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Physiological and Transcriptional Response of *Lactobacillus casei* ATCC 334 to Acid Stress▽†§

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This study investigated features of the acid tolerance response (ATR) in *Lactobacillus casei* ATCC 334. To optimize ATR induction, cells were acid adapted for 10 or 20 min at different pH values (range, 3.0 to 5.0) and then acid challenged at pH 2.0. Adaptation over a broad range of pHs improved acid tolerance, but the highest survival was noted in cells acid adapted for 10 or 20 min at pH 4.5. Analysis of cytoplasmic membrane fatty acids (CMFAs) in acid-adapted cells showed that they had significantly ($P < 0.05$) higher total percentages of saturated and cyclopropane fatty acids than did control cells. Specifically, large increases in the percentages of $C_{14:0}$, $C_{16:1n(9)}$, $C_{16:0}$, and $C_{19:0(11c)}$ were noted in the CMFAs of acid-adapted and acid-adapted, acid-challenged cells, while $C_{18:1n(9)}$ and $C_{18:1n(11)}$ showed the greatest decrease. Comparison of the transcriptome from control cells (grown at pH 6.0) against that from cells acid adapted for 20 min at pH 4.5 indicated that acid adaption invoked a stringent-type response that was accompanied by other functions which likely helped these cells resist acid damage, including malolactic fermentation and intracellular accumulation of His. Validation of microarray data was provided by experiments that showed that *L. casei* survival at pH 2.5 was improved at least 100-fold by chemical induction of the stringent response or by the addition of 30 mM malate or 30 mM histidine to the acid challenge medium. To our knowledge, this is the first report that intracellular histidine accumulation may be involved in bacterial acid resistance.

Lactobacillus casei is an aciduric, rod-shaped, facultatively heterofermentative lactic acid bacterium (LAB) that can be isolated from a variety of environments including raw and fermented milk and meat or plant products, as well as the oral, intestinal, and reproductive tracts of humans and animals (24). Like other LAB species, *L. casei* produces lactic acid as a major end product of carbohydrate fermentation during growth, and strains of *L. casei* are used as acid-producing starter cultures in the preparation of fermented foods, as health-enhancing probiotic cultures, and for the production of L(+)-lactic acid (18, 47, 52). In each of these applications, the industrial performance of *L. casei* strains is dependent, in one way or another, on their acidurance.

During fermentation, *L. casei* transports lactic acid outside the cell as lactate ion via an electrogenic proton-lactate symporter. As the pH of the medium (pH_o) decreases or the concentration of lactate increases, the concentration of protonated (undissociated) lactic acid in the medium also increases. The undissociated form of lactic acid is membrane soluble and thus can enter the cytoplasm by simple diffusion (27). Metabolically active bacteria maintain a pH gradient (ΔpH) where the intracellular pH (pH_i) is more alkaline than the pH_o (4, 25), so diffusion of acid into the cytoplasm results in rapid dissociation and release of protons and anions inside the cell. If the rate of proton accumulation exceeds the cytoplasmic buffering capacity and capabilities of efflux systems, the pH_i begins to fall and eventually reaches a critical point where the ΔpH can no longer be maintained and cellular functions are impaired (4, 22). Furthermore, intracellular accumulation of acid anions may be of greater importance than proton release in the inhibition of cell growth (8, 40, 41). Thus, low pH and organic acid resistance are vitally important physiological attributes of *L. casei* and other LAB species, in relation to both lifestyle and commercial utility, and there is considerable interest in the mechanisms used by these cells to combat acid-related stress conditions (4, 11, 22, 51).

Interestingly, differences in acidurance among LAB species have been linked to the activity and pH optima of the proton-translocating (H^+)-ATPase, and this enzyme complex plays a major role in pH_i regulation by these cells (11, 22, 48). However, many LAB species do not utilize the H^+-ATPase to maintain a near-neutral pH_i in response to acid stress. Instead, these species (including *L. casei*) maintain a relatively constant ΔpH as the pH_o falls by allowing the pH_i to decrease (25, 22, 40, 43, 45). This capability is thought to reduce the energy demand for proton translocation through the H^+-ATPase (45) and has been shown to help prevent intracellular accumulation of organic acid anions (40).

In addition to H^+-ATPase activity, acidurance in a LAB is also known to involve a variety of stress protection mechanisms (11, 30, 51). In particular, many cells display an inducible acid tolerance response (ATR) that protects them from acid killing. First reported by Goodson and Rowbury (19), the ATR is usually triggered by brief exposure to a sublethal pH_o ("acid adaptation"), which, like other stress responses, results in tran-

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sient induction of specific proteins and physiological changes that enhance cell survival during subsequent exposure to a lethal pH_o. The ATR has been detected in numerous bacteria, including *L. casei* and other species of LAB (20, 21, 30, 31, 50, 51).

Characterization of the ATR has facilitated the identification of enzymes, proteins, and macromolecular changes (e.g., alteration of cytoplasmic membrane lipid content) that allow bacteria to combat the negative consequences of cytoplasmic acidification (11, 30, 51). Work to dissect the *L. casei* ATR has detected 9 to 11 acid-regulated proteins (21) and shown that cytoplasmic membrane fatty acid (CMFA) content is altered to increase the proportion of $C_{18:1}$ and cyclopropane fatty acid (FA) $C_{19:0}$ in response to acidification (15). However, information on the mechanisms used by *L. casei* to modulate ATR and CMFA content, as well as the identities and functions of acid-regulated proteins, is still lacking.

In this study, we optimized conditions for ATR induction in *L. casei* ATCC 334 and then analyzed its effects on membrane lipid composition and global gene expression. As expected, the membrane lipid composition of acid-adapted cells showed a dramatic increase in the ratio of saturated to unsaturated membrane FAs and cyclopropane FA content. Comparisons between the transcriptome of cells grown at pH 6.0 (control) to that of acid-adapted (5 or 20 min at pH 4.5) or acid-adapted and then acid-challenged (20 min at pH 4.5 and then 10 min at pH 2.0) cells showed differential expression of numerous genes in acid-treated versus control cells. Overall, functional predictions for these genes indicated that acid adaptation induced a stringent-type response that was accompanied by other functions, particularly malolactic fermentation and intracellular accumulation of histidine, which were important for enhanced acidurance. Validation of microarray data was provided by follow-up experiments that showed that *L. casei* survival at pH 2.5 was improved at least 100-fold by chemical induction of the stringent response with α-methylglucoside or by the addition of 30 mM malate or 30 mM His to the acid challenge medium.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *L. casei* ATCC 334 (American Type Culture Collection, Manassas, VA) was maintained in a laboratory collection as a glycerol stock at −80°C and propagated at 37°C in MRS broth (Difco Laboratories, Detroit, MI). Working cultures were prepared from stock cultures through two successive transfers (1% inocula) in MRS broth at 37°C for 17 h.

Characterization of the acid tolerance response. Batch cultures were prepared by 1% (vol/vol) inoculation into 1 or 2 liters of MRS in a Bioflow III fermentor (New Brunswick Scientific, Edison, NJ) or a VirTis (Gardiner, NY) Omni culture fermenter, respectively, which was equipped with pH control. The cells were incubated at 37°C with an agitation rate of 75 to 100 rpm, and a constant pH of 6.0 was maintained by automatic addition of 15% (vol/vol) NH_4OH. When cells reached an A_{600} of 5.0 to 6.0, 200-ml samples of the suspension were aseptically harvested by centrifugation (9,000 × *g*, 10 min, 25°C), washed twice with 0.85% (wt/vol) NaCl (pH 7.0), and then suspended in 200 ml of fresh MRS adjusted to pH 6.0 (control), 5.0, 4.8, 4.5, 4.3, 4.0, 3.8, 3.5, or 3.0 using 10 N HCl. Cells were then acid adapted by incubation at the test pH for 10 or 20 min at 37°C and then harvested and washed as noted and suspended in 100 ml MRS adjusted to pH 2.0 with 10 N HCl (acid challenge). Acid-adapted cells were incubated under acid challenge conditions for up to 140 min with periodic sampling to evaluate viability. The bacterial suspensions were serially diluted in 0.1% Bacto peptone, plated on MRS agar, and enumerated after 48 to 72 h of anaerobic incubation at 37°C.

In some experiments, *L. casei* was grown in batch culture with pH control as described above and then acid adapted (20 min at pH 4.5) in the presence or absence of 30 mM sodium malate or 30 mM His. These cells were then acid challenged for up to 120 min at pH 2.0 or 2.5 in MRS that also did or did not contain an equivalent concentration of malate or His, respectively. Finally, the contribution of stringent-response induction to acid resistance was investigated by adding 1% (wt/vol) α-methylglucoside (36) to cells grown in batch culture with pH control 5 min before acid challenge at pH 2.5 in MRS that also did or did not contain 1% α-methylglucoside.

Data presented for the effects of different acid adaptation treatments on the viability of *L. casei* ATCC 334 during acid challenge represent mean values from two independent experiments.

Cell membrane lipid composition. *L. casei* ATCC 334 was grown in batch culture with pH control and then acid adapted and acid challenged as described above. FA methyl esters (FAMEs) of CMFAs were prepared by the method of Ingham et al. (23). All solvents were procured from Sigma-Aldrich (St. Louis, MO) and were high-performance liquid chromatography grade, and gases for the gas chromatograph (GC) were over 99.9% pure (AGA Gas, Madison, WI) and the glassware used in FA determinations was washed with 10% Micro cleaner (Cole Palmer Instrument Co., Vernon Hills, IL) and rinsed repeatedly with distilled water before use.

An internal standard of 0.1 mg/ml undecanoic acid and methyl eicosanoate (Sigma-Aldrich) was diluted in diethyl ether and stored at 4°C in a bottle with a gas-tight Mininert valve (Supelco, Bellefonte, PA). One milliliter of each standard was injected into each sample with a gas-tight syringe (Hamilton Co., Reno, NV) prior to methyl transesterification. A Hewlett-Packard (Avondale, PA) model 5890 GC equipped with a flame ionizing detector and a 30-m by 0.25-mm by 0.25-μm film DB23 50% cyanopropyl–50% dimethylpolysiloxane column (Agilent Technologies, Inc., Santa Clara, CA) was used for FAME separation. A sample (1.0 μl) was injected in the split mode with a ratio of 100:1. The flow rate of hydrogen, the carrier gas, was adjusted to 1.00 ml/min. The injector and detector temperatures were maintained at 250°C and 300°C, respectively. The column temperature was held at 100°C for 2 min, raised to 220°C at a rate of 10°C/min, and then held at 220°C for 5 min. FAMEs were identified by comparison of sample retention times to a qualitative standard bacterial FAME mixture (CP mix; Matreya, Inc., Pleasant Gap, PA) analyzed under the same conditions, and results represent mean values from two independent experiments.

Cell treatments for transcriptome studies. Cells were grown in batch culture with pH control as described above, and then five 50-ml samples were aseptically harvested into sterile 200-ml centrifuge bottles and administered one of five treatments designated as control, acid adaptation for 5 or 20 min (AA5 or AA20), acid challenged (AC), and acid adaptation and then acid challenge (AA20-AC). Control cells were immediately suspended in 100 ml of RNAprotect reagent (Qiagen, Inc., Valencia, CA) plus 900 μl rifampin (25 mg/ml in methanol; Sigma-Aldrich), mixed by vortex for 15 s, and then incubated for 10 min at room temperature. After this treatment, the cells were collected by centrifugation and then the supernatant was discarded and the pellet was stored at −20°C until needed for RNA isolation.

Cells for each of the other four treatments were collected by centrifugation for 10 min in a rotor that had been stabilized at 37°C to avoid confounding from temperature stress (46). The supernatant was discarded, and the pellets were suspended in 50 ml MRS warmed to 37°C and adjusted as necessary to deliver different treatments. Cells for treatments AA5 and AA20 were suspended in MRS adjusted to a pH of 4.5 and then incubated for 5 min or 20 min, respectively, at 37°C. For AC treatment, cells were suspended in MRS adjusted to pH 2.0 and then incubated at 37°C for 10 min. Finally, cells for AA20-AC were suspended in MRS adjusted to pH 4.5, incubated for 20 min at 37°C, and then collected and suspended in MRS at pH 2.0 and incubated for another 10 min at 37°C. At the conclusion of each treatment, 100 ml of RNAprotect and 900 μl rifampin were added and the suspensions were mixed by vortex for 15 s and incubated at room temperature for 10 min. The cells were collected by centrifugation for 10 min, and the pellet was stored at −20°C until needed for RNA isolation. Cell pellets from each of the five treatments were collected from four independent experiments for microarray studies.

RNA isolation. Cell pellets were thawed at room temperature and suspended in 1 ml of lysozyme solution (20 mg/ml in TE buffer) that also contained 20 U of mutanolysin (Sigma-Aldrich) and 75 μl of rifampin (25 mg/ml in methanol). This mixture was incubated at 37°C for 15 min in a shaker incubator at 240 rpm, and then total RNA was isolated from each of the cell samples using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA) following procedures recommended by the vendor, except for the following modifications. First, cells were mixed with 3.5 ml of Aurum lysis solution supplemented with 1% beta-mercaptoethanol by pipetting up and down several times to effect lysis. Next, 2.5 ml of 70% isopropanol was added and mixed until no bilayer was visible and the viscosity was substantially reduced. Cell lysates were then transferred (~700 μl) to a total of 10 Aurum RNA binding columns and centrifuged at 14,000 × *g* for

TABLE 1. Target genes and oligonucleotides used for RT-PCR

Protein function (gene ID)	Primer sequence		Amplicon size (bp)	Annealing temp (°C)
	Forward	Reverse		
Malolactic enzyme (LSEI_0740)	AGCAGAACGGAATCAGTATGG	CGGCTTGGTCTTTAACTGAGC	103	58
Two-component RR (LSEI_0460)	CTGATGAGGATAAAGTTCGTGG	GGCTTTCCCGTCTAAAGTGG	114	55
Sensor kinase DpiB (LSEI_2868)	CCATTGACGACATTGATTGC	TCACCATAATAGGTGCGGC	102	62
Transcription repressor HrcA (LSEI_1567)	CGTCTTAAAAGAGATCATCCGG	GCGAGAACTGACGTGACC	97	55
XRE family transcriptional regulator (LSEI_2759)	GTGGGCATGCAGTTTATTGG	GGAATCAGCAATGTAACGGC	140	61
Arsenate reductase (glutaredoxin) (LSEI_2761)	ACGTTCAGCACCTACACTGG	CTTCAATCTTATCGCGGACC	141	55
Peptide methionine sulfoxide reductase MsrA (LSEI_1393)	CCACCTATGAACAGGTTTCG	GGATAGCTAATGGTGTCGGC	85	57
Histidinol dehydrogenase (LSEI_1433)	GGGTCTATGACTTCGTTAAGCG	AGGGACACGTTTGATGATGG	115	61
Imidazole glycerol phosphate synthase, cyclase subunit (LSEI_1429)	GGATATGCACGCTTTGTTGC	AGCCAACGTGTATCAATCGC	117	62
30S ribosomal protein S2 (LSEI_1586)	GGTATCGAAGACATGCCTCG	CGGTGTTAGTATCAACCATCGC	127	58

30 s (or until all of the solution had passed through). The filtrate was discarded, and the column was placed in the same wash tube. From here, the protocol was followed precisely as described by manufacturer. Once the process was complete, the RNA concentration was measured with a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA), RNA quality was evaluated with an Agilent 2100 bioanalyzer, and then the RNA was stored at −80°C.

cDNA synthesis and labeling. cDNA was synthesized and labeled as recommended by the Affymetrix (Santa Clara, CA) protocol for prokaryotic target preparation in the GeneChip Expression Analysis Technical Manual. Briefly, cDNA was synthesized from 10 μg of total RNA using random primers (Invitrogen, Carlsbad, CA) and SuperScript II reverse transcriptase (Invitrogen). After synthesis, template RNA was digested with 1 N NaOH and neutralized with 1 N HCl, and cDNA was purified using the MinElute PCR purification kit (Qiagen) using a final elution volume of 12 μl rather than the 10 μl recommended by the manufacturer. cDNA was fragmented into approximately 50 to 100 bp using DNase I (GE Healthcare, Waukesha, WI) with fragmentation efficiency determined with an Agilent 2100 bioanalyzer. Fragmented cDNA was biotin labeled with GeneChip DNA labeling reagent (Affymetrix) and terminal deoxynucleotidyl transferase (Promega, Madison, WI), and labeling efficiency was measured by gel shift assay.

Microarray design, hybridization, and data extraction. Fragmented, labeled cDNA was taken to the Affymetrix core facility in the Center for Integrated Biosystems on the Utah State University campus for hybridization and data extraction. Hybridizations were performed against an Affymetrix custom microarray designed to include 2,674 (96%) chromosomal and 17 (85%) plasmid genes predicted to occur in *L. casei* ATCC 334 (7, 32). The only predicted coding sequences not included in the microarray design were redundant transposase and rRNA genes.

Data normalization and analysis. Statistical analysis of microarray data was performed using R software (www.r-project.org). Array images were reduced to intensity values for each probe and screened for acceptable quality control criteria before further analysis. Array preprocessing (background correction, normalization, and summarization) was performed with Bioconductor software (www.bioconductor.org) using the robust multiarray average method (3). The data from all four biological replications were pooled and then filtered to remove the genes with low expression values and/or low coefficients of variability in order to restrict our focus to genes that are highly expressed and genes that show a difference in expression between treatments (42). The significance of differential gene expression between controls and treatments was calculated with the limma (linear models for microarray data) statistical package in Bioconductor. Genes were considered significantly differentially expressed if the adjusted *P* values were less than 0.05.

Array validation. Validation of microarray data for 10 different genes (Table 1) was performed by real-time quantitative PCR (RT-PCR) as described previously (46), by using the same cDNA samples employed for AA5 (LSEI_0740), AA20-AC (LSEI_1586), or AA20 (all other targets) array hybridizations. Primers for RT-PCR were designed with GeneWorks software (IntelliGenetics, Inc., Mountain View, CA) and compared against the *L. casei* ATCC 334 genome using ERGO bioinformatic software (Integrated Genomics, Inc., Chicago, IL) to verify that each annealed to a single locus in the genome. Primer pairs were predicted to have annealing temperatures that ranged from 55 to 62°C and produce amplicons that ranged from 85 to 141 bp in length (Table 1). Template DNA from *L. casei* ATCC 334 was used to determine optimal RT-PCR conditions for each primer pair and to ensure the absence of nonspecific amplification, and then the fidelity of PCR products was confirmed by nucleotide sequence determination. Reactions were performed in an Opticon II thermal cycler (MJ Research, Reno, NV). Each reaction mixture consisted of 5 μl of either cDNA or water (negative controls), 5 μl of primer mix (1.2 μM each primer), and 10 μl of SYBR green mix (Bio-Rad Laboratories, Hercules, CA). Blanks contained 10 μl of SYBR green mix and 10 μl of water. RT-PCR was performed using two concentrations of cDNA (10 and 1 ng/μl) obtained from control, acid-challenged, acid-adapted, or acid-adapted and acid-challenged *L. casei* ATCC 334 cells as described above. Triplicate reactions were run in a 96-well plate. Amplicon quantification in RT-PCRs was performed by comparison with gene-specific standard curves constructed from known concentrations of individually purified amplicons. The obtained amplicon copy numbers were log transformed and used in the calculation of the expression change (*n*-fold) for a particular gene.

Microarray data accession number. Microarray hybridization data have been deposited in Gene Expression Omnibus under accession number GPL9271.

RESULTS AND DISCUSSION

The ability to survive in acidic environments is central to the lifestyle of *L. casei*, and it is also of paramount importance to the utility of this microorganism in bioprocessing. Although acidurance is considered an innate feature of *L. casei*, the molecular basis of this capability is only partly defined. *L. casei* has, for example, been shown to display an ATR, but few reports have addressed its effect on cell physiology (15, 21, 31, 50). Because ATR induction produces physiological changes that enhance acid resistance, more-detailed investigation of this response will reveal the molecular mechanisms used by *L. casei* to persist in highly acidic environments and may provide new strategies to enhance the industrial utility of this species.

Acid tolerance response of *L. casei* ATCC 334. Acid adaptation experiments with *L. casei* ATCC 334 demonstrated that ATR induction could be triggered by transient exposure to a wide range of sublethal pH values (Fig. 1). Although all of the acid-adapted *L. casei* ATCC 334 cultures suffered a greater-than-4-log decrease in viability after 140 min of acid challenge, numbers of viable control cells (acid adaptation treatment for 10 or 20 min at pH 6.0) fell below the limit of detection after only 40 min. In contrast, survival after 140 min of acid challenge by cells that were acid adapted for 10 min at lower pH values ranged from 0.7 to 2.4 log CFU/ml and from 0.7 to 3.4

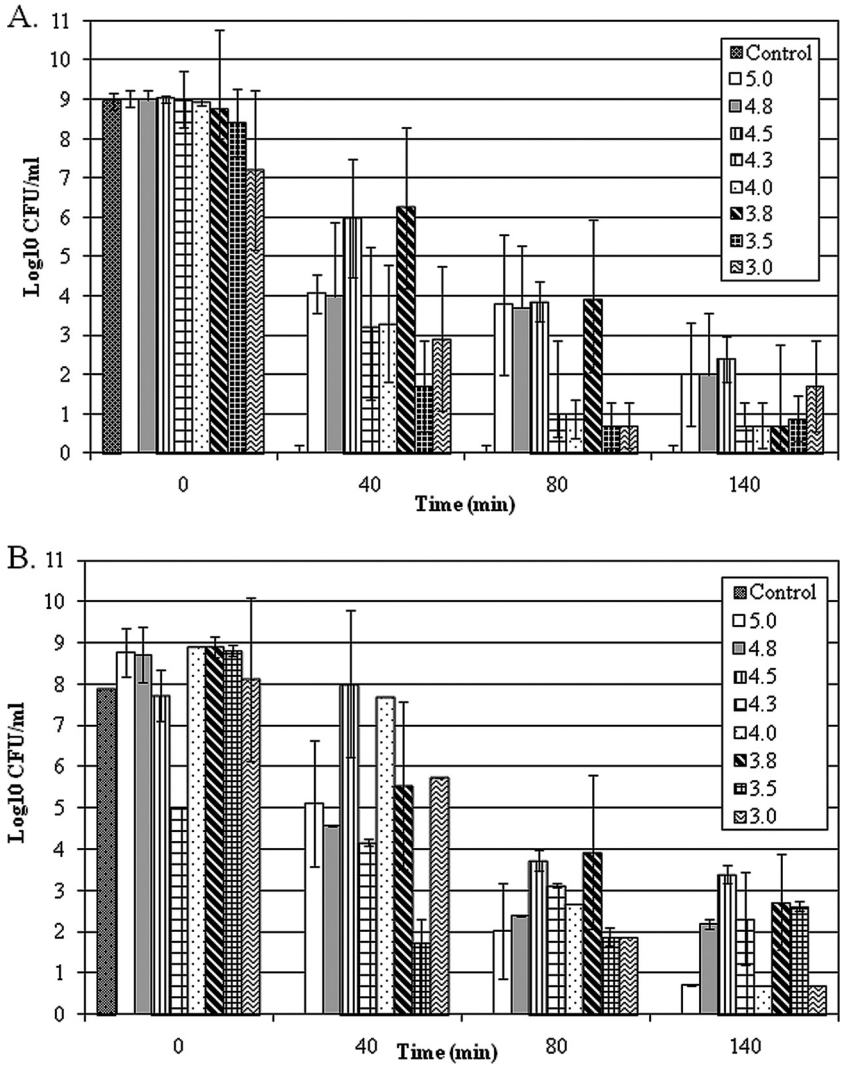

FIG. 1. Influence of different acid adaptation treatments on the viability of *L. casei* ATCC 334 during acid challenge at pH 2.0. *L. casei* ATCC 334 was propagated at pH 6.0 and then incubated for 10 min (panel A) or 20 min (panel B) at pH 6.0 (control), 5.0, 4.8, 4.5, 4.3, 4.0, 3.8, 3.5, or 3.0 and then acid challenged at pH 2.0. Values represent the mean number of recovered CFU calculated from duplicate analysis of duplicate experiments. Error bars indicate the standard error of the mean. If error bars are not visible, the standard error was less than 2%.

log CFU/ml for ATCC 334 cells that had been acid adapted at lower pH values for 20 min (Fig. 1). Data presented in Fig. 1 also illustrate the importance of optimizing conditions for ATR induction in a particular strain, as some adaptation treatments were less effective at protecting cells against a lethal acid challenge or simply showed greater variability in their results. Data presented in Fig. 1 revealed that cells that had been acid adapted at pH 4.5 for 10 or 20 min displayed superior survival (with good experimental reproducibility) during a 140-min acid challenge at pH 2.0.

Membrane lipid composition of acid-adapted *L. casei* ATCC 334. When confronted with acid stress, bacteria may act to counter proton influx by increasing the rigidity and compactness of the cytoplasmic membrane (11, 14, 51, 54). Such structural changes are accomplished, in part, by changing the FA species within the cytoplasmic membrane. The major CMFAs of *Lactobacillus* spp. are myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1n(9)}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1n(9)}$), *cis*-vaccinic acid ($C_{18:1n(11)}$), and the cyclopropanes dihydrosterculic acid [*cis*-9,10-methyleneoctadecanoic acid; $C_{19:0(9c)}$] and lactobacillic acid [*cis*-11,12-methyleneoctadecanoic acid; $C_{19:0(11c)}$] (38). As shown in Table 2, these acids made up over 87% of the total CMFA content of early-stationary-phase *L. casei* ATCC 334 propagated at pH 6.0 in MRS.

To decrease fluidity, cells may increase the concentration of saturated FAs or cyclopropane FAs (10, 15, 16), and both events were prominent in the CMFA profiles of acid-adapted versus control cells analyzed in this study (Table 2). The saturated-to-unsaturated FA ratio increased from 0.4 in non-adapted control cells to 4.2 or 5.3, respectively, after 10 or 20 min of acid adaptation at pH 4.5 because acid-adapted cells had a higher total percentage of saturated FAs and cyclopropane than did control cells (Table 2). Specifically, the FAs $C_{14:0}$ and $C_{19:0(11c)}$ exhibited the largest increase in acid-adapted cells, and proportions of $C_{16:1n(9)}$ and $C_{16:0}$ also in-

TABLE 2. Effect of acid adaptation and acid challenge on the cell membrane FA composition of early-stationary-phase *L. casei* ATCC 334[a]

FA or parameter	Mean % of each FA species in total cytoplasmic membrane lipid pool ± SEM after treatment				
	Control	AA10	AA20	AA10-AC	AA20-AC
$C_{14:0}$	3.7 ± 0.5	40.0 ± 0.1	39.0 ± 1.1	36.0 ± 1.1	20.0 ± 0.3
$C_{16:1n(9)}$	0.6 ± 0.3	3.3 ± 0.1	2.0 ± 0.0	3.0 ± 0.0	5.8 ± 1.0
$C_{16:0}$	15.0 ± 0.1	22.0 ± 0.1	27.0 ± 0.1	30.0 ± 0.1	25.0 ± 0.3
$C_{17:0(9c)}$	0.6 ± 0.5	BQL[b]	BQL	1.9 ± 0.0	4.2 ± 1.8
$C_{18:1n(9)}$	41.0 ± 0.3	7.6 ± 0.0	7.0 ± 0.1	6.8 ± 0.0	11.0 ± 0.1
$C_{18:1n(11)}$	23.0 ± 1.3	8.1 ± 0.0	7.0 ± 0.0	7.7 ± 0.0	8.3 ± 2.6
$C_{18:0}$	1.3 ± 1.1	1.7 ± 0.0	BQL	BQL	15.0 ± 0.6
$C_{19:0(11c)}$	5.0 ± 3.1	17.0 ± 0.0	18.0 ± 0.1	15.0 ± 0.1	15.0 ± 2.2
% Cyclopropane[c]	5.6	17	18	16.9	19.2
% Saturated[d]	26	80	84	83	79
% Unsaturated[e]	65	19	16	17	25
Saturated/unsaturated ratio	0.4	4.2	5.3	4.9	3.2

[a] Cells were propagated at pH 6.0 and then acid adapted by incubation for 10 min (AA10) or 20 min (AA20) at pH 4.5 or acid adapted for 10 or 20 min and then acid challenged at pH 2.0 for 140 min (AA10-AC or AA20-AC, respectively).
[b] BQL, below quantifiable limit (0.002%).
[c] Percentage of cyclopropane FAs [$C_{17:0(9c)}$ and $C_{19:0(11c)}$] in the membrane.
[d] Percentage of saturated FAs [$C_{14:0}$, $C_{16:0}$, $C_{17:0(9c)}$, $C_{18:0}$, and $C_{19:0(11c)}$] in the membrane.
[e] Percentage of unsaturated FAs ($C_{16:1}$ and $C_{18:1}$) in the membrane.

creased, while $C_{18:1n(9)}$ and $C_{18:1n(11)}$ exhibited the greatest decrease in comparison to control cells. Similar trends were noted for these FAs in cells that were acid adapted and acid challenged, where the ratio of saturated to unsaturated FAs was 4.9 or 3.2, respectively, for cells that had been acid adapted for 10 or 20 min prior to acid challenge (Table 2). Levels of $C_{17:0(9c)}$ were also higher in acid-challenged, but not acid-adapted, ATCC 334. However, interpretation of CMFA changes in acid-challenged cells is subject to question because these cells are rapidly losing viability and the observed changes may be a result of the erratic metabolic activity of dying cells.

Fozo et al. (15) have previously reported that the CMFA composition of *L. casei* was altered if cells were incubated at pH 5.0 rather than pH 7.0. Specifically, those authors noted that incubation at the lower pH resulted in increased proportions of $C_{18:1}$ and cyclopropane $C_{19:0}$, with concomitant decreases in $C_{16:0}$ and cyclopropane $C_{17:0}$ (15). As shown in Table 2, acid adaptation of *L. casei* ATCC 334 for 10 or 20 min at pH 4.5 also produced a sizable increase in the proportion of cyclopropane $C_{19:0}$ and a decrease in $C_{17:0}$. However, results from this work also differed from those of Fozo et al. (15) in that we observed a small increase in $C_{16:0}$ and a dramatic decrease in $C_{18:1n(9)}$ and $C_{18:1n(11)}$ in acid-adapted versus control cells. Moreover, the proportion of $C_{14:0}$ was substantially increased in acid-adapted cells, and increased levels of $C_{16:1n(9)}$ and $C_{16:0}$ were also noted (Table 2). Finally, the ratio of saturated to unsaturated FAs decreased from 4.6 to 2.4 when *L. casei* was incubated at pH 5.0 versus pH 7.0 in the work of Fozo et al. (15), but an opposite and more dramatic change was noted between cells incubated at pH 6.0 (control) and those that had been acid adapted for 10 or 20 min at pH 4.5 in this study (Table 2). The basis for these discrepancies is unclear, but they are most likely a reflection of the different growth media, pHs, and incubation conditions used in the two studies.

Influence of acid stress on global gene expression. The effect of ATR induction at pH 4.5 on transcription was investigated using an Affymetrix custom microarray designed for *L. casei* ATCC 334. Normalization and analysis of microarray hybridization signals revealed significant ($P < 0.05$) changes in the expression of 15 genes after comparisons between the transcriptomes of control and AA5-treated cell preparations (grown at pH 6.0, collected, washed, and acid adapted for 5 min in fresh MRS adjusted to pH 4.5). Twelve (80%) of the 15 genes had known or predicted functions (Table 3; for a complete list of the differentially expressed genes, see Table S1 in the supplemental material). One gene, encoding malolactic enzyme, was upregulated more than 16-fold, while 14 other genes for proteins involved in metabolism, information processing, and other processes were significantly downregulated in AA5 cells (Table 3).

After 20 min of acid adaptation at pH 4.5 (AA20), the number of genes whose expression was significantly ($P < 0.05$) altered had increased to 320, with 104 genes upregulated and 216 significantly downregulated (see Table S1 in the supplemental material). The 15 genes whose expression was significantly altered in the control-to-AA5 comparison were also differentially expressed in the same manner (up- or downregulated) by this treatment. Of the total of 320 genes affected by this treatment, 254 (79%) encode proteins with known or predicted functions (Table 3).

No significant changes in gene expression were detected in acid-challenged cells (pH 2.0 for 10 min) compared to control cells, but the transcriptome of cells that were acid adapted for 20 min at pH 4.5 before acid challenge (AA20-AC) showed differential expression of 66 genes. Forty-nine (74%) of these genes encode proteins with known or predicted functions (Table 3), and all but two (LSEI_0561 and LSEI_1487) had been detected and showed a similar response in the control-to-AA20 comparison (see Table S1 in the supplemental material).

RT-PCR validation. Of the 10 genes selected for analysis by RT-PCR, 7 were predicted by microarray data to be upregulated (LSEI_0740, LSEI_0460, LSEI_2868, LSEI_2759, LSEI_2761, LSEI_1433, and LSEI_1429) and 3 were downregulated (LSEI_1586, LSEI_1393, and LSEI_1567). As shown in Fig. 2, no contradictions between the two platforms were detected, and there was a strong positive correlation ($r = 0.84$)

TABLE 3. Numbers of genes with known or predicted functions that were significantly[a] up- or downregulated in acid-adapted *L. casei* ATCC 334[b]

General functional category	AA-5		AA-20		AA20-AC	
	Upregulated	Downregulated	Upregulated	Downregulated	Upregulated	Downregulated
Metabolism						
Energy production, conversion	1	1	3	2	1	1
Carbohydrate transport, metabolism	—[c]	1	12	9	2	3
Amino acid transport, metabolism	—	—	19	5	1	2
Nucleotide transport, metabolism	—	4	—	25	—	14
Coenzyme metabolism	—	—	—	1	—	1
Secondary metabolites biosynthesis, transport, catabolism	—	—	5	—	1	—
Information storage, processing						
Translation, ribosomal structure, biogenesis	—	2	—	58	—	3
Transcription	—	—	3	13	—	—
DNA replication, recombination, repair	—	—	—	5	—	2
Mobile DNA elements	—	—	12	1	1	—
Cellular processes						
Protein turnover, stress response	—	2	1	13	—	3
Signal transduction	—	—	2	1	—	—
Cell secretion	—	—	—	7	—	2
Cell envelope biogenesis	—	1	—	11	—	2
Cell division, chromosome partitioning	—	—	2	7	—	1
Poorly characterized, general function prediction only	—	—	13	25	2	7

[a] $P < 0.05$.
[b] Versus control cells grown at pH 6.0. AA5 or AA20, cells grown at pH 6.0 and then acid adapted for 5 or 20 min, respectively, in fresh MRS adjusted to pH 4.5; AA20-AC, cells grown at pH 6.0, incubated for 20 min in MRS adjusted to pH 4.5, and then suspended in MRS at pH 2.0 for 10 min. See Table S1 in the supplemental material for full details.
[c] —, no gene found.

between the fold change in gene induction or repression predicted from the microarray and the respective values determined by RT-PCR.

FA biosynthesis. Alteration of CMFA composition in response to acid stress has been observed in gram-positive and gram-negative bacteria (10, 11, 14, 15, 51) and likely occurs through a combination of *de novo* FA biosynthesis and modification of existing lipid membrane phospholipid acyl chains (54, 55). Because FA biosynthesis in bacteria is an energy-intensive process (55), the capability to modify existing CMFAs offers cells a less taxing means to adjust membrane structure, and at least three distinct enzymes have been found to catalyze these reactions in bacteria: phospholipid acyl desaturase, cyclopropane synthase (Cfa), and FA *cis-trans* isomerase (54). The *L. casei* ATCC 334 genome appears to encode only one of these enzymes, Cfa, which catalyzes the addition of a methylene residue across the *cis* double bond of $C_{16:1n(9)}$, $C_{18:1n(9)}$, or $C_{18:1n(11)}$ unsaturated FAs to form an unsaturated cyclopropane derivative (49). Thus, the concomitant decrease in $C_{18:1n(11)}$ and increase in $C_{19:0(11c)}$ detected in the cytoplasmic membrane content of acid-adapted cells (Table 2) can be directly attributed to Cfa activity on existing $C_{18:1n(11)}$. Other changes that occurred in the membrane lipid content of acid-adapted cells, however, are more likely explained by adjustments in the *de novo* synthesis of long fatty acyl chains and phospholipid turnover. The large reduction in $C_{18:1n(9)}$ noted in acid-adapted cells, for example, occurred without a corresponding increase in $C_{18:0}$ and supports our conclusion that *L. casei* ATCC 334 does not possess phospholipid acyl desaturase activity. Because most of the changes that were detected in the CMFA composition of acid-adapted *L. casei* appear to be dependent on *de novo* lipid synthesis, acid adaptation likely places a significant demand on the energy resources of the cell.

Inspection of the *L. casei* ATCC 334 genome for genes involved in FA (*fab*) and phospholipid (*pls*) biosynthesis revealed that *fab* genes are located in a large, 15-gene cluster (LSEI_2121 to LSEI_2107) that is structurally unique among all of the sequenced LAB species by virtue of its inclusion of the gene for Cfa. In contrast, genes that encode the three enzymes needed to transfer long-chain FAs to glycerol-3-phosphate and form phosphatidic acid (*plsX*, *plsY*, and *plsC*) are distributed across three different loci (LSEI_1614, LSEI_1407, and LSEI_1589, respectively). Although CMFA data indicate that enzymes for FA and phospholipid biosynthesis are involved in the *L. casei* ATR, microarray data indicate that the genes that encode those enzymes are not differentially expressed during ATR induction. This finding indicates that the constitutive expression levels of these genes are sufficient to meet cellular needs during ATR induction or that the activity of enzymes involved in these processes is not regulated at the transcriptional level. Even though the *fab* and *pls* genes were not affected by acid adaptation, significant upregulation of several genes that might be involved in phospholipid turnover was detected in AA20 cells. These included LSEI_0140, which encodes a putative membrane-associated phospholipid phosphatase, and LSEI_2773 and LSEI_1868, which are predicted to code for an esterase and an acetyltransferase, respectively (Table 4).

Acid adaptation invokes a stringent response. In addition to the cytoplasmic membrane, acid stress has a deleterious effect

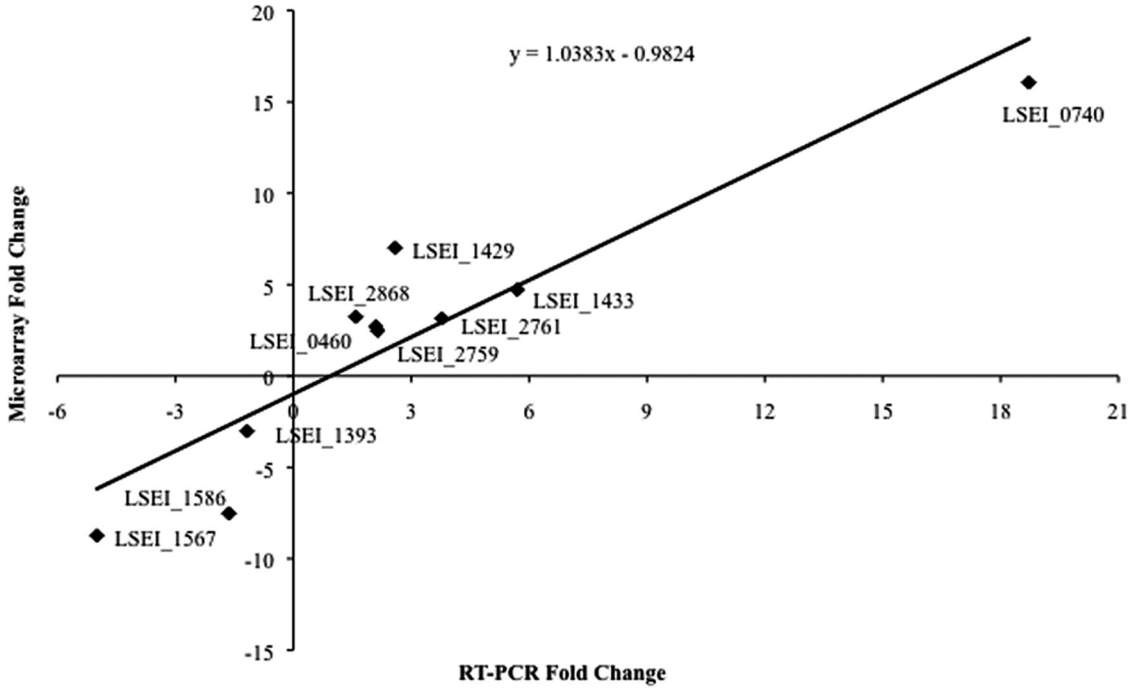

FIG. 2. Correlation of fold change values from microarray results and RT-PCR. Total RNA was extracted from both controls and all four acid-treated *L. casei* ATCC 334 cell groups and used as the template for cDNA synthesis to be used for microarray hybridizations and RT-PCR experiments. The fold change values were obtained for the 10 genes used in the RT-PCR experiments (Table 1). The best-fit curve is shown along with the calculated equation. The *r* value is 0.84.

on a variety of other cellular functions (20), so it was not unexpected to find significant changes between the transcriptomes of control and AA20-treated *L. casei* cells (see Table S1 in the supplemental material). The largest fraction (>35%) of downregulated genes was involved in cellular information processing, and those that contribute to the translational machinery of *L. casei* were especially targeted for repression (Table 4; Fig. 2). The latter observation is of particular interest because transcriptional repression of genes involved in the translational apparatus of cells is a hallmark of the bacterial stringent response to nutrient deprivation, which couples that action to the upregulation of genes associated with metabolic activities, especially amino acid biosynthesis, as well as some transport functions (9, 13). Other characteristics of the stringent response include the repression of genes needed for growth and reproduction (e.g., cell wall or DNA synthesis) and weak or no induction of chaperone/chaperonin proteins (9, 13). Very similar phenomena were observed in the transcriptional profile of AA20-treated versus control cells, except that the transcription of genes for chaperones, chaperonins, and other stress proteins was actually repressed (Table 4; Fig. 2).

Transcriptional changes that accompany the stringent response are triggered by Rel-dependent synthesis of (p)ppGpp, which interacts with RNA polymerase to effect gene repression or induction (9, 13). Rallu et al. (36) demonstrated that (p)ppGpp is also a signal for the induction of acid resistance in the LAB *Lactococcus lactis* and showed that insertion mutations in genes for purine biosynthesis such as *guaA*, which encodes GMP synthase (EC 6.3.5.2), dramatically enhanced acid resistance in this species. The *L. casei* ATCC 334 genome includes genes for two (p)ppGpp synthetases, *relA* (LSEI_1539) and *relQ* (LSEI_0901). Microarray data showed no significant change in the expression of *relA* or *relQ* in AA20 versus control cells, but transcriptional repression of 24 genes involved in nucleotide metabolism, including *guaA* (LSEI_1979), was detected (Table 4).

As a whole, these observations strongly suggested that (p)ppGpp also serves as the signal for ATR induction in *L. casei* and that the ATR in this species involves mechanisms that overlap the stringent response. To test this hypothesis, we performed acid challenge studies with cells that were treated with 1% (wt/vol) α-methylglucoside to induce the stringent response (36). As demonstrated in Fig. 3, exposure to α-methylglucoside before and during acid challenge resulted in a 100-fold increase in cell survival. This result confirmed that the stringent response confers acid resistance on *L. casei* and strongly suggested that (p)ppGpp accumulation is the signal for ATR induction in this species.

While the ATR and stringent responses of *L. casei* appear to employ the same effector molecule, logic dictates that differences must exist in the mechanisms used to trigger Rel-dependent (p)ppGpp synthesis and in the downstream physiological consequences of each stress response. In the classic example of nitrogen-starved cells, induction of the stringent response is precipitated by entry of deacylated tRNA into an empty ribosomal A site, which triggers RelA-dependent (p)ppGpp synthesis (9). Not surprisingly, one of the most important physiological consequences of the stringent response involves the upregulation of genes that encode functions (e.g., amino acid

TABLE 4. Examples of differentially regulated[a] genes in acid-adapted *L. casei* ATCC 334

General functional category and gene ID[c]	Predicted function	Fold change vs control[b]		
		AA5	AA20	AA20-AC
Metabolism				
Energy production and conversion				
LSEI_0740	Malolactic enzyme	16.06	7.35	NS
LSEI_0741	Malate/lactate antiporter	NS	5.99	NS
LSEI_1161	ATP synthase C chain (EC 3.6.3.14)	NS	−5.00	NS
Carbohydrate transport, metabolism: LSEI_2764	*sn*-Glycerol-3-phosphate transport system permease protein UgpA	NS	2.71	NS
Amino acid transport, metabolism				
LSEI_2061	Oligopeptide transport ATP-binding protein OppF	NS	3.27	NS
LSEI_2062	Oligopeptide transport ATP-binding protein OppD	NS	5.49	NS
LSEI_2063	Oligopeptide transport system permease protein OppC	NS	3.06	NS
LSEI_2064	Oligopeptide transport system permease protein OppB	NS	3.39	NS
LSEI_0175	Oligopeptide-binding protein OppA	NS	3.64	NS
LSEI_1890	Oligopeptide transport system permease protein OppB	NS	2.34	NS
LSEI_0046	Branched-chain amino acid transport protein AzlD	NS	3.01	NS
LSEI_1260	Histidine transport system permease protein HisM	NS	2.17	NS
LSEI_1426	Histidinol-phosphate aminotransferase (2.6.1.9)	NS	7.52	NS
LSEI_1427	Phosphoribosyl-ATP pyrophosphatase (EC 3.6.1.31)	NS	4.76	NS
LSEI_1429	Imidazole glycerol phosphate synthase, cyclase subunit (EC 4.1.3.-)	NS	7.01	NS
LSEI_1430	1-(5-Phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino]imidazole-4-carboxamide isomerase (EC 5.3.1.16)	NS	3.61	NS
LSEI_1431	Imidazole glycerol phosphate synthase, glutamine amidotransferase subunit (EC 2.4.2.-)	NS	5.43	NS
LSEI_1432	Imidazoleglycerol-phosphate dehydratase (EC 4.2.1.19)	NS	3.98	NS
LSEI_1433	Histidinol dehydrogenase (EC 1.1.1.23)	NS	4.72	NS
LSEI_1434	ATP phosphoribosyltransferase (EC 2.4.2.17)	NS	5.00	NS
LSEI_0078	Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	NS	2.68	NS
LSEI_0098	Diaminopimelate decarboxylase (EC 4.1.1.20)	NS	2.80	3.01
LSEI_2796	Acetylornithine deacetylase (EC 3.5.1.16)/succinyl-diaminopimelate desuccinylase (EC 3.5.1.18), related deacylases	NS	2.36	NS
LSEI_1345	Arginine transport ATP-binding protein ArtP	NS	−3.00	−3.83
LSEI_1486	Aspartate aminotransferase (EC 2.6.1.1)	NS	−3.21	NS
LSEI_1643	Xaa-Pro dipeptidase (EC 3.4.13.9)	NS	−3.23	NS
LSEI_1652	Glutamine synthetase (EC 6.3.1.2)	NS	−4.47	NS
LSEI_1653	Transcriptional regulator, MerR family	NS	−5.52	NS
LSEI_1288	5′-Methylthioadenosine nucleosidase (EC 3.2.2.16)/ *S*-adenosylhomocysteine nucleosidase (EC 3.2.2.9)	NS	−4.54	−4.40
Nucleotide transport and metabolism				
LSEI_1557	Adenine phosphoribosyltransferase (EC 2.4.2.7)	NS	−3.19	−3.26
LSEI_1118	Xanthine permease	NS	−3.16	−4.12
LSEI_1119	Phosphoribosylaminoimidazole carboxylase NCAIR mutase subunit (EC 4.1.1.21)	NS	−3.33	NS
LSEI_1120	Adenylosuccinate lyase (EC 4.3.2.2)	NS	−3.27	−3.99
LSEI_1286	ADP-ribose pyrophosphatase (EC 3.6.1.13)	−4.27	−3.72	−5.39
LSEI_1746	Phosphoribosylamine-glycine ligase (EC 6.3.4.13)	NS	−3.34	NS
LSEI_1747	Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3)/ IMP cyclohydrolase (EC 3.5.4.10)	NS	−9.35	−8.18
LSEI_1748	Phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)	NS	−5.18	NS
LSEI_1749	Phosphoribosylformylglycinamidine cycloligase (EC 6.3.3.1)	NS	−4.32	−4.65
LSEI_1750	Amidophosphoribosyltransferase (EC 2.4.2.14)	NS	−4.48	NS
LSEI_1751	Phosphoribosylformylglycinamidine synthase (EC 6.3.5.3)	NS	−4.47	NS
LSEI_1752	Phosphoribosylformylglycinamidine synthase (EC 6.3.5.3)	NS	−4.75	−4.77
LSEI_1753	Phosphoribosylformylglycinamidine synthase, PurS component (EC 6.3.5.3)	−5.71	−7.49	−8.75
LSEI_1754	Phosphoribosylamidoimidazole-succinocarboxamide synthase (EC 6.3.2.6)	NS	−7.16	−6.86
LSEI_1755	Phosphoribosylaminoimidazole carboxylase NCAIR mutase subunit (EC 4.1.1.21)	NS	−6.98	−7.55
LSEI_1756	Phosphoribosylaminoimidazole carboxyltransferase subunit (EC 4.1.1.21)	−5.09	−7.49	−8.35

Continued on following page

TABLE 4—*Continued*

General functional category and gene ID[c]	Predicted function	Fold change vs control[b]		
		AA5	AA20	AA20-AC
LSEI_1979	GMP synthase (glutamine hydrolyzing) (EC 6.3.5.2)	NS	−3.82	NS
LSEI_1468	Ribonucleoside-diphosphate reductase alpha chain (EC 1.17.4.1)	NS	−3.44	NS
LSEI_2482	Adenylate kinase (EC 2.7.4.3)/nucleoside-diphosphate kinase (EC 2.7.4.6)	NS	−10.06	NS
LSEI_1450	Orotidine 5′-phosphate decarboxylase (EC 4.1.1.23)	NS	−2.61	−3.08
LSEI_1806	Nicotinate phosphoribosyltransferase (EC 2.4.2.11)	NS	−2.80	−3.46
LSEI_1378	Cytidylate kinase (EC 2.7.4.14)	NS	−3.13	NS
LSEI_1669	Uridine kinase (EC 2.7.1.48)	−4.10	−3.16	−4.59
LSEI_1668	Transcription elongation factor	NS	−5.27	NS
LSEI_1159	Uracil phosphoribosyltransferase (EC 2.4.2.9)	NS	−3.29	NS
LSEI_1584	Uridylate kinase	NS	−4.94	NS
Information storage and processing				
Translation, ribosomal structure and biogenesis				
LSEI_0977	tmRNA-binding protein	NS	−2.62	NS
LSEI_1057	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C (EC 6.3.5.-)	NS	−3.32	NS
LSEI_1058	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit A (EC 6.3.5.-)	NS	-3.24	NS
LSEI_1292	tRNA (5-methylaminomethyl-2-thiouridylate)methyltransferase (EC 2.1.1.61)	NS	−4.24	−4.78
LSEI_1579	Prolyl-tRNA synthetase (EC 6.1.1.15)	NS	−2.96	NS
LSEI_1280	Isoleucyl-tRNA synthetase (EC 6.1.1.5)	NS	−2.97	NS
LSEI_1485	Asparaginyl-tRNA synthetase (EC 6.1.1.22)	NS	−3.37	NS
LSEI_1703	Threonyl-tRNA synthetase (EC 6.1.1.3)	NS	−3.80	NS
LSEI_0883	Leucyl-tRNA synthetase (EC 6.1.1.4)	NS	−4.05	NS
LSEI_2569	LSU[d] ribosomal protein L31P	NS	−2.78	NS
LSEI_1697	LSU ribosomal protein L20P	NS	−4.60	NS
LSEI_1642	Protein translation elongation factor P (EF-P)	NS	−5.55	NS
LSEI_1641	General stress protein, Gls24 family	NS	−5.18	NS
LSEI_1640	Transcription antitermination factor	NS	−4.38	NS
LSEI_1644	LSU ribosomal protein L27P	NS	−5.09	NS
LSEI_1645	Hypothetical ribosome-associated protein	NS	−8.30	NS
LSEI_1847	LSU ribosomal protein L33P	NS	−6.38	NS
LSEI_1572	Ribosome-binding factor	NS	−6.49	NS
LSEI_1573	Protein translation initiation factor 2 (IF-2)	NS	−4.76	NS
LSEI_1574	LSU ribosomal protein L7AE	−7.33	−7.55	−5.64
LSEI_1575	Hypothetical cytosolic protein	NS	−4.91	NS
LSEI_1576	Transcription termination factor	NS	−3.88	NS
LSEI_1577	Hypothetical cytosolic protein	NS	−3.84	NS
LSEI_2272	LSU ribosomal protein L12P (L7/L12)	NS	−13.08	NS
LSEI_2273	LSU ribosomal protein L10P	NS	−10.70	NS
LSEI_2476	LSU ribosomal protein L17P	NS	−7.52	NS
LSEI_2478	SSU[e] ribosomal protein S11P	NS	−6.88	NS
LSEI_2479	SSU ribosomal protein S13P	NS	−10.19	NS
LSEI_2480	LSU ribosomal protein L36P	NS	−19.62	NS
LSEI_2481	Protein translation initiation factor 1 (IF-1)	NS	−9.48	NS
LSEI_2484	LSU ribosomal protein L15P	NS	−11.94	NS
LSEI_2485	LSU ribosomal protein L30P	NS	−9.30	NS
LSEI_2486	SSU ribosomal protein S5P	NS	−8.51	NS
LSEI_2487	LSU ribosomal protein L18P	NS	−12.10	NS
LSEI_2488	LSU ribosomal protein L6P	NS	−8.62	NS
LSEI_2489	SSU ribosomal protein S8P	NS	−12.20	NS
LSEI_2490	SSU ribosomal protein S14P	NS	−12.31	NS
LSEI_2491	LSU ribosomal protein L5P	NS	−7.17	NS
LSEI_2492	LSU ribosomal protein L24P	−7.42	−17.81	NS
LSEI_2493	LSU ribosomal protein L14P	NS	−10.32	NS
LSEI_2494	SSU ribosomal protein S17P	NS	−14.50	NS
LSEI_2495	LSU ribosomal protein L29P	NS	−9.62	NS
LSEI_2496	LSU ribosomal protein L16P	NS	−8.59	NS
LSEI_2497	SSU ribosomal protein S3P	NS	−8.83	NS
LSEI_2499	SSU ribosomal protein S19P	NS	−7.34	NS
LSEI_2500	50S ribosomal protein L2	NS	−7.79	NS

Continued on following page

TABLE 4—*Continued*

General functional category and gene ID[c]	Predicted function	Fold change vs control[b]		
		AA5	AA20	AA20-AC
LSEI_2501	LSU ribosomal protein L23P	NS	−9.67	NS
LSEI_2502	LSU ribosomal protein L1E (= L4P)	NS	−6.11	NS
LSEI_2503	Ribosomal protein L3	NS	−5.57	NS
LSEI_2508	Protein translation elongation factor G (EF-G)	NS	−3.38	NS
LSEI_2509	SSU ribosomal protein S7P	NS	−7.19	NS
LSEI_1379	SSU ribosomal protein S1P	NS	−6.04	NS
LSEI_1244	SSU ribosomal protein S4P	NS	−7.14	NS
LSEI_1601	SSU ribosomal protein S16P	NS	−14.28	NS
LSEI_1327	SSU ribosomal protein S20P	NS	−15.68	NS
LSEI_1328	SSU ribosomal protein S15P	NS	−4.43	NS
LSEI_1332	Protein translation elongation factor Tu (EF-TU)	NS	−8.07	NS
LSEI_0009	SSU ribosomal protein S6P	NS	−8.45	NS
LSEI_1583	Ribosome recycling factor (RRF)	NS	−5.90	NS
LSEI_1585	Protein translation elongation factor Ts	NS	−8.36	NS
LSEI_1586	30S ribosomal protein S2	NS	−13.17	−7.52
LSEI_1303	Peptide deformylase (EC 3.5.1.88)	NS	−2.65	NS
LSEI_1393	Peptide methionine sulfoxide reductase MsrA (EC 1.8.4.11)	NS	−3.02	NS
Mobile DNA elements				
LSEI_1333	Transposase (transposase_12 superfamily)	NS	3.79	NS
LSEI_1101	Transposase (transposase_12 superfamily)	NS	3.20	NS
LSEI_1103	Transposase (transposase_12 superfamily)	NS	3.62	NS
LSEI_2689	Transposase, IS*30* family	NS	2.43	NS
LSEI_2691	Transposase, IS*30* family	NS	3.58	NS
LSEI_2008	Transposase, IS*30* family	NS	2.21	NS
LSEI_1907	Transposase, IS*3* family	NS	3.42	3.57
LSEI_0597	Transposase, IS*3* family	NS	3.03	NS
LSEI_0590	Transposase, IS*3* family	NS	2.65	NS
LSEI_0580	Transposase, IS*5* family	NS	3.37	NS
LSEI_0230	Transposase, IS*5* family	NS	2.28	NS
LSEI_2166	Transposase, IS*66* family	NS	2.56	NS
LSEI_0787	Resolvase	NS	−3.32	NS
Cellular processes				
Protein turnover, stress response				
LSEI_1617	General stress protein, Gls24 family	NS	−2.45	NS
LSEI_1281	Cold shock protein	−3.71	−2.80	−3.52
LSEI_1848	Superoxide dismutase (EC 1.15.1.1)	NS	−3.64	NS
LSEI_0963	ATP-dependent endopeptidase Clp proteolytic subunit ClpP (EC 3.4.21.92)	NS	−3.67	NS
LSEI_1467	Glutaredoxin	NS	−5.19	NS
LSEI_1762	ATP-dependent endopeptidase Clp ATP-binding subunit ClpE	−5.54	−6.19	−5.08
LSEI_1338	FKBP-type peptidyl-prolyl *cis-trans* isomerase (trigger factor) (EC 5.2.1.8)	NS	−9.26	NS
LSEI_2239	Chaperonin GroES	NS	−29.35	NS
LSEI_2238	60-kDa chaperonin GroEL	NS	−8.56	NS
LSEI_1567	Heat-inducible transcription repressor HrcA	NS	−8.73	−6.61
LSEI_1566	Molecular chaperone GrpE	NS	−16.72	−11.27
LSEI_1565	Molecular chaperone DnaK	NS	−13.71	NS
LSEI_1564	Small hypothetical protein	−14.36	−12.59	−9.71
LSEI_1563	Chaperone protein DnaJ	NS	−5.22	NS
Signal transduction				
LSEI_2868	Sensor kinase DpiB (EC 2.7.3.-)	NS	3.24	NS
LSEI_0460	Two-component RR	NS	2.71	NS
LSEI_1679	CsrR-like RR	NS	−2.77	NS
Cell envelope biogenesis				
LSEI_1033	Glycosyltransferase family 8	NS	−2.54	NS
LSEI_1808	*N*-Acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	NS	−2.85	NS
LSEI_2012	dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133)	NS	−3.00	NS
LSEI_2014	dTDP-4-dehydrorhamnose 3,5-epimerase (EC 5.1.3.13)	NS	−2.90	NS
LSEI_2015	Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24)	NS	−2.74	NS

Continued on following page

TABLE 4—*Continued*

General functional category and gene ID[c]	Predicted function	Fold change vs control[b]		
		AA5	AA20	AA20-AC
LSEI_1117	Xanthine phosphoribosyltransferase (EC 2.4.2.-)	NS	−3.04	NS
LSEI_1395	Carboxy-terminal processing protease precursor (EC 3.4.21.102)	NS	−3.80	NS
LSEI_0796	D-Alanyl carrier protein	NS	−4.53	−5.63
LSEI_0797	D-Alanyl transfer protein DltD precursor	NS	−4.07	NS
LSEI_0020	Cell wall hydrolase (amidase family)	NS	−5.57	NS
LSEI_2029	Cell wall hydrolase (amidase family)	−5.54	−11.28	−12.11
Cell division and chromosome partitioning				
LSEI_2441	CrcB family protein	NS	2.47	NS
LSEI_2442	CrcB family protein	NS	2.41	NS
LSEI_0932	Cell division protein FtsX	NS	−2.58	NS
LSEI_1268	Cell division protein FtsL	NS	−2.66	NS
LSEI_1274	Cell division protein FtsA	NS	−2.46	NS
LSEI_1275	Cell division protein FtsZ	NS	−4.89	NS
LSEI_1279	Cell division initiation protein DivIVA	NS	−4.27	NS
LSEI_1478	Cell division initiation protein DivIVA	NS	−7.37	−9.28
LSEI_0931	Cell division ATP-binding protein FtsE	NS	−4.89	NS
Poorly characterized, general function prediction only				
LSEI_1868	Acetyltransferase, GNAT family	NS	2.85	NS
LSEI_2773	Esterase	NS	2.64	NS
LSEI_0140	Putative membrane-associated phospholipid phosphatase	NS	2.57	NS

[a] $P < 0.05$.
[b] Fold change in normalized microarray signal intensity represents the average value calculated from four independent replicates. Control, cells grown at pH 6.0; AA5 or AA20, cells grown at pH 6.0 and then acid adapted for 5 or 20 min, respectively, in fresh MRS adjusted to pH 4.5; AA20-AC, cells grown at pH 6.0, incubated for 20 min in MRS adjusted to pH 4.5, and then suspended in MRS at pH 2.0 for 10 min.
[c] Gene ID represents the locus tag used in GenBank for chromosomal or plasmid-carried genes in *L. casei* ATCC 334 (NC 008526 and NC 008502, respectively).
[d] LSU, large subunit.
[e] SSU, small subunit.

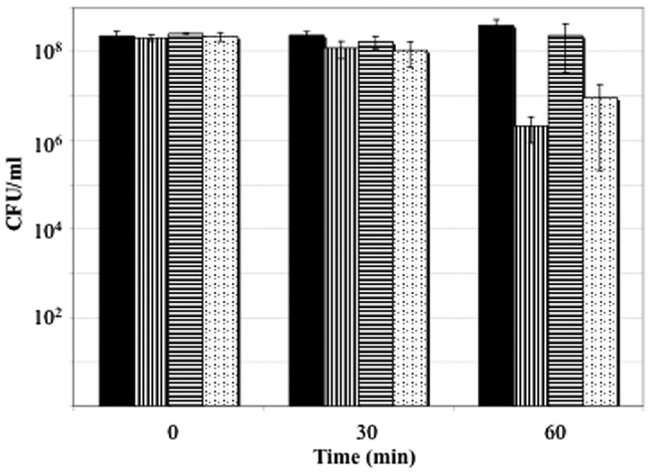

FIG. 3. Influence of a chemically induced stringent response on the viability of *L. casei* ATCC 334 during acid challenge at pH 2.5. Cells were grown in batch culture with pH control (pH of 6.0) and incubated for 5 min in the presence (▤, ▨) or absence (▥) of 1% (wt/vol) α-methylglucoside to induce the stringent response and then acid challenged in MRS adjusted to pH 2.5 that also did (▤) or did not (▨, ▥) contain 1% α-methylglucoside. Control cells (■) were incubated in MRS adjusted to pH 6.0 for 60 min. Data represent the mean values from two independent experiments.

biosynthesis and transport) that can help the cell escape amino acid starvation.

The trigger for ATR induction in *L. casei* is unknown, but a role for deacylated tRNA seems unlikely since cells were subject to acid adaptation treatment in a nutritionally rich laboratory medium (MRS). Because the *L. casei* ATR is accompanied by rapid and wholesale changes in CMFA composition (Table 2), and most of those changes appear to be dependent on *de novo* CMFA synthesis, one plausible hypothesis is that (p)ppGpp synthesis is triggered by a transient limitation for FAs (35). Although *fab* or *pls* gene expression was not affected by acid adaptation treatment, significant upregulation of the gene that encodes UgpA (LSEI_2764), the permease for an *sn*-glycerol-3-phosphate transport system, was detected in AA20 versus control cells (Table 4). The latter observation may be significant because glycerol-3-phosphate is a key precursor for phospholipid biosynthesis, but the relationship of this molecule or CMFA synthesis in general to ATR induction in *L. casei* requires further investigation.

Malate and His contribute to acid adaptation in *L. casei*. If the pool of genes that were upregulated as a consequence of ATR induction in *L. casei* are viewed in parallel to those induced in other bacteria by nitrogen starvation, then malolactic fermentation (MLF) and intracellular pools of His may also play key roles in acid adaptation (Table 4). In MLF, L-malate is decarboxylated in the cytoplasm by the malolactic enzyme to

produce L-lactate and CO_2 (37). Decarboxylation contributes to alkalinization of the cytoplasm and allows ATP generation through H^+-ATPase (34). The electrogenic potential created by lactate efflux through a malate/lactate antiporter, whose gene is commonly organized in an operon structure with that which encodes malolactic enzyme, may also facilitate energy production (34). MLF has not been associated with the stringent response, but it has been linked to LAB survival under acidic conditions (17, 34, 37, 44).

The sequenced genomes of *L. casei* and several other *Lactobacillus* species demonstrate that genes for malolactic enzyme (*mleS*) and malate/lactate antiporter (*mleP*) are arranged in tandem and presumably cotranscribed under the control of a LysR-type regulatory protein, MleR, whose gene is present immediately upstream and in divergent orientation with respect to *mleS*. An identical structure for MLF genes has been described for *Oenococcus oeni*, where *mleSP* cotranscription has been demonstrated by Northern hybridization (26).

mleS (LSEI_0740) was the only significantly upregulated gene in *L. casei* ATCC 334 AA5 cells, while both *mleS* and *mleP* (LSEI_0741) were among the most strongly upregulated genes in AA20 cells (Table 4; Fig. 2). No change was detected in the expression level of *mleR* (LSEI_0739) after any treatment, and differential expression of *mleS* or *mleP* was not detected in AA20-AC cells. Transient, high-level induction of *mleSP* in response to acid adaptation suggests that MLF is a critical component of the *L. casei* ATR, and its known physiological consequences are consistent with our hypothesis that acid adaptation requires a significant energy investment from cells.

The importance of MLF to *L. casei* biology is also illustrated by recent comparative genome hybridization data which demonstrated that *mleS*, *mleP*, and *mleR* were present in all of the 21 *L. casei* strains examined, which were isolated from cheese, plant material, or human sources (6, 7). Furthermore, Sheng and Marquis (44) found that *L. casei* had the highest specific MLF activity and the lowest pH optimum among five different species of oral LAB. More importantly, malate addition has been shown to enhance the survival of *Lactobacillus plantarum* and *Streptococcus mutans* during acid challenge at low pH values (17, 44).

Unlike MLF, induction of genes for His biosynthesis has been reported as part of the stringent response in gram-negative and gram-positive bacteria (5, 39). In this study, the transcriptome of AA20-treated cells exhibited a stringent-response-like response that included significant upregulation of an eight-gene cluster for His biosynthesis (LSEI_1426 to LSEI_1434) (Table 4; Fig. 2). Significant upregulation of a gene predicted to encode HisM (LSEI_1260), a permease that forms part of a His transport system, was also detected in these cells. In view of the fact that acid adaptation was performed in a nutritionally rich medium, these observations suggest that intracellular pools of His may also contribute to the ATR in *L. casei*.

One possibility is that His contributes to intracellular buffering capacity. With a pK_a value near 6.0, the imidazole groups of His and His-containing peptides have been shown to contribute to intracellular buffering in higher cells (1). Len et al. (29) have suggested that acid-induced genes for branched-chain amino acid synthesis in *S. mutans* might contribute to alkalinization of the cytoplasm as a consequence of NH_3 production. In *L. casei*, His biosynthesis may have a comparable function by virtue of its ability to function as a base.

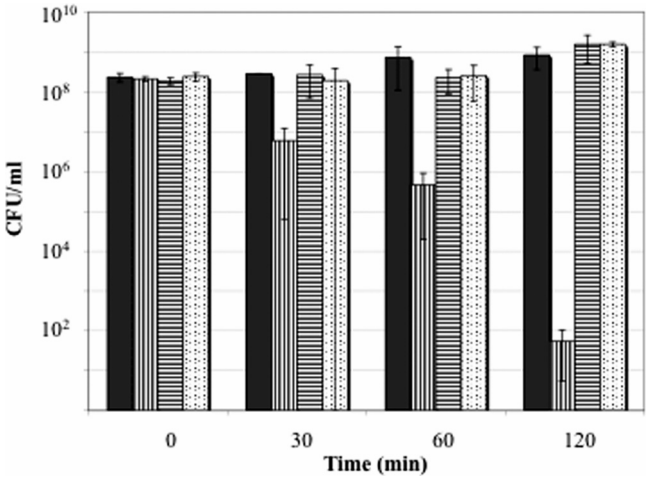

FIG. 4. Influence of malate or histidine addition on the viability of *L. casei* ATCC 334 during acid challenge at pH 2.5. Cells were acid adapted for 20 min in MRS broth adjusted to pH 4.5 and then acid challenged at pH 2.5 in regular MRS (▥) or MRS that contained 30 mM sodium malate (▤) or 30 mM histidine (▨). Control cells (■) were incubated in MRS adjusted to pH 6.0. Data represent the mean values from two independent experiments.

To determine whether the presence of malate or His would influence acid tolerance in *L. casei*, we performed acid challenge studies at pHs 2.0 and 2.5 using cells that were either acid adapted (20 min at pH 4.5) or acid challenged in MRS that contained 30 mM sodium malate or 30 mM His. The viability of *L. casei* ATCC 334 was not improved by the addition of malate or His at either phase of treatment when acid challenge was performed at pH 2.0, and results were highly variable during acid challenge at pH 2.5 when the compounds were present during acid adaptation (data not shown). However, the presence of either malate or His during acid challenge at pH 2.5 resulted in a more-than-100-fold increase in cell survival after 60 min of incubation and a greater-than-10^7-fold improvement after 2 h (Fig. 4). To our knowledge, this is the first reported evidence that intracellular His accumulation may contribute to acid resistance in bacteria.

***atpC* is downregulated during acid adaptation.** Acidurance in several LAB species has been directly linked to the activity and pH optima of H^+-ATPase (11, 22, 48), and increased activity has sometimes been correlated with elevated transcription of *atp* genes (33). However, ATR induction in some species is not accompanied by a significant change in H^+-ATPase activity or transcription of *atp* genes that encode its cognate proteins (33). In *L. casei* ATCC 4646, H^+-ATPase activity was significantly reduced when cells were grown at pH 5.0 versus pH 7.0 (R. A. Burne, personal communication), and microarray data from this study demonstrated that *atpC* (LSEI_1161) was actually downregulated in AA20 versus control cells (Table 4). Though perhaps unexpected, these observations are, in fact, consistent with the view that H^+-ATPase activity is more important for energy production (via MLF) than proton extru-

sion in acid-adapted *L. casei*, assuming that the former need can be readily met by existing enzymes.

Differential expression of two-component systems. The molecular sensor(s) and regulators that modulate the *L. casei* ATR remain unknown, but AA20 treatment did affect the expression levels of genes associated with two-component regulatory systems (2CRS) and ABC-type oligopeptide transport proteins (Opp), which are known to function as sensors for environmental change (12, 28), as well as several transcriptional regulators (Table 4; Fig. 2). The *L. casei* ATCC 334 genome is predicted to encode 16 complete 2CRS, more than are found in any other sequenced *Lactobacillus* sp., as well as at least 11 ABC-type transporters for the uptake of peptides or amino acids and 124 transcriptional regulators (7, 32). While some of the 2CRS, Opp, and transcriptional regulators whose cognate genes were induced or repressed by acid adaptation could be involved in ATR modulation, their specific functions have not yet been determined. One possible exception is LSEI_1679, which encodes the response regulator (RR) for a 2CRS implicated in the acid resistance of *Lactobacillus acidophilus* (2). In that study, inactivation of the associated histidine kinase (HK) dramatically increased acid sensitivity in logarithmic-phase *L. acidophilus* and produced greater-than-2-fold changes in the expression of 80 genes. In particular, the authors noted upregulation in HK mutants incubated at pH 5.5 of numerous genes associated with the LAB proteolytic enzyme system, including two Opp operons (2).

In this work, significant downregulation of the RR (LSEI_1679) was detected in AA20-treated *L. casei* (Table 4). Because inactivation of the cognate HK gene in *L. acidophilus* increased the acid sensitivity of log-phase cells, and loss of HK activity would be predicted to cause RR downregulation at the protein level, transcriptional repression of LSEI_1679 might not be an expected component of ATR induction. However, repression of LSEI_1679 during acid adaptation of *L. casei* was only transient (Table 4), and Azcarate-Peril et al. (2) found that an *L. acidophilus* HK mutant could still mount an ATR that was just as effective as that produced by wild-type cells for protection against lethal acid challenge at pH 3.5 over a 2-h period. Although viability of the mutant began to decline faster than that of wild-type cells between 2 to 2.5 h of acid challenge, the results clearly demonstrated that ATR induction in *L. acidophilus* involves mechanisms that are not exclusively controlled by this particular 2CRS (2). Thus, our discovery that LSEI_1679 was transiently repressed during acid adaptation of *L. casei* is not inconsistent with the findings of Azcarate-Peril et al. (2) and supports a role for this 2CRS in the acid resistance of *L. casei*.

Differential expression of transposase genes. An unexpected finding from this work involved the transient upregulation of 12 genes that encode transposase proteins from four different insertion sequence families in AA20 cells (Table 4). None of these genes was differentially expressed in AA5-treated cells, and only one (LSEI_1907) was still upregulated in AA20-AC cells. We are unaware of any similar observation in LAB species, but the histone-like nucleoid structuring protein of gram-negative bacteria, which is an important regulator of stress responses, has been shown to promote the transposition of Tn*10*, IS*903*, Tn*552*, and IS*1* (53). Cellular mechanisms to stimulate transposition in response to environmental change could provide an evolutionary advantage to the host and the mobile DNA element. From that perspective, it is very interesting that even though transposon-related genes are quite abundant in *L. casei* ATCC 334 (over 3% of the total open reading frames) (32), 9 of the 12 transposase genes that were significantly upregulated in AA20 cells are either located in (LSEI_1101, LSEI_1103, LSEI_2008, and LSEI_0580) or proximate to (LSEI_2689, LSEI_2691, LSEI_1907, LSEI_0597, and LSEI_0590) discrete genomic regions that were likely acquired through horizontal gene transfer (7).

In summary, survival in acidic environments is critical to both the lifestyle and industrial performance of *L. casei*, so detailed understanding of its ATR holds fundamental and applied value. Physiological and transcriptional data presented in this report describe important features of the *L. casei* ATR and provide compelling evidence that acid adaption invokes a stringent-type response which is accompanied by other changes, including MLF and intracellular His accumulation, which promote cell survival at lower pH values. In combination, results from cell incubations with α-methylglucoside, malate, and His provide strong validation of our interpretation of microarray data and underscore the value of this technology for research on complex physiological processes such as acid resistance.

The overall nature of acid-induced changes in *L. casei* suggests that the ATR is an energy-intensive process whose cost may be driven by wholesale changes in CMFA composition that largely appear to rely on *de novo* FA biosynthesis. Efforts are currently under way in our laboratories to develop effective tools for gene inactivation in *L. casei* ATCC 334 so that we can further validate and characterize the contributions of enzymes and metabolic pathways described in this report to acid resistance in this species.

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REFERENCES

1. **Abe, H.** 2000. Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. Biochemistry **65:**757–765.
2. **Azcarate-Peril, M. A., O. McAuliffe, E. Altermann, S. Lick, W. M. Russell, and T. R. Klaenhammer.** 2005. Microarray analysis of a two-component regulatory system involved in acid resistance and proteolytic activity in *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **71:**5794–5804.
3. **Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed.** 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics **19:**185–193.
4. **Booth, I. R.** 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49:**359–378.
5. **Brockmann-Gretza, O., and J. Kalinowski.** 2006. Global gene expression during stringent response in *Corynebacterium glutamicum* in presence and absence of the *rel* gene encoding (p)ppGpp synthase. BMC Genomics **7:**230.
6. **Cai, H., B. T. Rodríguez, W. Zhang, J. R. Broadbent, and J. L. Steele.** 2007. Genotypic and phenotypic characterization of *Lactobacillus casei* strains isolated from different ecological niches suggests frequent recombination and niche specificity. Microbiology **153:**2655–2665.
7. **Cai, H., R. Thompson, M. Budinich, J. R. Broadbent, and J. L. Steele.** 2009. Genome sequence and comparative genome analysis of *Lactobacillus casei*: insights into their niche-associated evolution. Genome Biol. Evol. **1:**239–257.
8. **Carpenter, C. E., and J. R. Broadbent.** 2009. External concentration of organic acid anions and pH: key independent variables for studying how

organic acids inhibit growth of bacteria in mildly acidic foods. J. Food Sci. **74:**R12–R15.
9. **Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella.** 1996. The stringent response, p. 1458–1496. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
10. **Chang, Y. Y., and J. E. Cronan, Jr.** 1999. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol. Microbiol. **33:**249–259.
11. **Cotter, P. D., and C. Hill.** 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol. Mol. Biol. Rev. **67:**429–453.
12. **El-Sharoud, W. M.** 2005. Two-component signal transduction systems as key players in stress responses of lactic acid bacteria. Sci. Prog. **88:**203–228.
13. **Eymann, C., G. Homuth, C. Scharf, and M. Hecker.** 2002. *Bacillus subtilis* functional genomics: global characterization of the stringent response by proteome and transcriptome analysis. J. Bacteriol. **184:**2500–2520.
14. **Foster, J. W.** 1995. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Crit. Rev. Microbiol. **21:**215–237.
15. **Fozo, E. M., J. K. Kajfasz, and R. G. Quivey, Jr.** 2004. Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol. Lett. **238:**291–295.
16. **Fozo, E. M., and R. G. Quivey, Jr.** 2004. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl. Environ. Microbiol. **70:**929–936.
17. **Garcia, M. J., M. Zúñiga, and H. Kobayashi.** 1992. Energy production from L-malic acid degradation and protection against acidic external pH in *Lactobacillus plantarum* CECT 220. J. Gen. Microbiol. **138:**2519–2524.
18. **Goldin, B. R., and S. L. Gorbach.** 1992. Probiotics for humans, p. 355–376. *In* R. Fuller (ed.), Probiotics: the scientific basis. Chapman & Hall, London, England.
19. **Goodson, M., and R. J. Rowbury.** 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sub-lethal acid pH. Lett. Appl. Microbiol. **8:**77–79.
20. **Hall, H. K., K. L. Karen, and J. W. Foster.** 1995. Molecular responses of microbes to environmental pH stress. Adv. Microb. Physiol. **37:**229–272.
21. **Hamilton, I. R., and G. Svensäter.** 1998. Acid-regulated proteins induced by *Streptococcus mutans* and other oral bacteria during acid shock. Oral Microbiol. Immunol. **13:**292–300.
22. **Hutkins, R. W., and N. L. Nannen.** 1993. pH homeostasis in lactic acid bacteria. J. Dairy Sci. **76:**2354–2365.
23. **Ingham, S., J. Hassler, Y. Tsai, and B. H. Ingham.** 1998. Differentiation of lactate-fermenting, gas-producing *Clostridium* spp. isolated from milk. Int. J. Food Microbiol. **43:**173–183.
24. **Kandler, O., and N. Weiss.** 1986. Genus *Lactobacillus*, p. 1209–1234. *In* P. H. A. Sheath, N. S. Maiz, M. E. Sharp, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore, MD.
25. **Kashket, E. R.** 1987. Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. FEMS Microbiol. Rev. **46:**233–244.
26. **Labarre, C., C. Diviès, and J. Guzzo.** 1996. Genetic organization of the *mle* locus and identification of a *mleR*-like gene from *Leuconostoc oenos*. Appl. Environ. Microbiol. **62:**4493–4498.
27. **Lambert, R. J., and M. Stratford.** 1999. Weak-acid preservatives: modeling microbial inhibition and response. J. Appl. Microbiol. **86:**157–164.
28. **Lazazzera, B. A.** 2001. The intracellular function of extracellular signaling peptides. Peptides **22:**1519–1527.
29. **Len, A. C., D. W. Harty, and N. A. Jacques.** 2004. Proteome analysis of *Streptococcus mutans* metabolic phenotype during acid tolerance. Microbiology **150:**1353–1366.
30. **Lorca, G. L., and G. Font de Valdez.** 2009. *Lactobacillus* stress responses, p. 115–137. *In* A. Ljungh, and T. Wadstro˝m (ed.), *Lactobacillus* molecular biology: from genomics to probiotics. Calister Academic Press, Norfolk, United Kingdom.
31. **Ma, Y., T. M. Curran, and R. E. Marquis.** 1997. Rapid procedure for acid adaptation of oral lactic-acid bacteria and further characterization of the response. Can. J. Microbiol. **43:**143–148.
32. **Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J.-H. Lee, I. Díaz-Muñiz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, L. McKay, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills.** 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. U. S. A. **103:**15611–15616.
33. **Nascimento, M. M., J. A. Lemos, J. Abranches, R. B. Gonçalves, and R. A. Burne.** 2004. Adaptive acid tolerance response of *Streptococcus sobrinus*. J. Bacteriol. **186:**6383–6390.
34. **Poolman, B., D. Molenaar, E. J. Smid, T. Ubbink, T. Abee, P. P. Renault, and W. N. Konings.** 1991. Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. J. Bacteriol. **173:**6030–6037.
35. **Potrykus, K., and M. Cashel.** 2008. (p)ppGpp: still magical? Annu. Rev. Microbiol. **62:**35–51.
36. **Rallu, F., A. Gruss, S. D. Ehrlich, and E. Maguin.** 2000. Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. Mol. Microbiol. **35:**517–528.
37. **Renault, P., C. Gaillardin, and H. Heslot.** 1988. Role of malolactic fermentation in lactic acid bacteria. Biochimie **70:**375–379.
38. **Rizzo, A. F., H. Korkeala, and I. Mononen.** 1987. Gas chromatography analysis of cellular fatty acids and neutral monosaccharides in the identification of lactobacilli. Appl. Environ. Microbiol. **53:**2883–2888.
39. **Rudd, K. E., B. R. Bochner, M. Cashel, and J. R. Roth.** 1985. Mutations in the *spoT* gene of *Salmonella typhimurium*: effects on his operon expression. J. Bacteriol. **163:**534–542.
40. **Russell, J. B.** 1991. Resistance of *Streptococcus bovis* to acetic acid at low pH: relationship between intracellular pH and anion accumulation. Appl. Environ. Microbiol. **57:**255–259.
41. **Russell, J. B.** 1992. Another explanation for the toxicity of fermentation acids at low pH; anion accumulation verses uncoupling. J. Appl. Microbiol. **73:**363–370.
42. **Scholtens, D., and A. von Heydebreck.** 2005. Analysis of differential gene expression studies, p. 229–248. *In* R. Gentleman, V. Carey, W. Huber, R. Irizarry, and S. Dudoit (ed.), Bioinformatics and computational biology solutions using R and Bioconductor. Springer Science+Business Media, Inc., New York, NY.
43. **Shabala, L., T. McMeekin, B. B. Budde, and H. Siegumfeldt.** 2006. *Listeria innocua* and *Lactobacillus delbrueckii* subsp. *bulgaricus* employ different strategies to cope with acid stress. Int. J. Food Microbiol. **110:**1–7.
44. **Sheng, J., and R. E. Marquis.** 2007. Malolactic fermentation by *Streptococcus mutans*. FEMS Microbiol. Lett. **272:**196–201.
45. **Siegumfeldt, H., K. Björn Rechinger, and M. Jakobsen.** 2000. Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. Appl. Environ. Microbiol. **66:**2330–2335.
46. **Smeianov, V. V., P. Wechter, J. R. Broadbent, J. E. Hughes, B. Rodriguez, T. K. Christensen, Y. Ardo, and J. L. Steele.** 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. Appl. Environ. Microbiol. **73:**2661–2672.
47. **Stiles, M. E., and W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. **36:**1–29.
48. **Sturr, M. G., and R. E. Marquis.** 1992. Comparative acid tolerances and inhibitor sensitivities of isolated F-ATPases of oral lactic acid bacteria. Appl. Environ. Microbiol. **58:**2287–2291.
49. **Suutari, M., and S. Laakso.** 1992. Temperature adaptation in *Lactobacillus fermentum*: interconversions of oleic, vaccenic and dihydrosterulic acids. J. Gen. Microbiol. **138:**445–450.
50. **Svensäter, G., U. B. Larsson, E. C. Greif, D. G. Cvitkovitch, and I. R. Hamilton.** 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol. Immunol. **12:**266–273.
51. **van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin.** 2002. Stress responses in lactic acid bacteria. Antonie Van Leeuwenhoek **82:**187–216.
52. **Vijayakumar, J., R. Aravindan, and T. Viruthagiri.** 2008. Recent trends in the production, purification and application of lactic acid. Chem. Biochem. Eng. Q. **22:**245–264.
53. **Wardle, S. J., M. O'Carroll, K. M. Derbyshire, and D. B. Haniford.** 2005. The global regulator H-NS acts directly on the transpososome to promote Tn*10* transposition. Genes Dev. **19:**2224–2235.
54. **Zhang, Y.-M., and C. O. Rock.** 2008. Membrane lipid homeostasis in bacteria. Nat. Rev. Microbiol. **6:**222–233.
55. **Zhang, Y.-M., and C. O. Rock.** 2009. Transcriptional regulation in bacterial membrane lipid synthesis. J. Lipid Res. **50**(Suppl.)**:**S115–S119.