0-adapted cells to elucidate the role of active induced stress proteins (Figure 2). Three analogues, DL-7-azatryptophan (a tryptophan analog), *S*-2-aminoethyl-L-cysteine (a lysine analog), and *p*-fluoro-DL-phenylalanine (a phenylalanine analog) were tested. When added individually at 50 μg/ml, each of these analogues affected the ATR differently. 7-azatryptophan, like chloramphenicol, abolished the ATR response. *S*-2-aminoethyl-L-cysteine and *p*-fluoro-DL-phenylalanine reduced the ATR by 90% (Figure 2). This deleterious effect may be related to a decrease in the rate of protein synthesis and/or the synthesis of faulty proteins. To evaluate these hypotheses, the incorporation of [^{3}H]leucine in the presence of the amino acid analogues (Figure 3A), and the activity of intracellular enzymes were determined using β-gal and PepN as physiological markers (Figure 3B). Although the rate of protein synthesis did not decrease in the presence of amino acid analogues (Figure 3A) a reduction of 96 to 98% in the specific activity of PepN and β-gal was observed (Figure 3B) compared to adapted cells without

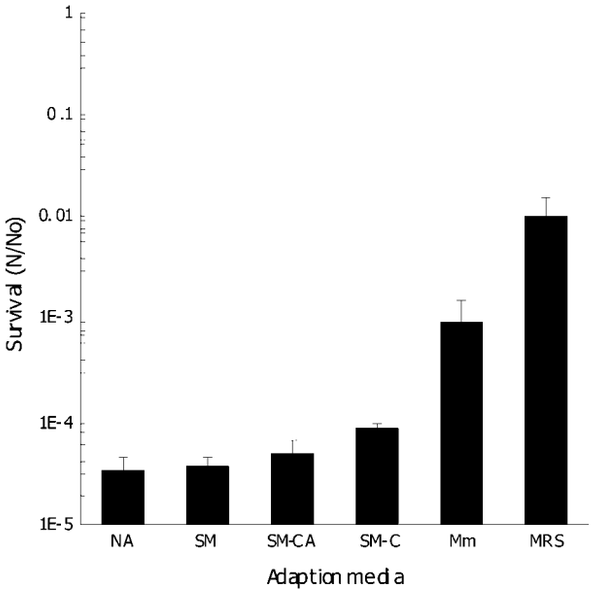

Figure 1. ATR of *L. acidophilus* CRL 639 in different culture media. Cells at an early exponential phase ($A_{560} = 0.25$) were adapted at pH 5.0 during 60 min prior to acid challenge in MRS_5 at pH 3.0. NA = Non adapted. The different adaption media used were: MRS_5, Mm (modified MRS_5), SM (synthetic medium), SM-C (SM with 0.5% casein hydrolysate) and SM-CA (SM with 0.5% casamino acids).

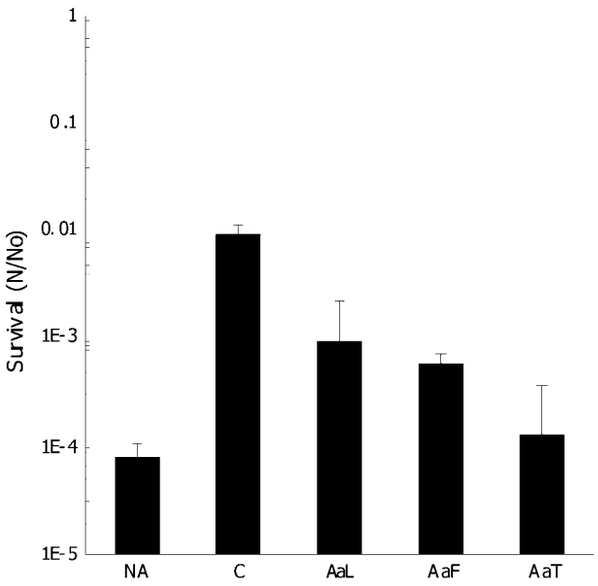

Figure 2. Effect of amino acid analogues on the development of acid tolerance by *L. acidophilus* CRL 639. The analogues, DL-7-azatryptophan (AaT), *S*-2-aminoethyl-L-cysteine (AaL), and *p*-fluoro-DL-phenylalanine (AaF), were included during adaption of the cells at pH 5.0. Cells adapted at pH 5.0 without analogs (C); non adapted cells (NA).

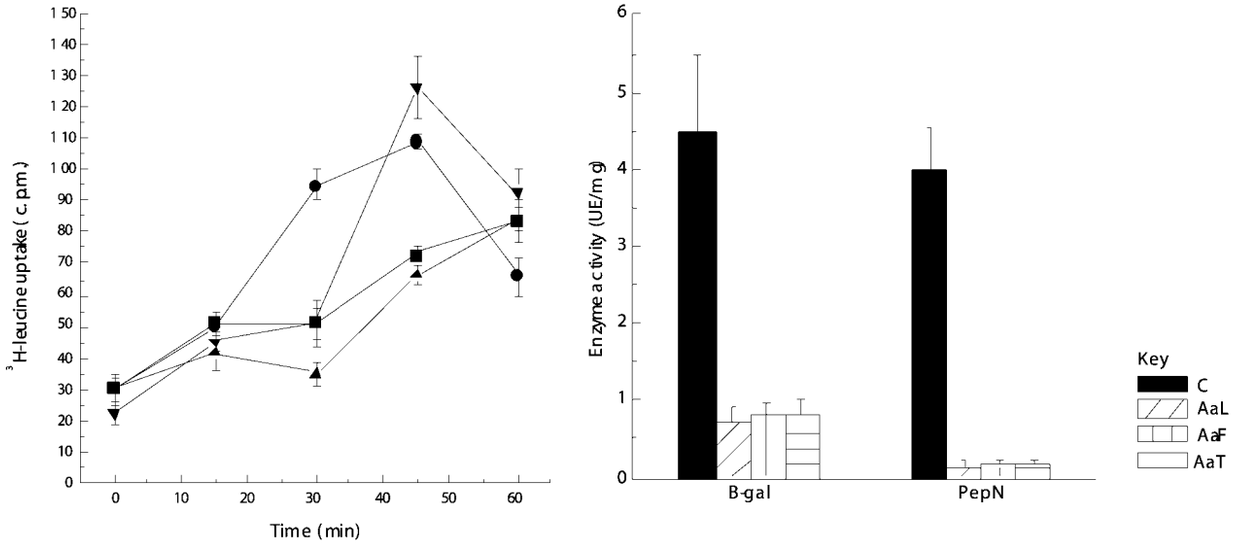

Figure 3. Effect of amino acid analogues on the rate of protein synthesis (A) or in β- gal and PepN activities (B). The analogues, DL-7-azatryptophan (▲), *S*-2-aminoethyl-L-cysteine (▼), and *p*-fluoro-DL- phenylalanine (●), were included during the adaption in MRS_5 at pH 5.0 (■). Cells adapted at pH 5.0 without analogs (C); non adapted cells (NA).

analogues. In addition, the protein degradation rate in adapted cells was lower (3.0%/h) than non-adapted cells (4.7%/h) that were kept at pH 6.0. In the presence of chloramphenicol, the adapted cells displayed the same degradation rate as the non-adapted ones, indicating that the synthesis of acid-induced specific proteins (ASP) during adaptation might be involved in protein stability.

Pattern of Protein Synthesis During Adaptation

The synthesis of proteins by *L. acidophilus* CRL 639 labeled with [^{35}S]methionine during acid adaption in MRS_5 was examined by SDS-PAGE. In one dimensional SDS-PAGE, overexpression of 30 proteins with apparent molecular weights ranging from 11.9 to 117.0 kDa were visualized (Table 1). Comparing the protein pattern of MRS_5- and Mm-adapted cells, a higher (1.6 to 3.8 fold) induction and the specific expression of 14 proteins (12.3, 13.3, 20.1, 23.0, 25.4, 27.6, 28.3, 38.8, 47.7, 59.9, 85.5, 93.0, 96.4 and 117.0 kDa) was observed in the former medium. These proteins might be involved in the higher ATR developed by the cells adapted in MRS_5.

^{35}S-labeled proteins of *L. acidophilus* CRL 639 adapted in MRS_5 were examined by 2D-PAGE (Figure 4) which gave a better estimation of the characteristics of the proteins synthesized (based on the isoelectric point, pI). If it is assumed that a linear pH gradient from pH 4.0 to 7.0 is formed over the entire range of the gel (as claimed by the manufacturer), a crude estimate of the pI value for a polypeptide can be obtained. It was only observed proteins with a pI between 4.4 and 5.6 (Table 2) since all proteins with neutral or basic characteristics were repressed. The expression of proteins of 10.1, 15.2, 25.4, 27.5, 28.3, and 68.1 kDa were specifically induced under acid adaptation, while proteins of 12.3, 20.1 and 59.9 kDa were overexpressed. All proteins obtained by 2D-SDS-PAGE were also detected by 1D SDS-PAGE but at a higher induction rate (Table 1).

The rate of protein formed in MRS_5-adapted cells was determined by: (I) pulse labeling at the beginning of the adaptation period and sampling after 15, 30, 45 and 60 min (Figure 5A) and (II) pulse labeling after 15, 30, and 45 min of adaptation and sampling at the end of the incubation period (60 min) (Figure 5B). In the first case, the protein synthesis reached its maximum after 45 min and remained unchanged for 60 min (Figure 5A). In the second case, the synthesis of almost all proteins from 10.1 to 117.0 kDa took place when the pulse labeling with ^{35}S-methionine was applied after 15 min; the synthesis rate decreased after 30 min and no labeled proteins were detected thereafter (Figure 5B).

Identification of Heat Shock Proteins

To identify known heat shock proteins (HSP) among the polypeptides expressed under acid adaption, Western blotting was performed using antibodies against *E. coli* HSP:s (DnaK, DnaJ, GrpE, GroEL and GroES).

All the HSP homologues were only detected in acid-adapted cultures of *L. acidophilus* CRL 639 (Figure 6). Two proteins of 43 and 50 kDa cross-reacted with rabbit anti-DnaJ (Figure 6, lines 7 and 8), the smaller one being in accordance with other DnaJ proteins already described (Van Asseldonk *et al.*, 1993). Proteins of 24 and 70 kDa cross-reacted with rabbit anti-GrpE (lines 5 and 6); the first one could be related to the GrpE protein while the 70 kDa-immunoreactive molecule would be either an impurity of the proteins used for antibodies production or related to

Table 1. Influence of culture medium in the protein overexpression and specific induction during adaption of *L. acidophilus* CRL 639 at pH 5.0

kDa	$I.I._5/I.I._6$* in MRS_5	$I.I._5/I.I._6$* in Mm
11.9	3.1	2.0
12.3	2.9	NI[†]
13.3	4.0	NI
14.1	4.0	2.0
15.2	3.8	2.0
16.6	3.4	2.2
18.6	6.3	2.2
19.5	3.2	3.0
20.1	2.7	NI
22.0	2.5	2.7
23.0	2.9	NI
23.6	7.1	2.6
25.4	SE (0.41)[‡]	NE[§]
26.5	4.5	2.2
27.6	2.4	NI
28.3	5.9	NI
30.2	3.6	2.2
36.1	3.4	3.0
38.8	SE (3.26)	NE
47.7	SE (2.27)	NE
49.6	3.3	2.5
59.9	SE (3.22)	NE
64.0	8.5	2.2
68.1	2.0	2.5
79.1	9.3	2.6
85.0	4.4	NI
93.0	SE (0.16)	NE
96.4	12.3	NI
99.2	5.5	2.7
117.0	12.5	NI

* I.I., integrated intensity; 5, cell adapted at pH 5.0 during 60 min; 6, non-adapted cells maintained at pH 6.0, indicated as subscripts.
[†]NI, not induced.
[‡]SE, specifically expressed at pH 5.0, numbers in brackets represent integrated intensity value from the densitograms.
[§]NE, not expressed.

the DnaK protein. Western blots with polyclonal rabbit anti-GroES serum (lines 1 and 2) showed reactivity with a protein of 10 kDa and a faint band of 55 kDa. The former has a molecular weight similar to GroES of *E. coli* and *Lac. lactis* (Georgopoulos *et al.*, 1994; Eaton *et al.*, 1993). No positive bands were found using monoclonal antibodies against *E. coli* DnaK or GroEL (lines 9–10 and 3–4, respectively).

Discussion

Specific proteins play a role in the ATR of *L. acidophilus* CRL 639 to further survive acid stress conditions. Two theories have been postulated in relation to stress tolerance and protein synthesis. According to Mandelstam (1958), the protein synthesis would be a stabilizing factor which prevents ribosomal RNA degradation under environmental stress conditions (e.g., starvation) while Reeve *et al.* (1987) consider the role that specific proteins play in stress resistance. To choose between these alternatives, amino acid analogues were added to cells subjected to acid adaption (Figure 2). These conditions allow continued protein synthesis but the peptide formed might be defective (Fowden *et al.*, 1967). The presence of amino acid analogues markedly decreased acid tolerance, probably due to the synthesis of faulty proteins rather than their effect in decreasing the rate of protein synthesis. To our knowledge, we are presenting the first evidence that LAB require active proteins for survival under acid stress. Similar results have been demonstrated in *E. coli* for long term survival of carbon-starved cells (Reeve *et al.*, 1984). The decrease in protein degradation rate (from 4.7%/h to 3.0%/h) of bulk proteins in *L. acidophilus* CRL 639 during acid adaptation at pH 5.0 was dependent on protein synthesis indicating that specific proteins (e.g., chaperones) would be necessary for stabilizing some of the preexisting proteins.

Given this data, we wondered if *L. acidophilus* CRL 639 triggered similar mechanisms for survival in minimal and complex media. The ATR was only developed in complex media (MRS_5 or Mm), and even then at different rates, while the cells in SM remained acid sensitive. It is not known which of the components present in MRS_5 are required for the full induction of the ATR system. In enteric bacteria, many genes require co-induction by low pH or specific molecules that presumably serve as substrates for the gene product (Bearson *et al.*, 1997). Examples are mannose for *aniG* (Foster, 1993), lysine for *cadBA* (Auger *et al.*, 1989), and formate for *hyd* (Birkman and Böck, 1989). Melnykovych and Snell (1958) reported that the addition of arginine, methionine, tyrosine, asparagine, glutamate, and iron to minimal media would allow full induction of arginine decarboxylase in *E. coli* which is involved in the cell homeostasis. This evidence reflects the strikingly different acid survival strategies within the bacteria kingdom according to their ecological niches.

The L-[^{35}S]methionine incorporation during acid adaptation of *L. acidophilus* CRL 639 made it possible to link the stress response to the induction of particular proteins. A good relationship between the 1D- and 2D-SDS PAGE:s was observed in the autoradiograms. The lower number of proteins (nine in total) separated in the latter case could be due the concentration of proteins used for loading in 2D-PAGE compared to 1D-PAGE. The protein profiles obtained for other microorganisms subjected to acid stress are quite different. In *Streptococcus thermophilus,* only ten proteins were expressed after acid shock (González-Marques *et al.* 1997) while *Leuconostoc oenos* only overexpressed one protein of 42 kDa which has been related to an acid tolerant phenotype (Guzzo *et al.*, 1994). In contrast, *Lac. lactis* displays about 30 independent mutations which confer acid stress resistance (Rallu *et al.*, 1996).

It has been proposed that the DnaK-DnaJ-GrpE chaperon team maintains nascent or other preexisting proteins in un-folded states, while the GroEL-GroES chaperone complex can interact with partially folded polypeptides and assist in additional folding (Hartl, 1992). Environmental stresses that induce some or all HSP:s are heat, ethanol, certain heavy metals,

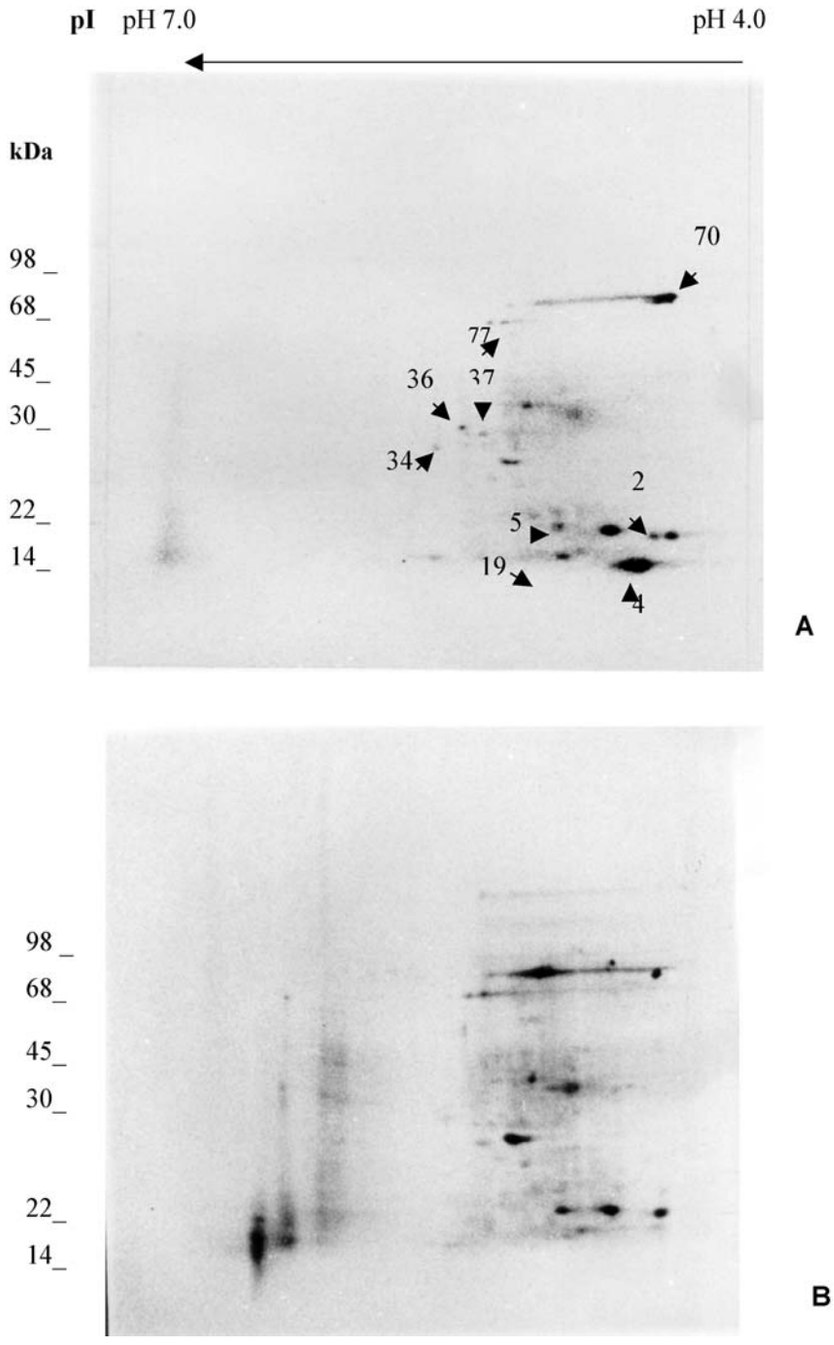

Figure 4. Autoradiograms of 2D-PAGE analysis of extracts of *L. acidophilus* labeled with [^{35}S]methionine during acid adaption in MRS_5 at pH 5.0 (A) and MRS_5 at pH 6.0 (B) (control). The first dimension is the isoelectric separation in a range of pH 4.0 to pH 7.0. The second dimension is a SDS-PAGE with a linear polyacrylamide gradient from 10 to 15%. Arrows numbers indicate the spot number in Table 2.

puromycin, nalidixic acid, hydrogen peroxide as well as alkaline and acidic conditions (Van Bogelen *et al.*, 1987). However, chaperone induction has only been described in lactobacilli in response to heat (Broadbent *et al.*, 1997). In this study we have identified proteins of 10, 24, and 43 kDa that were immunologically reactive to the co-chaperons: GroES, GrpE, and DnaJ, respectively. Two faint bands of 70 and 55 kDa cross-reacted with GrpE and GroES antibodies, respectively, which could be linked to the proteins DnaK and GroEL. Hendrick and Hartl (1993) found significant similarity between the sequences of Cpn10 and Cpn60 (GroES and GroEL homologues). These observations could explain the cross reactivity of polyclonal antisera against GrpE (to DnaK) and GroES (to GroEL) found in *L. acidophilus* CRL 639.

Interestingly, HSP's were only detected after adaptation at pH 5.0. Since DnaJ, GrpE and DnaK

Table 2. Properties of the induced proteins at pH 5.0 separated by 2D-PAGE.

Spot	Estimated value		Concentration		Induction V_A/V_{NA}
	PI*	MW (kDa)	AU†	V‡	
A2	4.5	15.2	1.46	2.72	SI[d]
A4	4.6	10.1	3.07	31.29	SI
A5	5.0	12.3	1.66	5.0	5.3
A19	5.2	20.1	1.5	3.41	1.7
A34	5.6	25.4	0.82	0.03	SI
A36	5.6	28.3	1.45	0.71	SI
A37	5.5	27.5	0.93	0.52	SI
A70	4.4	68.1	2.89	10.55	SI
A77	5.4	59.9	1.03	0.29	2.9

*pI, estimated isoelectric point.
†AU, maximal absorbance.
‡V, volume (AU x mm^2); A, spot volume of acid adapted cells at pH 5.0 during 60 min; NA, spot volume of non-adapted cells (pH 6.0); as subscripts.
§SI, specific induction during acid adaptation.

act in concert, the genes for these proteins are often grouped on an operon that forms part of the heat regulon (HR). Genetic studies performed in *Lac. lactis* demonstrated that the structural gene encoding DnaK is situated in the *hrcA-grpE-dnaK* operon (Eaton *et al.*, 1993) while GroEL is encoded in the *groES-groEL* operon (Kim and Batt, 1993). The *dnaJ* is not included in the *grpE-dnaK* operon of this bacteria, but both operons are still included in the HR (Eaton *et al.*, 1993). Our preliminary data indicate that the *dnaJ* gene is encoded in the same operon as *dnaK* in *L. acidophilus* CRL 639 (unpublished results). This finding would explain why *dnaK* and *dnaJ* are induced

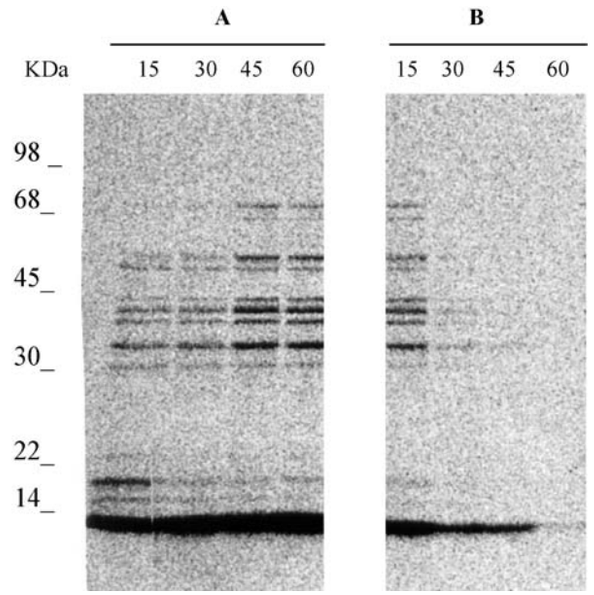

Figure 5. Autoradiograms of 1D-SDS-PAGE of adapted cells pulse labeled at pH 5.0 with [^{35}S]methionine during 15, 30, 45, or 60 min (A) or after 15, 30, or 45 min (B).

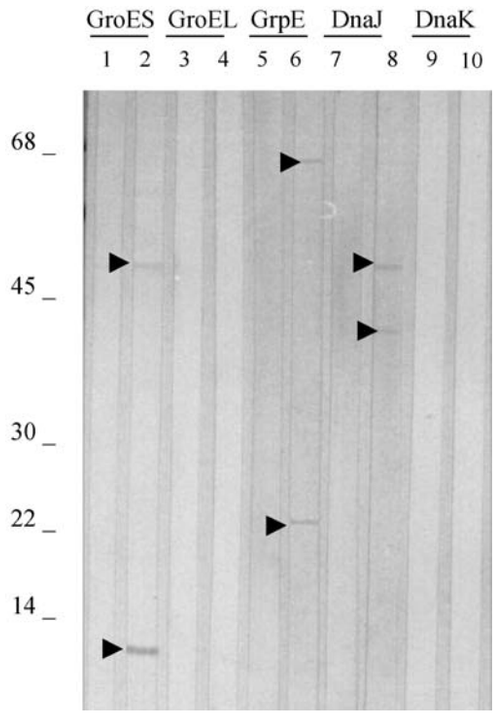

Figure 6. Western blot analysis of extracts separated by 1D-SDS-PAGE from *L. acidophilus* CRL 639 with polyclonal rabbit antibodies raised against recombinant DnaJ (lanes 7 and 8), GrpE (lanes 5 and 6) and GroES (lanes 1 and 2) of *Escherichia coli* and mouse monoclonal antibodies raised against GroEL (lanes 3 and 4) and DnaK (lanes 9 and 10) of *E. coli*. Non-adapted cells (lanes 1, 3, 5, 7 and 9); cells adapted at pH 5.0 during 60 min (lanes 2, 4, 6, 8 and 10) in MRS_5.

together under acid adaptation (Figure 6) while in other lactobacilli *dnaJ* has been found to be constitutively induced (Broadbent *et al.*, 1997).

It appears that low pH serves as an important signal for the induction of mechanisms that protect *L. acidophilus* against several environmental stresses. The molecular regulation of HSP in *L. acidophilus* CRL 639 during acid adaptation is under study.

Experimental Procedures

Bacterial Strain and Growth Conditions

The strain *L. acidophilus* CRL 639, isolated from fermented dairy products, was obtained from the Culture Collection of CERELA. Cultures were routinely carried out at 37°C in MRS broth (De Man, 1960) containing 0.5% (w/v) glucose (MRS_5) at an initial pH of 6.5 under CO_2 (5%). Incorporation of labelled (^{3}H and ^{35}S) amino acids were performed in the following media: MRS_5, Mm (modified MRS_5 broth in which meat peptone and meat extract were replaced by 0.5% (w/v) casein hydrolysate) and a synthetic medium, SM (Hébert *et al.*, 2000). When necessary, 0.5% casein hydrolysate (Sigma) and 0.5% (w/v) casamino acids (Sigma) were added.

Acid Adaption

A 16h-old culture of *L. acidophilus* CRL 639 in MRS_5 was used to inoculate the same media at an A_{560} of 0.1 in MRS_5 broth, unless otherwise stated. The cells were grown to an $A_{560} = 0.25$ (early exponential phase), harvested by centrifugation at 5000 g and resuspended at 37°C for 60 min in the different media (SM, Mm and MRS_5) adjusted to pH 5.0 with concentrated lactic acid. Non-adapted

cells resuspended at pH 6.0 were used as control (Lorca *et al*., 1998). Chloramphenicol and the amino acid analogues DL-7-azatryptophan, p-fluoro-DL-phenylalanine, and *S*-2-aminoethyl-L-cysteine (ICN) were added (50 μg/ml each) at the beginning of acid adaption.

Challenge Conditions and Viability Determinations

For challenge assays, cells were harvested by centrifugation (5000 g for 10 min) and resuspended in fresh MRS_5 at pH 3.0 (adjusted with lactic acid) for 60 min at 37°C. Serial dilutions of each sample were plated in mass in MRS agar (MRS broth with 1.5% agar) by the plate dilution method, and plates were incubated at 37°C for 72 h. Results were expressed as colony forming units (c.f.u./ml) and the survival rate was determined as N/No where N is the c.f.u./ml after a given incubation time and No is the c.f.u./ml at zero time (without acid shift).

Enzyme Activities

The β-galactosidase (β-gal) activity was determined according to Noh and Gilliland (1993) with *o*-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co.) as substrate. One unit (UE) of β-gal activity was defined as the amount of enzyme that released 1 μmol of o-nitrophenol per min. Specific activity was expressed as UE per milligram of protein (UE/mg).

Leucyl-aminopeptidase (PepN) activity was determined at 40°C using L-leucine-*p*-nitroaniline (20 mM final concentration; Sigma) as substrate (Hébert *et al*., 2000). Reaction mixtures contained 100 μl of substrate stock solution, 850 μl of 0.2 M Tris-HCl buffer (pH 7.0) and 50 μl of enzyme extract. The reaction was stopped by the addition of 0.5 ml of 80% acetic acid. The samples were centrifuged and the released nitroaniline was followed spectrophotometrically at 410 nm. One unit (UE) of PepN activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min. Specific activity was expressed as UE per milligram of protein (UE/mg).

Determination of Protein Degradation

An overnight culture of *L acidophilus* CRL 639 was inoculated in MRS_5 at an $A_{560} = 0.1$. The culture was grown for one generation, at which time 1 μCi (9.25 mBq) of L-[4,5-^{3}H]leucine/ml was added. The culture was grown for another generation in the presence of the radioactive label and then harvested, washed twice with 0.1 M Tris-HCl buffer (pH 7.0) and calibrated to cell dry weight (0.06 g/l) in MRS_5 broth previously adjusted at pH 5.0 or 6.0 with lactic acid (Sigma).

At zero time and at intervals during adaptation, duplicate trichloroacetic acid (TCA) precipitates were obtained by adding 0.5 ml of culture to 50 μl of 50% TCA (w/v), incubation at room temperature for 1 h, and centrifugation for 5 min. An aliquot (0.25 ml) of the supernatant was counted in 5 ml of scintillation fluid (Merck). At zero time only, total TCA-precipitable counts were determined as follows: the TCA precipitates were washed with 100 μl of 1% bovine serum albumin plus 1 ml of ice cold 5% TCA (w/v) and then with 1 ml of ice cold 1:1 (v/v) ethanol-anhydrous ethyl ether. The precipitates were then dried and dissolved in 50 μl of 1 N NaOH. After the addition of 0.4 ml of water and 50 μl of 1N HCl, 0.25 ml of the dissolved precipitate was counted in 5 ml of scintillant (Merck). The rate of protein degradation was expressed as the percentage of the TCA-precipitable counts at zero time released as TCA-soluble material per hour (Reeve *et al*., 1984).

Determination of Protein Synthesis

An overnight culture of *L acidophilus* CRL 639 was inoculated in MRS_5 at an $A_{560} = 0.1$. Cultures were grown to an $A_{560} = 0.25$. The cells were harvested, washed twice with 0.1 M Tris-HCl buffer (pH 7.0) and resuspended to 0.06 g/l in MRS_5 broth previously adjusted at pH 5.0 or 6.0 with lactic acid (Sigma). At zero time of adaptation, 5 μCi (9.25 mBq) of L-[4,5-^{3}H]leucine/ml was added to the culture. Then, at zero time and at desired intervals thereafter, duplicate TCA precipitates were formed and counted as described above. Protein synthesis was expressed as the increase in TCA-precipitable counts with time (Reeve *et al*., 1984).

[^{35}S]methionine Labelling of Polypeptides

L. acidophilus CRL 639 was grown exponentially at 37°C in MRS_5 medium to an $A_{560} = 0.25$. Cells were harvested, washed twice with 0.1 M Tris-HCl buffer (pH 7.0), and resuspended in the adaption media Mm and MRS_5 which were adjusted at pH 5.0 or 6.0. At various time points, 150 μl of culture were transferred to an Eppendorf tube containing 15 μCi (355 kBq) of [^{35}S]methionine. After 10 min, unlabelled methionine was added at a final concentration of 0.8 mg/ml to complete synthesis of labelled proteins (Kilstrup *et al*., 1997). After 2 min of incubation, 10 μl of chloramphenicol (20 mg/ml) /ml was added, and the cells were pelleted by centrifugation, washed twice with 0.1 M Tris-HCl buffer (pH 7.0) and disrupted by grinding with glass beads.

Preparation of Cell Extracts and 1-D 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 (1976). The cell extract was used for protein analysis by sodium dodecyl sulphate-polyacrylamide gels electrophoresis (SDS-PAGE) (30 μg proteins per line) according to Laemmli (1970), modified by using a 10–15% acrylamide gel gradient (BioRad) and molecular weight markers in a range of 14 to 94 kDa (BioRad). Polyacrylamide gels were silver stained for total protein detection (Oakley *et al*., 1980). Quantitative measurements were based on peak areas of the densitograms obtained by using an Ultroscan XL densitometer with the Gel Scan XL 2.1 software (Pharmacia LKB, Uppsala, Sweeden). The protein bands were considered as significantly overexpressed when the integrated intensity ratios were greater than 2.0.

2D-PAGE

The first isoelectric focusing was performed using the Multiphor II horizontal electrophoresis apparatus (Amersham Pharmacia Biotech). Immobiline DryStrip linear immobilised pH gradient (IPG) gel strips (11 cm) with a pH range of 4–7 were rehydrated in 250 μl sample buffer (SB) containing 8 M urea, 2% (v/v) Nonidet P-40, 10 mM DTT (Sigma) and 2% (v/v) Pharmalyte range 4–7 as recommended by the manufacturer (Amersham Pharmacia Biotech) and the sample (50 μg) was included during this period. Rehydration was carried out overnight at room temperature in a reswelling cassette with strips covered with silicone oil to avoid evaporation. The proteins were focused overnight at 15°C under a protective layer of silicone oil using 300 V for 1 min, a linear raise from 300 to 3500 V during 1.5 h and 3500 V for 3.5 h. After focusing, the IPG gel strips were immediately subjected to a second dimension electrophoresis. The gel and separation conditions were as described above. Immediately before SDS-PAGE, the IPG strips were gently shaken in 10 ml of 50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 26% (v/v) glycerol and bromophenolblue for 2 × 15 min. DTT (100 mg) was added to the first equilibration solution, and iodoacetamide (250 mg) to the second. The IPG strips were quickly loaded on top of the gel, and the separation was performed at 15°C at 50 V until the bromophenol blue dye front reached the bottom of the gels. After SDS-electrophoresis the proteins were transferred to Immobilon PVDF membranes (0.45 μm, Micron Separations Inc) using a semi-dry electroblotter (Ancos) for 1.5 h at a constant current of 0.8 mA/cm^2. Molecular weight standards (BioRad) including proteins ranging from 14.3 to 97.4 kDa were treated identically. After transferring, the membranes were dried and exposed to X-ray films (Hyperfilm b-max, Amersham) for 21 days and films were developed with Kodak D19. The spots were quantified by densitometry as described above.

Western Blot Assays

Protein extracts from *L. acidophilus* CRL 639 were separated by SDS-PAGE as described above and then electroblotted with 25 mM Tris-HCl (pH 8.3)- 192 mM glycine-20% (v/v) acetone transfer buffer onto Immobilon PVDF membranes (0.45 μm, Micron Separations Inc) using a semi-dry electroblotter (Ancos) for 1.5 h at a constant current of 0.8 mA/cm^2.

The PVDF membranes were blocked for 15 min with PBS buffer (pH 7.2) containing 5% (w/v) skim milk and 0.005% (v/v) Tween 20, washed three times for 5 min each with the same buffer, and then incubated for 16 h at 4°C with a 1:200 dilution of either polyclonal rabbit antibodies raised against recombinant DnaJ, GrpE and GroES of *E. coli* or mouse monoclonal antibodies raised against GroEL and DnaK of *E. coli* (StressGen, Biotechnologies Corp.). The membranes were washed three times and incubated with the secondary antibody conjugated with horseradish peroxidase (HRP) at a dilution of 1:2000, and incubated at 20°C for 2 h. After repeated washing, bound antibodies were detected by reaction in 50 mM sodium acetate (pH 5.0) with 0.04% (w/v) 3-amino-9-ethylcarbazole (Merck) and 0.015% (v/v) H_2O_2. When necessary the strips were incubated without the first antibody as specificity control.

Reproducibility

All experiments were performed in triplicate. The means of the data and standard deviation are presented.

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References

Auger, E.A., Redding, K.E., Plumb, T., Childs, L.C., Meng, S-Y., and Bennett, G.N. 1989. Construction of *lac* fusions to the inducible arginine and lysine decarboxilase genes of *Escherichia coli* K-12. Mol. Microbiol. 3: 609–620.

Bearson, S., Bearson, B., and Foster, J.W. 1997. Acid stress responses in enterobacteria. FEMS Microbiol. Lett. 147: 173–180.

Birkman, A., and Böck, A. 1989. Characterization of a *cis* regulatory DNA element necessary for formate induction of the formate dehydrogenase gene (*fdhF*) of *Escherichia coli*. Mol. Microbiol. 3: 187–195.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation an microgram concentrations of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Broadbent, J.R., Oberg, C.J., Wang, H., and Wei, L. 1997. Attributes of the heat shock response in three species of dairy *Lactobacillus*. System. Appl. Microbiol. 20: 12–19.

De Man, J.C., Rogosa, M., and Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 42: 123–125.

Eaton, T., Shearman, C., and Gasson, M. 1993. Cloning and sequence analysis of the *dnaK* gene region of *Lactococcus lactis* subsp. *lactis*. J. Gen. Microbiol. 139: 253–3264.

Foster, J.W. 1993. The acid tolerance response of *Salmonella typhimurium* involves the transient synthesis of key acid shock proteins. J. Bacteriol. 175: 1981–1987.

Fowden, L., Lewis, D., and Tristram, H. 1967. Toxic aminoacids: their action as antimetabolites. Adv. Enzymol. 29: 89–163.

Georgopoulos, C., Liberek, K., Zylicz, M., and Ang, D. 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. In: The Biology of Heat Shock Proteins and Molecular Chaperons. R.I. Morimoto, A. Tissieres, and C. Georgopoulos, (eds). Cold Spring Harbor Laboratory Press. p. 209–249.

González-Márques, H., Perrin, C., Bracquart, P., Guimont, C., and Linden, G. 1997. A 16 kDa protein family overexpressed by *Streptococcus thermophilus* PB18 in acid environments. Microbiology 143: 1587–1594.

Guzzo, J., Cavin, J.F., and Divies, C. 1994. Induction of stress proteins in *Leuconostoc oenos* to permit direct inoculation of wine. Biotechnol. Lett. 16: 1189–1194.

Hartke, A., Frére, J., Boutibonnes, P., and Auffray, Y. 1997. Differential induction of the chaperonin GroEL and the co-chaperonin GroES by heat, acid, and UV-irradiation in *Lactococcus lactis* subsp *lactis*. Curr. Microbiol. 34: 23–26.

Hartl, F.U., Martin, J., and Neupert, W. 1992. Protein folding in the cell: the role of molecular chaperons Hsp70 and Hsp60. Annu. Rev. Biophys. Biomol. Struct. 21: 293–322.

Hébert, E.M., Raya, R.R., and de Giori, G.S. 2000. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. Appl. Environ. Microbiol. 66: 5316–5321.

Hecker, M., Schumamm W., and Völker, U. 1996. Heat-shock and general stress response in *Bacillus subtilis*. Mol. Microbiol. 19: 417–428.

Hendrick, J.P., and Hartl F.-U. 1993. Molecular chaperone functions of heat shock proteins. Annu. Rev. Biochem. 62: 349–384.

Kilstrup, M, Jakobsen, S., Hammer, K., and Vogensen, F.K. 1997. Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*. Appl. Environ. Microbiol. 63: 1826–1837.

Kim, G.S., and Batt, C.A. 1993. Cloning and sequencing of the *groEL* operon in *Lactococcus lactis* subsp. *lactis*. Gene 127: 121–126.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 227: 680–685.

Lin, J., Lee, I.S., Frey, J., Slonczeweski, J.L., and Foster, J.W. 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*. J. Bacteriol. 177: 4097–4104.

Lorca, G.L., and Font de Valdez, G. 2001. Acid tolerance mediated by membrane ATPases in *Lactobacillus acidophilus*. Biotechnol. Lett. 23: 777–780.

Lorca, G.L., and Font de Valdez, G. 2001. A-low-pH-inducible stationary-phase acid tolerance response in *Lactobacillus acidophilus* CRL 639. Curr. Microbiol. 42: 21–25.

Lorca, G.L., Raya, R.R., Taranto, M.P., and de Valdez G. F. 1998. Adaptative acid tolerance response in *Lactobacillus acidophilus*. Biotechnol. Lett. 20: 239–241.

Mandelstam, J. 1958. Turnover of protein in growing and non-growing populations of *Escherichia coli*. Biochem. J. 69: 110–119.

Melnykovych, G., and Snell, E.S. 1958. Nutritional requirements for the formation of arginine decarboxilase in *Escherichia coli*. J. Bacteriol. 76: 518–523.

Noh, D.O., and Gilliland, S.E. 1993. Influence of bile on cellular integrity and β-galactosidase activity of *Lactobacillus acidophilus*. J. Dairy Sci. 76: 1253–1259.

Oakley, B.R., Kirsch, D.R., and Morris, N.R. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. 105: 361–363.

Rallu, F., Gruss, A., and Maguin, E. 1996. *Lactococcus lactis* and stress. Antonie Leeuwenhock 70: 243–251.

Reeve, C.A., Bockman, A.T., and Matin, A. 1984. Role of protein degradation in the survival of carbon-starved *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. 157: 758–763.

Van Asseldonk, M., Simons, A., Visser, H., De Vos, W., and Simons, G. 1993. Cloning, nucleotide sequence, and regulatory analysis of the *Lactococcus lactis dnaJ* gene. J. Bacteriol. 175: 1637–1644.

Van Bogelen, R.A., Kelley, P.M., and Neidhardt, F.C. 1987. Differential induction of heat shock, SOS, and oxidative stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. 169: 26–32.