The acid-stress response in *Lactobacillus sanfranciscensis* CB1

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***Lactobacillus sanfranciscensis* CB1, an important sourdough lactic acid bacterium, can withstand low pH after initial exposure to sublethal acidic conditions. The sensitivity to low pH varied according to the type of acid used. Treatment of *Lb. sanfranciscensis* CB1 with chloramphenicol during acid adaptation almost completely eliminated the protective effect, suggesting that induction of protein synthesis was required for the acid-tolerance response. Two constitutively acid-tolerant mutants, CB1-5R and CB1-7R, were isolated using natural selection techniques after sequential exposure to lactic acid (pH 3.2). Two-dimensional gel electrophoresis analysis of protein expression by non-adapted, acid-adapted and acid-tolerant mutant cells of *Lb. sanfranciscensis* showed changes in the levels of 63 proteins. While some of the modifications were common to the acid-adapted and acid-tolerant mutant cells, several differences, especially regarding the induced proteins, were determined. The two mutants showed a very similar level of protein expression. Antibodies were used to identify heat-shock proteins DnaJ, DnaK, GroES and GrpE. Only GrpE showed an increased level of expression in the acid-adapted and acid-tolerant mutants as compared with non-adapted cells. The N-terminal sequence was determined for two proteins, one induced in both the acid-adapted and mutant cells and the other showing the highest induction factor of those proteins specifically induced in the acid-adapted cells. This second protein has 60% identity with the N-terminal portion of YhaH, a transmembrane protein of *Bacillus subtilis*, which has 54 and 47% homology with stress proteins identified in *Listeria monocytogenes* and *Bacillus halodurans*. The constitutively acid-tolerant mutants showed other different phenotypic features compared to the parental strain: (i) the aminopeptidase activity of CB1-5R decreased and that of CB1-7R markedly increased, especially in acid conditions; (ii) the growth in culture medium at 10 °C and in the presence of 5% NaCl was greater (the same was found for acid-adapted cells); and (iii) the acidification rate during sourdough fermentation in acid conditions was faster and greater.**

Keywords: lactic acid bacteria, acid-tolerant mutant, protein expression, sourdough

INTRODUCTION

It is well known that microbial growth is a self-limiting process and is often suboptimal in nature compared to controlled conditions in the laboratory. Natural stresses like acidity and sometimes starvation are generated by

cell growth itself, while other stresses (e.g. temperature, osmotic shock or oxygen) are induced by the environment (Foster, 1999).

Studies on the adaptive physiology of lactic acid bacteria facilitate efforts to improve their activity in industrial applications. Most studies have considered *Lactococcus lactis* subsp. *lactis* because of its extensive use in dairy products. Heat-shock proteins DnaK, DnaJ and GrpE, which constitute a three-component chaperone system

Abbreviations: 2D, two-dimensional; NA, nitroanilide.

in *Escherichia coli* (Diamant & Goloubinoff, 1998), as well as GroES and GroEL, have been identified in *Lc. lactis* (Rallu *et al.*, 1996), where they are necessary for proper refolding of proteins, and/or for targeting denatured proteins for degradation by cytoplasmic proteases. The RecA protein, which is important in the response to several environmental stresses such as DNA damage, oxygen and heat stress (Duwat *et al.*, 1995), and a large protein complex which includes ClpP (caseinolytic protease) and is involved in degrading misfolded proteins (Frees & Igmer, 1999), were also studied in *Lc. lactis*. H⁺-ATPase is another of the acid tolerance response proteins induced by acid intracellular pH (O'Sullivan & Condon, 1999). Cellular metabolic pathways are closely related to stress response, and the flux of particular metabolites, notably guanine and phosphate, may be implicated in stress responses of lactococci (Duwat *et al.*, 1999). The adaptive response to several stresses varied between *Lc. lactis* subsp. *lactis* and subsp. *cremoris*; the former can adapt better to acid, bile-salt and freezing stresses (Kim *et al.*, 1999). As a conclusion, while some of the stress-induced genes seem to be genuinely specific, others are induced by a wide variety of stresses, and are thus thought to be general stress-response genes (Hecker *et al.*, 1996).

Lactobacilli have received less attention than *Lc. lactis*. The *htrA* gene, encoding a stress-inducible HtrA-like protein, has been cloned, sequenced and characterized in *Lactobacillus helveticus* CNRZ32 (Smeds *et al.*, 1998). After thermotolerance experiments, heat-shock proteins such as DnaK, GroEL, ClpB and GrpE were detected in *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lb. helveticus* (Broadbent *et al.*, 1997). The growth of *Lactobacillus plantarum* is more severely inhibited by salt stress than by equi-osmolar concentrations of sugars, which reflects the inability of the cells to accumulate enough K⁺ (or Na⁺) to restore turgor, as well as to prevent deleterious effects of the electrolytes intracellularly (Glaasker *et al.*, 1998). Acid- and bile-resistant variants of *Lb. acidophilus* capable of growing at pH 3.5 with 0.3% bile were isolated using natural selection techniques after sequential exposure to hydrochloric acid (Chou & Weimer, 1998).

To our knowledge, no studies have been published on the stress responses of sourdough lactobacilli. The study of the stress responses of sourdough lactic acid bacteria is worthwhile, to improve the use of this natural starter, to have a more complete knowledge about the physiology of these industrial bacteria and to make a comparison between lactic acid bacteria which populate different environments and dairy strains. During routine use, sourdough bacteria are mainly subjected to cold and acid stresses which are inherent to refrigerated storage (about 24–48 h at 4 °C) of sourdough (pH about 3.4–4.0) after fermentation and before being used. Such stresses interfere with the constant microbial composition and with the performance of the sourdough (Gobbetti, 1998).

In this paper, we describe the acid-stress response of *Lactobacillus sanfranciscensis* CB1, the isolation of

constitutively acid-tolerant mutants, the improved growth properties of acid-adapted and mutant cells in restrictive environmental conditions (10 °C and 5% NaCl), the modification of the protein levels after acid adaptation, the identification in the mutants of DnaK, DnaJ, GrpE and GroES proteins, the N-terminal sequencing of proteins and their homology with previously identified stress proteins, and the use of mutants to improve sourdough fermentation in specific conditions.

METHODS

Bacterial strain and growth conditions. *Lb. sanfranciscensis* CB1 from the Culture Collection of the Dipartimento di Scienze degli Alimenti, Sezione di Microbiologia Agro-alimentare, University of Perugia, Italy, was used. Cultures were grown at 30 °C in modified MRS, which contained 0.5% maltose, 1.0% peptone, 0.5% yeast extract, 1 ml Tween 80 l⁻¹, 0.2% K₂HPO₄, 0.2% ammonium citrate, 0.02% MgSO₄ and 0.005% MnSO₄. The pH of the modified MRS was 6.4 and, unless otherwise stated, it was maintained constant by the on-line addition of 1 M NaOH.

Adaptation conditions and measurement of acid tolerance. Cells grown at a constant pH of 6.4 were harvested in the mid-exponential growth phase (OD₆₂₀ 0.6). To induce acid adaptation (adapted cells), cells were resuspended at the same OD₆₂₀ value in fresh medium, which prior to use was adjusted to pH 5.0 by approximately 14 mM lactic acid. Control cells were resuspended in fresh medium at pH 6.4 (non-adapted cells). After incubation at 30 °C for 1 h, control and adapted cells were harvested by centrifugation, washed and resuspended in modified MRS acidified to pH 3.2, 3.4, 3.6, 3.8 and 4.0 with concentrations of lactic acid which ranged from approximately 43 to 132 mM. The cell density was then adjusted to OD₆₂₀ 1.0 and the cultures were incubated for 10 h at 30 °C. Constitutively acid-tolerant mutant cells (see below), were used in the same conditions as the control. Challenges at pH 3.4 were also carried out by setting the pH with approximately 41 mM HCl and with a mixture of approximately 86 mM lactic acid and approximately 38 mM acetic acid (molar ratio approx. 2:3). The pH range (3.2–4.0) and the concentrations of lactic acid or a mixture of lactic and acetic acids were in line with the pH values and the concentrations of metabolic end-products which are usually found after sourdough fermentation (Gobbetti, 1998). When the acid tolerance was measured in the presence of acid or mixtures other than lactic acid alone, the adaptation of cells at pH 5.0 was also induced with the corresponding acid or mixture of acids.

Chloramphenicol (100 µg ml⁻¹) was used in the experiments according to Hartke *et al.* (1996). It was added to mid-exponential-phase cells which were harvested and resuspended in fresh medium at pH 6.4 and held at 30 °C for 10–60 min. After incubation, the cells were harvested by centrifugation, washed with fresh medium, subjected to acid adaptation and used for acid challenges. Chloramphenicol was also added to cells directly during adaptation.

Cell numbers were determined by plating on SDB agar medium (Kline & Sugihara, 1971) immediately after resuspension and at different time intervals. Numbers of c.f.u. were determined after 48 h incubation at 30 °C. The number of surviving micro-organisms was calculated as a percentage of the cell number at time zero. The tolerance factor corresponded to the ratio of the survival of adapted cells to that of control cells.

Isolation of acid-tolerant mutants of *Lb. sanfranciscensis*

CB1. The treatment for isolation of acid-tolerant mutants was according to the protocol of O'Driscoll *et al.* (1996). Exponential-phase cells of *Lb. sanfranciscensis* CB1 grown in modified MRS at constant pH of 6.4 were harvested by centrifugation and resuspended in the same medium at pH 3.2. After incubation for 24 h at 30 °C, the cells were harvested, washed in quarter-strength Ringer's solution and plated in modified MRS agar, pH 5.6, at 30 °C for 36 h. Individual survivors were further grown in modified MRS broth at constant pH of 6.4 (exponential growth phase) and subsequently subjected to five repeated treatments to increase the tolerance at the lethal pH. In this manner, seven mutants constitutively resistant to low pH in the absence of induction were isolated. Two of them, designated as CB1-5R and CB1-7R, were further analysed.

Two-dimensional (2D) electrophoresis. Control cells were grown in modified MRS at constant pH 6.4 until the mid-exponential growth phase (OD_{620} 0.6) was reached and used for 2D electrophoresis without acid adaptation. The same growth conditions were used for the constitutively acid-tolerant mutants. Acid-adapted cells were obtained after 60 min exposure of mid-exponential-phase cells in modified MRS adjusted to pH 5.0 with approximately 14 mM lactic acid. Non-adapted (control) cells, lactic-acid-adapted cells and the acid-tolerant mutant cells were chilled on ice, diluted 1:10 with stop solution (0.1 M Tris/HCl pH 7.5 containing 1 mg chloramphenicol ml⁻¹) and the suspensions were centrifuged at 4 °C and 15000 g for 10 min. Harvested cells were washed in 0.05 M Tris/HCl pH 7.5, containing 0.1 mg chloramphenicol ml⁻¹, centrifuged (15000 g for 10 min), and frozen or directly resuspended in denaturing buffer composed of 8 M urea, 4% CHAPS, 40 mM Tris base and 65 mM dithioerythritol (DTE). Cells were disrupted with a Branson model B15 sonifier by four cycles of sonication (20 s each). After pelleting of unbroken cells (15000 g for 15 min at 4 °C) the protein content was measured by the method of Bradford (1976). Two-dimensional gel electrophoresis was performed using the immobililine/polyacrylamide system, essentially as described by Görg *et al.* (1988) and Hochstrasser *et al.* (1988). The same amount of 60 µg (analytical runs) or about 1 mg (preparative runs for immunoblotting analysis) of total protein was used for each electrophoretic run. Isoelectric focusing was carried out on immobililine strips providing a non-linear 3–10 pH gradient (IPG strips, Amersham Pharmacia Biotech) by IPG-phor, at 15 °C. Voltage was increased from 300 to 5000 V during the first 5 h, then stabilized at 8000 V for 8 h. After electrophoresis, IPG strips were equilibrated for 12 min against 6 M urea, 30% (w/v) glycerol, 2% SDS, 0.05 M Tris/HCl, pH 6.8 and 2% DTE, and for 5 min against 6 M urea, 30% (w/v) glycerol, 2% SDS, 0.05 M Tris/HCl, pH 6.8, 2.5% iodoacetamide and 0.5% bromophenol blue. The second dimension was carried out in a Laemmli (1970) system on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm), at 40 mA per gel constant current and 10 °C for approximately 5 h until the dye front reached the bottom of the gel. Preliminary SDS-PAGE runs at different linear concentrations and gradients of polyacrylamide were carried out to find the optimal protein separation. Under our experimental conditions, the gradient 9–16% polyacrylamide gave the best separation for high to medium molecular mass proteins. Gels were calibrated with two molecular mass markers: co-migration of the cell extracts with human serum proteins for the molecular mass range 200–10 kDa and markers from Pharmacia Biotech for low molecular mass range (16.9, 14.4, 10.7, 8.2, 6.2 and 2.5 kDa). The electro-

phoretic co-ordinates used for serum proteins were according to Bjellqvist *et al.* (1993). Analytical gels were silver stained as described by Hochstrasser *et al.* (1988) and Oakley *et al.* (1980). The protein maps were scanned with a laser densitometer (Molecular Dynamics 300s) and analysed with the Melanie II computer software (Bio-Rad). Four gels of independently grown replicates were analysed and spot intensities were normalized as reported by Bini *et al.* (1997). In particular, the spot quantification for each gel was calculated as relative volume (% VOL): the volume of each spot divided by the total volume over the whole image. In this way, differences of the colour intensities among the gels were eliminated (Appel & Hochstrasser, 1999). The induction factor was defined as the ratio between the spot intensity of the same protein in the adapted cells or mutant strains and in the non-adapted cells. All the induction factors were calculated based on the mean of the spot intensities of all four gels, with standard deviations. Only induction factors with a statistical significance at the $P < 0.05$ level of probability are reported in Table 1. Since induction factors were calculated as means of four gels, some of the spot intensities of Figs 3(a–d), which each show only one gel, may not visually agree with the induction factor values reported in Table 1.

Immunoblot analysis. Gels were electroblotted on nitrocellulose membranes according to Towbin *et al.* (1979) and further processed by standard procedures, modified as described by Bini *et al.* (1999) and Magi *et al.* (1999). Briefly, before immunodetection, the membranes were stained with 0.2% (w/v) PonceauS in 3% (w/v) trichloroacetic acid for 3 min and the positions of selected landmark spots were marked on the membrane to assist subsequent matching of the immunoblots with the silver-stained map. Immunoreactive spots were detected by overnight incubation at room temperature with 1:2000 antibodies for DnaK, DnaJ and GrpE; 1:1000 for GroES, followed by incubation with 1:7000 conjugated peroxidase (Sigma), and revealed with a chemiluminescence based kit (ECL Pharmacia Amersham Biotech).

N-terminal amino acid sequence determination. 2D maps were prepared as described above starting from 1 mg sample per run, and blotted onto polyvinylidene difluoride membranes (Bio-Rad; 20 × 20 cm, 0.2 µm pore size) according to Matsudaira (1987). The blots were stained with 0.1% (w/v) Coomassie brilliant blue R250 in 50% methanol for 5 min, and destained in 40% methanol, 10% acetic acid. Membranes were air-dried at 37 °C and stored at –20 °C for further analysis. Selected protein spots were cut out and submitted to amino acid sequencing by Edman degradation using an automatic protein/peptide sequencer (model 470A; Applied Biosystems) connected on-line with a phenylthiohydantoin-amino acid analyser model 120A and a control/data module model 900A (Applied Biosystems). Typically three or four equivalent spots from similar blots were used, according to the estimated relative molar amount of protein in the spot. The programs included in the GCG package (Genetics Computer Group, Madison, WI, USA) were used for sequence analysis. BLAST and FASTA sequence comparisons were performed in the SWALL database.

Peptidase activity. Both non-adapted cells and constitutively acid-tolerant mutants were assayed for proteinase, aminopeptidase and iminopeptidase activities by using fluorescent casein, Lys-Leu-, Iso- and Pro-*p*-nitroanilide (NA) substrates, respectively (Gobbetti *et al.*, 1997). The enzyme assays were carried out in 0.05 M phosphate buffer pH 7.0 and 5.5. One unit (U) of proteinase activity was expressed as an increase of 0.1 unit of fluorescence per 10 min. One unit of aminopeptidase and iminopeptidase activities was defined as an

Table 1. Properties of acid-induced proteins after lactic acid adaptation or which are present in elevated amounts in the acid-tolerant mutants

Analysis was performed with the Melanie II computer software (Bio-Rad). Four gels of independently grown replicates were analysed. For spot quantification see Methods.

Spot designation*	Estimated pI	Estimated mol. mass (kDa)	Induction factor†		
			Adapted cells	Mutant strain	
				CB1-5R	CB1-7R
5	4.52	62.4	n	10 ± 0.3	10 ± 0.5
8	5.6	60.5	3 ± 0.1	2 ± 0.05	n
16	6.2	36.9	2 ± 0.06	n	n
17	4.49	36.9	2.5 ± 0.08	2 ± 0.06	n
18	5.68	34.4	n	5 ± 0.08	5 ± 0.15
19	4.68	34.4	2 ± 0.07	2 ± 0.05	2 ± 0.06
22	7.93	33.4	n	10 ± 0.5	10 ± 0.4
23	5.08	33.3	n	5 ± 0.08	5 ± 0.1
27	5.87	28.7	2.5 ± 0.1	10 ± 0.4	8 ± 0.2
28	8.02	28.6	3 ± 0.1	4 ± 0.15	3 ± 0.2
33	4.35	25.5	n	10 ± 0.25	10 ± 0.2
34	6.5	23.0	2 ± 0.07	n	n
36	5.01	22.5	3.5 ± 0.1	4 ± 0.06	4 ± 0.05
37	5.60	21.8	n	10 ± 0.3	10 ± 0.5
39	7.38	21.7	n	10 ± 0.5	10 ± 0.4
40	4.35	21.7	5 ± 0.15	2 ± 0.01	n
41	8.03	21.7	4.5 ± 0.2	10 ± 0.25	10 ± 0.4
42	4.92	19.4	n	10 ± 0.3	10 ± 0.5
43	4.47	19.4	2 ± 0.09	n	n
44	6.74	18.1	2 ± 0.08	2 ± 0.05	3 ± 0.1
45	5.26	18.1	n	10 ± 0.4	10 ± 0.5
46	5.37	17.2	n	7.0 ± 0.1	7.5 ± 0.3
47	4.49	17.2	2.5 ± 0.1	n	n
50	6.09	16.0	n	5 ± 0.18	4 ± 0.2
51	5.07	15.9	n	2 ± 0.05	2 ± 0.06
53	5.36	15.3	n	10 ± 0.5	10 ± 0.4
54	5.31	13.7	2 ± 0.06	4 ± 0.07	3 ± 0.1
55	5.57	13.0	n	3 ± 0.1	2 ± 0.08
58	7.85	13.0	2 ± 0.08	3.0 ± 0.25	3.5 ± 0.1
60	5.23	10.9	n	8.0 ± 0.37	7.5 ± 0.1

* The numbers of the proteins correspond to those on the gels in Fig. 3(a–d).

† The induction factor was defined as the ratio between the spot intensity of the same protein in the adapted cells or mutant strains and in the non adapted cells. All the induction factors were calculated based on the mean of the spot intensities of each four gels and are shown ± SD. Only induction factors with a statistical significance at the $P < 0.05$ level of probability were reported. n, No increase of the spot intensity with respect to the non-adapted cells detected.

increase of 0.01 unit of A_{410} per min. Specific activities for all enzymes were determined as units of total activity per mg biomass. All the proteolytic activity results were expressed as the mean of at least five independent assays.

Partial purification of an intracellular aminopeptidase from *Lb. sanfranciscensis* CB1. Five litres of a *Lb. sanfranciscensis* CB1 culture grown in modified MRS medium, pH 6.4, at 30 °C for 24 h were harvested by centrifugation and used for subcellular fractionation by lysozyme treatment in 50 mM Tris/HCl (pH 7.5) buffer, containing 24% sucrose (Gobbetti

et al., 1996). The cytoplasmic fraction was freeze-dried (MOD E1PTB, Edwards, Milan, Italy), concentrated 20-fold by resuspending in 20 mM phosphate buffer (pH 6.5) and dialysed for 24 h at 4 °C against the same buffer. This preparation was applied to a Q-Sepharose HR 16/50 column (Pharmacia-Biotech), previously equilibrated with 20 mM phosphate buffer (pH 6.5). After loading, proteins were eluted with a linear NaCl gradient, 0–0.6 M, at a flow rate of 18 ml h⁻¹. The fractions with the highest aminopeptidase activity were pooled, dialysed for 24 h at 4 °C against distilled water, concentrated 10-fold by freeze-drying, redissolved in a small

volume of 50 mM phosphate buffer (pH 6.5) and further purified by gel filtration. A Sephacryl 200 column (Pharmacia-Biotech), equilibrated with 50 mM phosphate buffer (pH 6.5), was used and elution with the same buffer was performed at a flow rate of 11 ml h⁻¹. The most active fractions were pooled, dialysed, freeze-dried and resuspended in 20 mM Bistris-propane (pH 6.5). The last step of purification was carried out on an FPLC Mono Q HR 5/5 column (Pharmacia-Biotech). After loading of the sample, elution was performed with a linear gradient of 0–0.3 M NaCl at a flow rate of 0.75 ml min⁻¹ with 20 mM Bistris-propane (pH 6.5). The only fraction corresponding to the peak of aminopeptidase activity was dialysed, freeze-dried and used for 2D electrophoresis (see below). Aminopeptidase activity assays during purification steps were carried out by using Lys-p-NA as reported elsewhere.

Growth at 10 °C and in the presence of 5 % NaCl. Growth at 10 °C was assayed by sudden transfer of mid-exponential-phase control, lactic-acid-adapted and constitutively acid-tolerant mutant cells of *Lb. sanfranciscensis* to modified MRS medium (initial OD₆₂₀ 0.2) at 10 °C and further incubation for 24 h at the same temperature. The ability of cultures to grow with high salt concentrations was examined by inoculating, in the same conditions, the control, lactic-acid-adapted and constitutively acid-tolerant mutant cells on modified MRS medium containing 5 % NaCl and incubating further at 30 °C for 24 h.

Sourdough fermentation. The characteristics of the wheat flour used were the same as those previously described (Gobbetti *et al.*, 1999). Wheat flour (250 g), 110 ml tap water and 40 ml cell suspension containing approximately log 9.0 c.f.u. ml⁻¹ of *Lb. sanfranciscensis* CB1, CB1-5R or CB1-7R were used to produce 400 g dough (dough yield = 160) with a continuous high-speed mixer (60 g) and mixing time of 5 min. The cell suspensions of strains CB1, CB1-5R and CB1-7R were made by using mid-exponential-phase cells cultivated at constant pH 6.4. The sourdough fermentation was carried out for 6 h at 30 °C and the initial pH of the dough was 5.6 or 4.4. The pH 4.4 dough was obtained by adding a mixture of lactic and acetic acid in a molar ratio of 2:3. The initial cell concentration of lactobacilli in the dough was approximately log 7.0 c.f.u. g⁻¹ as determined by plating on SDB agar medium. All the sourdough fermentation results were expressed as the mean of at least four independent assays.

Statistical analysis. All the data were analysed by a one-way analysis of variance. Statistical analyses were carried out using the SAS package (SAS Institute, 1985). Significant differences were defined at $P < 0.05$.

RESULTS

Acid sensitivity of *Lb. sanfranciscensis* CB1

A mid-exponential-phase culture of strain CB1 was transferred to modified MRS medium at pH 4.0, 3.8, 3.6, 3.4 and 3.2, which was set by adding lactic acid or HCl. Whereas the decrease in cell survival in the pH range 3.6–4.0 was limited to two or less orders of magnitude, at both pH 3.4 and 3.2 cell survival markedly decreased with increased incubation time (Fig. 1). At pH values higher than 4.2 cell survival did not decrease (data not shown). *Lb. sanfranciscensis* CB1 was less sensitive at pH 3.4 when the pH was set by adding HCl. A pH value of 3.4 set with lactic acid was chosen for subsequent experiments.

Fig. 1. Resistance of *Lb. sanfranciscensis* CB1 to a lactic acid stress at pH 4.0 (●), 3.8 (○), 3.6 (▲), 3.4 (△) and 3.2 (◆), and to HCl stress at pH 3.4 (◇). The data are means of four to six independent experiments; the bars indicate SD.

Fig. 2. The diamonds and squares show stress resistance of non-adapted (filled symbols) and pH 5.0-adapted (empty symbols) cells of *Lb. sanfranciscensis* CB1 to a lactic acid (◆, ◇), and lactic plus acetic acid (molar ratio 2:3) (■, □) stress at pH 3.4. ▲, △ Stress resistance of mutant cells: CB1-5R (▲) and CB1-7R (△) to lactic acid stress at pH 3.4. ●, Cells adapted at pH 5.0 in the presence of 100 µg chloramphenicol ml⁻¹. The data are means of four to six independent experiments; bars indicate SD.

Adaptive response of *Lb. sanfranciscensis* CB1 to acid pH

Preliminary experiments at different pH values (4.5, 5.0, 5.5 and 6.0, adjusted with lactic acid) for different times (30, 60 and 90 min) showed that mid-exponential-phase cells (pH 6.4) shifted to pH 5.0 for 1 h had the highest degree of resistance to subsequent acid challenges (data not shown). The tolerance factor induced by adaptation at pH 5.0 for 1 h was about 4×10^3 after 10 h exposure to the challenge pH (Fig. 2). When a mixture of lactic and acetic acid (molar ratio approx. 2:3) was used to

produce the challenge pH 3.4, the tolerance factor increased to about 3×10^4 . In agreement with other authors (O'Driscoll *et al.*, 1997), the same tolerance factor was found by adaptation of cells to pH 5.0 either with the corresponding mixture of organic acids or with lactic acid alone. All these results did not vary when the challenge pH was 3.2 (data not shown). When 100 µg chloramphenicol ml⁻¹, which is bacteriostatic and blocks protein synthesis, were added during the adaptive shift to pH 5.0 (1 h), the tolerance factor decreased to less than 10 (Fig. 2). The incubation of *Lb. sanfranciscensis* CB1 cells with chloramphenicol at pH 6.4 for 10–60 min before adaptation at pH 5.0 did not prevent the adaptive response to the acid challenge (data not shown).

Isolation of acid-tolerant mutants of *Lb. sanfranciscensis* CB1

Constitutively acid-tolerant mutants of *Lb. sanfranciscensis* CB1 were isolated following prolonged exposure (24 h) to the challenge pH 3.2. Survivors were grown at pH 6.4 and repeatedly screened for acid resistance. Seven such isolates exhibited an acid-tolerant phenotype; two of them, CB1-5R and CB1-7R, were further used in this study. The mutants were acid tolerant as the adapted parental cells (Fig. 2), although CB1-7R had a slightly lower survival than CB1-5R during the last few hours of incubation.

2D analysis of acid-induced modifications in protein synthesis

Compared to the whole-cell protein extracts of the non-adapted cells, the modifications found in the acid-adapted CB1 and the constitutively acid-tolerant mutants (CB1-5R and CB1-7R) concerned 63 proteins (Fig. 3a–d, Table 1). The synthesis of 17 proteins, which were mainly located at pI around 6.7 and molecular mass of approximately 10–28 kDa, decreased in the acid-adapted cells. Eight of these proteins also showed reduction in the constitutively acid-tolerant mutants CB1-5R and CB1-7R, which also showed a decrease in the expression of 16 (CB1-5R) or 14 (CB1-7R) other proteins, which mainly had molecular masses greater than 33 kDa. On the other hand, 15 proteins in the acid-adapted cells and 26 proteins in the acid-tolerant mutant CB1-5R (11 of which were common) increased in amount compared to non-adapted cells. Protein 40 was markedly expressed in the acid-adapted cells, showing the highest induction factor among the proteins that were specifically induced in this strain. Interestingly, the constitutively acid-tolerant mutants had a very similar level of protein expression, which only differed for proteins 8, 17, 40, 52 and 59.

Identification of heat-shock proteins by Western blotting

Antibodies against DnaK, DnaJ and GroES detected single proteins with experimental molecular masses of 70, 60 and 10.7 kDa and pIs of 4.4, 7.82 and 7.5,

respectively (Fig. 3a–d). There were no appreciable differences in the level of expression of these three proteins among the non-adapted, acid-adapted and acid-tolerant mutant cells. Antibodies against GrpE reacted with three proteins. Based on the 2D-electrophoretic coordinates of *Lb. acidophilus*, *Lb. casei*, *Lb. helveticus* and *Lc. lactis* homologues (Broadbent *et al.*, 1997; Hartke *et al.*, 1996), protein 36 was identified as GrpE (Fig. 3a–d, Table 1). It showed an induction factor of 3.5 and 4 in the acid-adapted cells and the acid-tolerant mutants, respectively.

Identification of proteins separated by 2D electrophoresis by N-terminal amino acid sequencing

Protein 19, which showed a similar induction factor in both the acid-adapted cells and acid-tolerant mutants, and protein 40, which had the highest induction factor among those proteins specifically induced in the acid-adapted cells and was also expressed in the constitutively acid-tolerant mutant CB1-5R, were subjected to N-terminal sequencing. The sequences were XKEYND and SFKKGLFLGTILGGAA, respectively. Regarding protein 40, comparison of the 15 amino acid sequence with protein databases revealed a 60% identity with the N-terminal portion of YhaH, a 13.1 kDa hypothetical protein of *Bacillus subtilis* (Kunst *et al.*, 1997).

The SWISS-PROT accession numbers for the acid-shock proteins are P82648 for spot 40 and P82655 for spot 19.

Peptidase activity of acid-tolerant mutants and partial purification of an intracellular aminopeptidase

As stated by other authors (Chou & Weimer, 1998), in addition to the main character selected (acid tolerance), the presence of multiple phenotypic changes is frequent in selected mutants. Compared to non-adapted cells of strain CB1, the mutants CB1-5R and CB1-7R had a variable aminopeptidase and iminopeptidase activity: 10.1 ± 0.8 and 23.6 ± 1.2 vs 16.8 ± 1.0 , and 6.2 ± 0.5 and 11.4 ± 0.7 vs 6.3 ± 0.2 U mg⁻¹, respectively. In particular, the difference in the aminopeptidase activity between mutant CB1-7R and the non-adapted cells was enhanced when the enzyme assays was conducted at pH 5.5: 37.2 ± 1.5 vs 9.5 ± 0.7 U mg⁻¹. The aminopeptidase activity did not vary when Lys-, Leu- and Iso-*p*-NA substrates were used. The proteinase activity did not differ between the three strains.

An intracellular aminopeptidase from the cytoplasmic extract of *Lb. sanfranciscensis* CB1 was partially purified by three chromatographic steps. The fraction which contained the highest aminopeptidase activity after elution on an FPLC Mono Q column was subjected to 2D electrophoresis. It contained four proteins with experimental molecular masses of 75, 23, 22.1 and 17.8 kDa and pIs of 4.88, 5.6, 6.74 and 4.95, respectively (data not shown). These proteins corresponded to spots located in the same position of the whole-cell protein extracts of

Fig. 3. 2D electrophoresis analysis of protein expression in *Lb. sanfranciscensis* CB1. (a) Cells grown at constant pH 6.4 (non-adapted cells); (b) cells adapted at pH 5.0 for 1 h with lactic acid (acid-adapted); (c, d) constitutively acid-tolerant mutants CB1-5R (c) and CB1-7R (d). Numbered squares and circles refer to proteins which are present in decreased and increased amounts, respectively, compared to non-adapted cells. The positions of proteins which reacted with antibodies against DnaJ, DnaK, GroES and GrpE are indicated.

non-adapted, acid-adapted and constitutively acid-tolerant mutant cells. In particular, the protein with a molecular mass of 75 kDa and pI 4.88 corresponds to spot 1 of Fig. 3(a–d). In a previous paper dealing with the proteolytic system of *Lb. sanfranciscensis* CB1 (Gobbetti *et al.*, 1996), a 75 kDa intracellular aminopeptidase was purified to homogeneity and characterized.

Growth at 10 °C and in the presence of 5 % NaCl

Non-adapted, acid-adapted and constitutively acid-tolerant mutant cells of *Lb. sanfranciscensis* CB1 were also assayed for growth at 10 °C and in the presence of 5 % NaCl, which represent restrictive conditions for the storage and use of sourdough lactobacilli. Both acid-adapted and mutant cells showed a moderately higher

Fig. 4. Growth of non-adapted (□), pH 5.0-adapted (■) and non-adapted mutant cells [CB1-5R (○) and CB1-7R (△)] of *Lb. sanfranciscensis* CB1 in modified MRS at 10 °C (a) and in the presence of 5% NaCl at 30 °C (b). ▲, Typical growth curve of *Lb. sanfranciscensis* CB1 in the absence of NaCl and at 30 °C. The data are means of four to six independent experiments; bars indicate SD.

growth at 10 °C compared to non-adapted cells (Fig. 4a). The final cell concentration was approximately 8.6 vs 8.1 $\log \text{ c.f.u. ml}^{-1}$, respectively. These differences were enhanced after growth in modified MRS containing 5% NaCl, which seemed to be lethal for non-adapted cells (Fig. 4b). Acid-adapted and, especially, mutant cells grew in this culture condition, whereas non-adapted cells of *Lb. sanfranciscensis* CB1 did not grow during the first 10 h of incubation, then showed a decrease of viability. After 24 h of incubation, the constitutively acid-tolerant mutant CB1-5R had a cell concentration of approximately 8.6 vs 7.0 c.f.u. ml^{-1} for the non-adapted cells.

Sourdough fermentation

Sourdoughs produced by strains CB1, CB1-5R and CB1-7R were compared. If the initial pH of the dough was 5.6, CB1 lowered the pH to 3.86 ± 0.02 as compared to 3.97 ± 0.02 with strain CB1-7R after 6 h of fermentation. However, if the initial pH was 4.4 the sourdough

produced with acid-tolerant mutant CB1-7R reached $\text{pH } 3.64 \pm 0.02$ compared to 3.97 ± 0.03 with the parental strain CB1. While no differences in the cell concentration were found during the sourdough fermentation started at pH 5.6 (approx. $\log 8.5 \text{ c.f.u. g}^{-1}$), when the initial pH was 4.4 the cell concentration of lactobacilli increased from approximately $\log 7.0$ to $8.2 \text{ c.f.u. g}^{-1}$ in the dough started with CB1-7R and there was no growth when the parental strain CB1 was used. Mutant CB1-5R behaved similarly to CB1-7R.

DISCUSSION

During routine use, sourdough bacteria are subjected to several stresses (e.g. osmotic, starvation and low temperature). Acid stress is probably the most relevant to the sourdough process because it may interfere with the microbial composition and therefore with the performance of the sourdough during straight or repeated use (Gobbetti, 1998). The availability of acid-adapted cells or acid-tolerant mutants could greatly improve the efficacy and consistency of industrial sourdough production. To our knowledge, no studies have yet been published on the stress responses of sourdough lactobacilli. Furthermore, studies on the acid-stress responses of lactobacilli, which have only considered *Lb. acidophilus* (Chou & Weimer, 1998) and *Lb. plantarum* (McDonald *et al.*, 1990), did not analyse changes in their protein expression patterns.

Acid-stress response

Overall, *Lb. sanfranciscensis* CB1 showed an inherent acid tolerance higher than that of dairy lactic acid bacteria. Whereas lactococcal strains were characterized by a very low acid resistance to a pH of approximately 4.0 (Hartke *et al.*, 1996; Rallu *et al.*, 1996), the same pH value had only a slight effect on the survival of this sourdough bacterium. Although the pH of fermented foods does not necessarily correlate with cell survival, in the case of *Lb. sanfranciscensis* CB1 it seems in agreement with the very high acidity (pH 3.3–3.7) which usually characterizes sourdough at the end of fermentation (Gobbetti, 1998).

The survival of *Lb. sanfranciscensis* CB1 decreased dramatically when cells grown at constant pH 6.4 were suddenly subjected to pH 3.2–3.4. The effect of various acids on the survival and therefore on the intracellular pH of *Lb. sanfranciscensis* CB1 varied.

The acid-stress tolerance of *Lb. sanfranciscensis* CB1 depended on the induction of protein synthesis. The tolerance markedly decreased when a bacteriostatic concentration of chloramphenicol ($100 \mu\text{g ml}^{-1}$) was added during adaptation. The incubation of *Lb. sanfranciscensis* with chloramphenicol for 10–60 min at pH 6.4, subsequent washing of the cells and further adaptation did not prevent adaptive response to the acid challenge, meaning that probably proteins synthesized before exposure to the sublethal pH 5.0 were not directly

responsible for the acid-stress tolerance. Similar results were obtained by studying the acid-stress response of *Lc. lactis* (O'Sullivan & Condon, 1997, 1999; Rallu *et al.*, 1996) and *Listeria monocytogenes* (O'Driscoll *et al.*, 1997).

The acid-adapted cells and the acid-tolerant mutant showed similarities by 2D analysis: 8 proteins which decreased as well as 8 or 11 proteins (depending on the mutant) which increased their level of expression as compared to the non-adapted cells were common. Several proteins were highly induced only in the lactic-acid-adapted cells or in the acid-tolerant mutant (induction factors from 5 to 10). A comparison between the two mutants revealed that among a total of 27 proteins (CB1-5R plus CB1-7R) overexpressed compared to the parental CB1, 21 showed no significant differences in the level of expression between the two mutants, 3 varied slightly and only 3 were overexpressed in either one or the other strain (Table 1). This suggests that most of these overexpressed proteins in the two mutants are involved in the acid-stress response.

The N-terminal sequence of protein 19, whose expression showed about the same induction factor in the acid-adapted cells and in the acid-tolerant mutants of *Lb. sanfranciscensis* (Fig. 3a–d, Table 1), did not show significant homology with any proteins in the BLAST and FASTA sequence comparisons of the SWALL database. The N-terminal sequence of protein 40, whose expression showed the highest induction factor among the proteins specifically induced in the lactic-acid-adapted cells and was also induced in mutant CB1-5R, has 60% identity with the sequence of YhaH, a 13.1 kDa protein deduced from the genome sequence of *B. subtilis*. The 15 amino acid overlap alignment is located in the N-terminal portion, where YhaH shows a potential transmembrane helix. Although the function of YhaH is still unknown, this *B. subtilis* protein shares 54% homology with a 174 amino acid stress protein identified in *Listeria monocytogenes* (EMBL accession number AF102167) and 47% with a 144 amino acid general stress protein from *Bacillus halodurans* (Takami *et al.*, 2000).

Genes involved in the heat-shock response are highly conserved and the related DnaK–DnaJ–GrpE and GroEL–GroES chaperone complexes are not only induced by heat shock but also by other types of stress, including acidity (Hartke *et al.*, 1996, 1997; Rallu *et al.*, 1996). In the lactobacilli *Lb. acidophilus*, *Lb. casei* and *Lb. helveticus*, DnaK was the only chaperonin whose expression increased in all three species (expression of GrpE increased only in *Lb. helveticus* and DnaJ did not vary its expression) when heat-shocked cells were compared to control cells (Broadbent *et al.*, 1997). Antibodies against DnaJ, DnaK, GroES and GrpE were used in this study. In contrast with most of the reports cited, the proteins which reacted with DnaJ, DnaK and GroES antisera did not vary in their levels of expression when the whole-cell protein extracts of acid-adapted or acid-tolerant mutant cells were compared with those of the non-adapted cells of *Lb. sanfranciscensis* CB1. Only

GrpE seemed to be a general stress-response protein in *Lb. sanfranciscensis* CB1 since it was induced by a factor of 3.5–4.0.

Other phenotypic features of the constitutively acid-tolerant mutants

Certain *Lb. acidophilus* mutants have been proposed as suitable probiotic strains because they have enhanced acid and bile tolerance (Chou & Weimer, 1998). These *Lb. acidophilus* mutants also differed from the parental strain in having higher stability to freezing, and especially in their lower efficient lactose utilization, and peptidase and proteinase activities (Chou & Weimer, 1998). Compared to the parental strain, *Lb. sanfranciscensis* CB1-5R and CB1-7R had an inherent acid tolerance and also varied in two peptidase activities. While CB1-5R had a lower aminopeptidase activity, CB1-7R showed much higher aminopeptidase and iminopeptidase activities compared to the parental strain CB1, especially in acid conditions (pH 5.5). Peptidase activities during sourdough fermentation are of great importance since they are involved in the microbial growth and in the synthesis of amino acids which are directly or indirectly responsible for bread flavour formation (Gobbetti, 1998).

Looking for aminopeptidase activity, the cytoplasmic extract of *Lb. sanfranciscensis* CB1 was purified by three chromatographic steps. The 2D electrophoresis gel showed the presence of four proteins, one of which had an experimental molecular mass of 75 kDa and pI of 4.88. This protein coincided with spot 1 of Fig. 3(a–d) and with the aminopeptidase activity. Indeed in a previous report (Gobbetti *et al.*, 1996) we purified a monomeric 75 kDa intracellular metallo-aminopeptidase from *Lb. sanfranciscensis* CB1 which, as in this report, had the same activity on Lys-, Leu- and Iso-p-NA as preferential substrates. The level of expression of this protein was the same in all the strains, except for mutant CB1-5R, where the expression was about three times less. This may be related to the great difference found in the aminopeptidase activities between the two mutants. In contrast, the higher aminopeptidase activity of mutant CB1-7R compared to the parent CB1 is not related to the level of protein expression but probably based on the inherent adaptability of the mutant to acid conditions.

Lc. lactis subsp. *cremoris* (O'Sullivan & Condon, 1997) and *Listeria monocytogenes* (O'Driscoll *et al.*, 1996) cells showed an acid-induced multistress tolerance against heat, ethanol, NaCl and H₂O₂. Under our conditions, acid-adapted and, especially, mutant cells grew better than non-adapted cells at 10 °C and in the presence of 5% NaCl. The prolonged incubation in modified MRS, pH 6.4, containing 5% NaCl was found to be lethal for non-adapted cells. The storage of sourdough at refrigeration temperatures before subsequent use is a common practice as is the production of salted (approx. 2–5% NaCl) sourdough breads. The

addition of salt to the dough can moderately decrease the acidification rate of sourdough lactic acid bacteria, and to have selected acid and salt-tolerant strains certainly improves the sourdough fermentation.

When used in sourdough fermentation, the acid-tolerant mutants *Lb. sanfranciscensis* CB1-5R and CB1-7R had some advantages. The sourdough fermentation may require several and different processing steps, one of which is the addition of fresh lactic acid bacteria cells to a partially acidified dough (pH 4.5–5.0) in order to promote a rapid fermentation before baking. A fast acidification rate in these conditions reduces the risk of microbial contamination and also makes the sourdough competitive compared to bakers' yeast by decreasing the fermentation time (Gobbetti, 1998). Under our conditions, when the initial pH of the dough was 4.5, the use of the acid-tolerant mutants CB1-5R and CB1-7R gave a faster and higher acidification rate than the parental strain CB1.

Conclusions

The acid-stress response in *Lb. sanfranciscensis* CB1, a key sourdough lactic acid bacterium, was investigated: (i) it tolerated low pH after initial exposure to sublethal acid conditions; (ii) protein synthesis was required for the acid tolerance; (iii) two constitutively acid-tolerant mutants were isolated which also differed for peptidase activity; (iv) 2D electrophoresis analyses revealed large changes in the level of protein expression of acid-adapted and mutant cells compared to the parental strain; (v) the level of protein expression in two mutants was very similar; (vi) among proteins involved in heat-shock response, only GrpE was induced in acid conditions; (vii) the N-terminal sequence of a protein with the highest induction factor in the acid-adapted cells showed some similarity with known bacterial acid or general stress proteins; and (viii) the use of acid-tolerant mutants during sourdough fermentation in acid conditions gave some advantages. The current findings are the basis for further molecular cloning studies to enhance the exploitation of the physiology of sourdough lactobacilli.

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