Identification and Inactivation of Genetic Loci Involved with *Lactobacillus acidophilus* Acid Tolerance

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Amino acid decarboxylation-antiporter reactions are one of the most important systems for maintaining intracellular pH between physiological limits under acid stress. We analyzed the *Lactobacillus acidophilus* NCFM complete genome sequence and selected four open reading frames with similarities to genes involved with decarboxylation reactions involved in acid tolerance in several microorganisms. Putative genes encoding an ornithine decarboxylase, an amino acid permease, a glutamate γ-aminobutyrate antiporter, and a transcriptional regulator were disrupted by insertional inactivation. The ability of *L. acidophilus* to survive low-pH conditions, such as those encountered in the stomach or fermented dairy foods, was investigated and compared to the abilities of early- and late-stationary-phase cells of the mutants by challenging them with a variety of acidic conditions. All of the integrants were more sensitive to low pH than the parental strain. Interestingly, each integrant also exhibited an adaptive acid response during logarithmic growth, indicating that multiple mechanisms are present and orchestrated in *L. acidophilus* in response to acid challenge.

During the last decade the use of microorganisms considered probiotic (health promoting) has increased dramatically. Specifically, some lactic acid bacteria have been shown to provide protection against gastrointestinal disorders by stimulating the immune system (8, 20, 23, 36). *Lactobacillus acidophilus* NCFM is a strain widely used in the manufacture of yogurt and fermented milk products (for a review see reference 29). The theoretical basis for selection of probiotic microorganisms includes safety, functionality (survival, adherence, colonization, etc.), and technology (sensory properties, growth, stability, and viability during manufacture [26]). The ability of microorganisms to survive in an acidic environment is important for both in vivo function and fermentation stability. Therefore, mechanisms contributing to the capacity of a microorganism to tolerate acidic pH are essential to the production and functionality of a probiotic culture.

Mechanisms underlying acid tolerance utilized by gram-positive bacteria include proton pumps, proteins involved in repair or degradation of damaged cell components, incremental expression of regulators that promote minor or global responses, and alterations in the composition of the cell envelope (6, 9). The multisubunit F_1F_0 ATPase, which facilitates the extrusion of protons from the cytoplasm by proton motive force, and the amino acid decarboxylation-antiporter systems are the main proton pumps utilized by these microorganisms. The F_1F_0-ATPase system of *L. acidophilus* has been well characterized (16). The *atp* operon of *L. acidophilus* contains eight genes. The inferred amino acid sequences of the subunits are similar to those of the ATPases of *Enterococcus hirae* and *Streptococcus mutans*. Exposure to low pH causes an increase in the abundance of ATPase-specific mRNA, indicating regulation at the level of transcription.

In amino acid decarboxylation-antiporter reactions, an amino acid is transported into the cell, where decarboxylation occurs. A proton is consumed in the reaction, and the product is exported from the cell via an antiporter. The net result is a rise in the intracellular pH (pH_i). Several inducible amino acid decarboxylases, including lysine, ornithine, and arginine decarboxylases, that contribute to raising the pH_i have been described for *Salmonella enterica* serovar Typhimurium. Inducible arginine decarboxylase and glutamate decarboxylase (GAD) have been described for both *Escherichia coli* and *Shigella flexneri* (3). Among gram-positive bacteria, a GAD system has been described for *Listeria monocytogenes* (7) and *Lactococcus lactis* (22).

The draft genome sequences of many microorganisms traditionally used in bioprocessing have become accessible recently (15). The analysis of the complete *L. acidophilus* NCFM genome sequence allowed us to identify putative genes potentially involved in decarboxylation-antiporter reactions within the genome. In this study, four open reading frames (ORFs) were investigated for their contribution to overall acid tolerance of this probiotic bacterium.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was propagated at 37°C in Luria-Bertani (Difco Laboratories Inc., Detroit, Mich.) broth with shaking. When appropriate, *E. coli* cultures were propagated or selected on brain heart infusion agar (Difco) supplemented with 150 μg of erythromycin/ml. Lactobacilli were propagated statically at 37°C in MRS broth (Difco) or on MRS agar supplemented with 1.5% agar. When appropriate, erythromycin (5.0 μg/ml) or/and chloramphenicol was added to MRS broth or agar plates.

Standard DNA techniques. Total *Lactobacillus* DNA was isolated as previously described (37). Standard protocols were used for ligations, endonuclease restrictions, DNA modification, and transformation as described by Sambrook et al. (27). Plasmid DNA from *E. coli* was isolated using the QIAprep spin kit

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
Strain		
E. coli EC1000	RepA$^+$ MC1000; Km^r; host for pOR128-based plasmids	18
L. acidophilus NCFM	Human intestinal isolate	2
L. acidophilus NCK1398	NCFM integrant *lacL*::pTRK685	25
L. acidophilus NCK1392	NCFM containing pTRK669	25
L. acidophilus NCK1678	NCFM integrant ORF La57 (glutamate/GABA antiporter)::pTRK803	This study
L. acidophilus NCK1680	NCFM integrant ORF La867 (transcriptional regulator)::pTRK804	This study
L. acidophilus NCK1682	NCFM integrant ORF La995 (amino acid permease)::pTRK805	This study
L. acidophilus NCK1684	NCFM integrant ORF La996 (ornithine decarboxylase)::pTRK806	This study
Plasmid		
pTRK803	576-bp internal region of ORF La57 cloned into the BgIII/Xbal sites of pOR128	This study
pTRK804	593-bp internal region of ORF La867 cloned into the BgIII/Xbal sites of pOR128	This study
pTRK805	604-bp internal region of ORF La995 cloned into the BgIII/Xbal sites of pOR128	This study
pTRK806	624-bp internal region of ORF La996 cloned into the BgIII/Xbal sites of pOR128	This study

according to the manufacturer's instructions (QIAGEN Inc., Valencia, Calif.). PCR was performed according to standard protocols (13). Southern hybridization of genomic DNA was performed using the Roche Molecular Biochemicals DIG nonradioactive nucleic acid labeling and detection system according to the manufacturer's specifications at 42°C in the standard hybridization buffer (containing 50% formamide). Detection of hybridization was performed with a CSPD chemiluminescent substrate.

Generation of site-specific integrations in *L. acidophilus*. An internal fragment of each ORF targeted for inactivation was amplified using *L. acidophilus* NCFM chromosomal DNA as template. The corresponding primers used are listed in Table 2. Internal fragments were cloned in the integrative vector pORI28 (18) and introduced by electroporation into *L. acidophilus* NCFM containing pTRK669 (25). Subsequent steps were carried out according to the protocol described by Russell and Klaenhammer (25). The position of the plasmid insertion within suspected integrants was confirmed by both PCR and Southern hybridization analysis to identify junction fragments.

Survival of stationary- and logarithmic-phase cultures in acid. To establish the acid sensitivity of stationary-phase cultures, cells were propagated in MRS at 37°C for 16 h. Four milliliters of cells were then centrifuged and resuspended in the same volume of MRS acidified to pH values ranging from 2.7 to 4.0, with lactic acid concentrations ranging from 220 to 1,520 mM. Survival was determined at 30-min intervals by serial dilutions in 10% MRS and enumeration on MRS agar using a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, West Yorkshire, England). Specific numbers of deaths per hour (*K*) were calculated by plotting the natural logarithm of survivors over time. The mean of four values was calculated for each sample at each interval, and *K* was calculated as the slope of the death curve.

To determine the acid sensitivity of log-phase cells, cultures were grown to an optical density at 600 nm (OD_{600}) of 0.25 to 0.3 (pH > 5.8) from a 2% inoculum in MRS broth. Cultures were centrifuged and resuspended in the same volume of MRS adjusted to pH 3.5 with lactic acid (at a concentration of 320 mM). Survival was determined at 30- or 40-min intervals by plating on MRS.

Acid adaptation assay. Cultures were propagated to an OD_{600} of 0.25 to 0.3 (pH > 5.8). Cells were then centrifuged and resuspended in the same volume of MRS, pH 5.5 (adjusted with lactate or HCl), and were then incubated for 1 h at 37°C. Controls were resuspended in MRS at pH 6.8. The cells were centrifuged again and subsequently resuspended in fresh MRS at pH 3.5 (adjusted with lactic acid) or pH 3.0 (adjusted with HCl). Samples were taken at 30-min intervals and were plated on MRS agar.

Bile, salt, ethanol, and heat stress assays. Adapted (pH 5.5 for 1 h, adjusted with lactate) and nonadapted log-phase cells (OD_{600} = 0.3) were centrifuged and resuspended in the same volume of MRS for heat stress (55°C) or in MRS containing 5% (wt/vol) bile (Oxgall; Difco), 10% (wt/vol) NaCl, or 20% (vol/vol) ethanol. Survival for each treatment was determined after 2 h by serial dilutions in 10% MRS and enumeration on MRS agar.

Computational analysis. BLASTP 2.2.7 (1) was used to align sequences. Protein conserved domains were defined based on Pfam (http://pfam.wustl.edu) and COG (Clusters of Orthologous Groups; http://www.ncbi.nlm.nih.gov/COG) collections of conserved patterns. Functional assignments for potential target genes were determined manually. TMHMM (http://www.cbs.dtu.dk/services/TMHMM) was used to predict transmembrane helices in proteins.

Nucleotide sequence accession numbers. The nucleotide sequences of *L. acidophilus* NCFM ORFs La57, La867, La995, and La996 have been deposited in the GenBank database under the accession numbers AY542887, AY542888, AY542889, and AY542890, respectively.

RESULTS

Tolerance of *L. acidophilus* to acid. To evaluate the resistance of *L. acidophilus* NCFM to acid, log-phase cells of NCK1398, a *lacL* integrant used as a parental control for these experiments (25), were exposed to pH 3.0 (adjusted with HCl) (Fig. 1). No loss of viability was detected over 5 h of exposure to pH 3.0, indicating a naturally high level of acid resistance in *L. acidophilus* to hydrochloric acid. In contrast, exposure of

TABLE 2. Primers used for PCR amplification

ORF	Internal primers[a]	External primers[b]
La57	Forward GATCTCTAGA-CCAGCAATCCAGTT	Forward GCATGCCAGCGATAAAGAAT
	Reverse GATCAGATCT-CTACACCGCTGATG	Reverse AAGGTGGTTCGCTCAGAAAT
La867	Forward GATCTCTAGA-ATATTGCGGTTGG	Forward GCACCTCAACAAAGTGATCAG
	Reverse GATCAGATCT-AGTGGGAAACATCG	Reverse AAAGGCCCTTAGATGGAAC
La996	Forward GATCTCTAGA-AGCCTGAGCCATAC	Forward ACAGTGTAGCCCTTGTAG
	Reverse GATCAGATCT-AGCGATACCGTTCC	Reverse CCATACTTGGAGGAGAAC
La995	Forward GATCTCTAGA-TGCTGCGCCTTACA	Forward AGTTGGCCAGTCATTGCT
	Reverse GATCAGATCT-TAGGGCACCCGTTC	Reverse TCGCCAAGATCACGAAGA

[a] Primer sets used to amplify the internal region for gene inactivation.
[b] Primer sets used to amplify junction fragments.

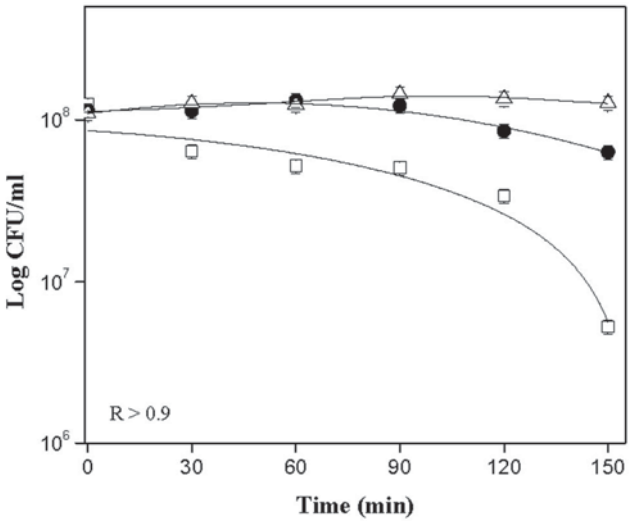

FIG. 1. Survival of *L. acidophilus* NCK1398 exposed to MRS broth adjusted to pHs 3.0 (with HCl) and 3.5 (with lactic acid) and incubated at 37°C. Symbols: ●, adapted (1 h, pH 5.5) cells exposed to pH 3.5; □, nonadapted cells exposed to pH 3.5; △, nonadapted cells exposed to pH 3.0.

NCK1398 to lactic acid (pH 3.5) eliminated more than 90% of the population within 2.5 h. When these cells were first exposed to pH 5.5 for 1 h and then were challenged by exposure to pH 3.5 (lactate), only a minor loss of viability was observed (Fig. 1). The data indicate that *L. acidophilus* induces an adaptive response at pH 5.5 that provides elevated acid tolerance to the cells.

Sequence analysis of regions implicated in acid tolerance and generation of site-specific integrations. We identified three regions in the *L. acidophilus* genome carrying putative genes that we suspected may provide acid tolerance to NCFM via amino acid decarboxylation. First, a gene similar to an ornithine decarboxylase (ORF La996; COG1982) was identified. Additionally, 12 ORFs were identified that contained a conserved amino acid permease module, COG0513. Among these, we selected an amino acid antiporter (La57) and the amino acid permease (La995), which was located adjacent to the ornithine decarboxylase (La996). Finally, a transcriptional regulator (La867) showing weak similarity to GadR, the regulator of the GadBC system in *L. lactis* (22), was selected. The annotations of these four genes and their surrounding regions are shown in Fig. 2.

Amino acid antiporter. No genes with high similarities to glutamate decarboxylase genes were found in the NCFM genome sequence. The putative protein encoded by ORF La57 showed (Fig. 2A) high identity (37 and 35%) with and similarity (60 and 58%) to a glutamate γ-aminobutyrate antiporter, designated *gadC*, in *Clostridium perfringens* (31) and *L. lactis* (5), respectively. In *L. lactis*, *gadCB* forms an operon present in one copy in the chromosome (22). La57 also shares similarity with the glutamate and glutamate γ-aminobutyrate antiporters in *Listeria innocua*, *L. monocytogenes*, *S. flexneri*, and *E. coli*. In *L. acidophilus* the antiporter is flanked by two terminators

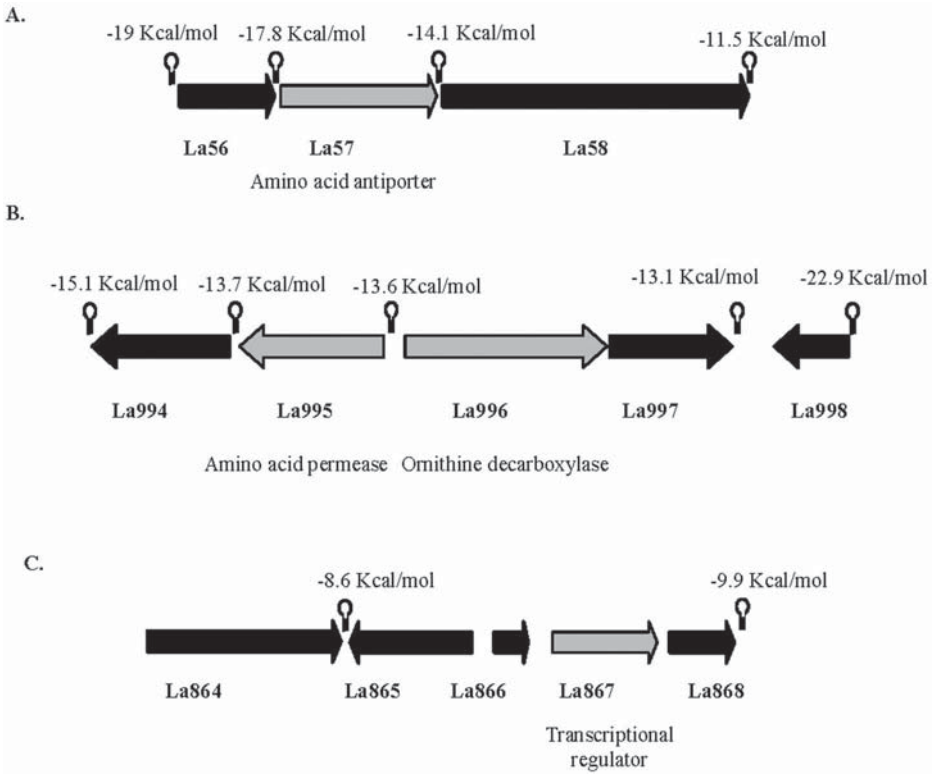

FIG. 2. Acid stress-related genes in *L. acidophilus*. (A) ORF La57 and surrounding genes. (B) Gene organization of the region containing ORFs La995 and La996. (C) Gene organization of the region containing ORF La867. Disrupted genes are represented by gray arrows. Putative rho-independent terminators and their calculated free energy are indicated. Potential functions based on homologies are indicated.

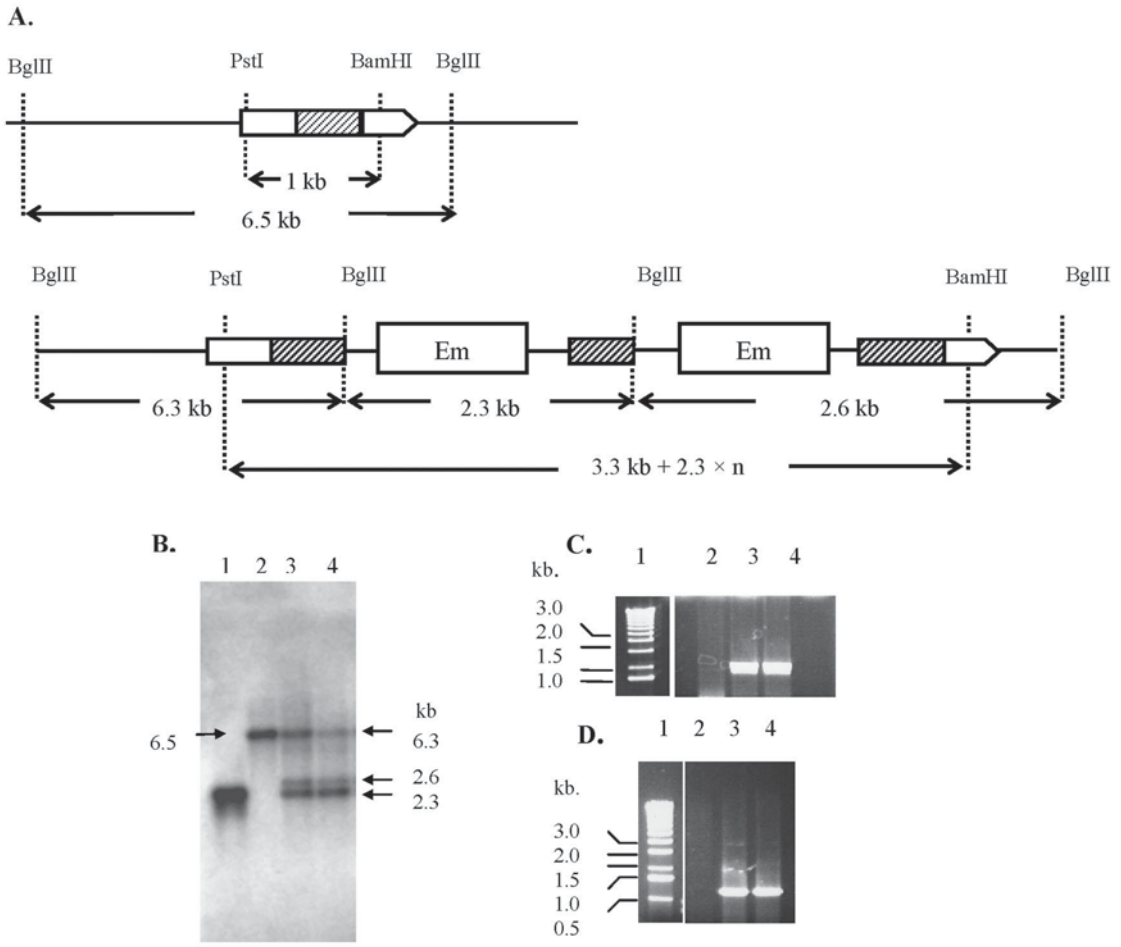

FIG. 3. Insertional inactivation of ORF La57 in *L. acidophilus* NCFM. (A) Diagram of the La57 locus of NCFM and NCK1678 chromosomes. La57 is represented by an arrow, and the internal fragment is denoted by a shaded box. The restriction sites PstI, BamHI, and BglII are indicated. The repeating unit represents the plasmid DNA present in various copies (n). (B) Southern hybridization analysis of NCFM and NCK1678. Chromosomal DNA was digested with BglII (lane 1, plasmid pTRK803; lane 2, NCFM; lanes 3 and 4, NCK1678). The internal fragment was used as the probe. (C and D) PCR amplification of the left (C) and right (D) junction fragments in NCK1678. Lane 1, 1-kb ladder; lane 2, NCFM; lanes 3 and 4, NCK1678. Em, erythromycin.

and is located downstream of a putative exodeoxyribonuclease (ORF La56; EC 3.1.11.2) and upstream of a putative helicase (La58). An amino acid permease-conserved domain (pfam00324) was found in La57, but a key motif that is predicted to play a role in the recognition of glutamate (FHLVF FLLLGG) was absent (38). The absence of the conserved motif necessary for glutamate recognition in La57 indicates that this gene is not likely to encode an antiporter for glutamate.

To determine if La57 contributed to acid tolerance in *L. acidophilus* NCFM, the gene was insertionally inactivated. A 576-bp internal region was amplified by PCR (Table 2). This fragment was then cloned into pORI28, and the resulting plasmid was transferred by electroporation into *L. acidophilus* NCK1392, which is NCFM harboring pTRK669. The integration strategy was carried out as described by Russell and Klaenhammer (25), and putative integrants were selected. Disruption of La57 was confirmed by both PCR analysis and Southern hybridization to detect junction fragments (Fig. 3). The mutant with the disruption of ORF La57 was designated NCK1678.

Ornithine/arginine/lysine decarboxylase and amino acid permease. ORF La995 (Fig. 2B) was similar to that of a permease from the amine-polyamine-choline superfamily. Polyamines (putrescine, spermidine, and spermine) are necessary for survival in *E. coli*, and they result from amino acid decarboxylation events (32). As mentioned above, a module related to amino acid transport (COG0531) is highly conserved within the protein sequence. In addition, La995 shows 12 strong transmembrane segments, suggesting that the N terminus is located in the cytoplasm. One of the most conserved bacterial members of this family is PotE, in *E. coli*. PotE is a 46-kDa protein that also contains 12 transmembrane segments, linked by hydrophilic segments of various lengths with the N and C termini, both located in the cytoplasm. Together with the gene encoding an inducible ornithine decarboxylase (*speF*), *potE* constitutes an operon in *E. coli*. Interestingly, the excretion of putrescine is catalyzed by the putrescine/ornithine antiporter activity of PotE (12).

ORF La996 (Fig. 2B) showed 48% identity (64% similarity) with the ornithine decarboxylase (EC 4.1.1.17) from *Lactoba-*

cillus sp. strain 30a (10), a representative of the large, pyridoxal-5′-phosphate (PLP)-dependent decarboxylases that act on lysine, arginine, or ornithine. This enzyme decarboxylates ornithine to putrescine and CO_2. Two major conserved domains are present in La996: the Orn/Lys/Arg decarboxylase major domain (pfam0127.4) and the Orn/Lys/Arg decarboxylase C-terminal domain (pfam 03711.2).

A putative aluminum resistance protein (La997) that showed similarity to the same protein in *Arthrobacter viscosus* (AF043609; 50% identity, 70% similarity) was located downstream of the ornithine decarboxylase. La997 also showed high similarity to an enzyme involved in cysteine/methionine metabolism in *Bacillus anthracis* A2012 (49% identity, 69% similarity) (24). Both proteins have the conserved motif from pfam1053 (Cys/Met metabolism PLP-dependent enzyme). This family includes enzymes involved in cysteine and methionine metabolism that also use PLP as a cofactor.

Transcriptional regulator. ORF La867 encodes a 267-amino-acid putative positive transcriptional regulator (Fig. 2C). It contains the conserved helix-turn-helix domain present in the XRE family-like proteins (smart00530.4). La867 has low similarity (22% identity) to the positive regulator GadR in *L. lactis*, part of the *gadCB* operon (28). In *L. lactis*, *gadR* is located upstream of *gadCB* and encodes a protein of 276 residues, similar to Rgg, a positive regulator involved in the expression of glucosyltransferase in *Streptococcus gordonii* (34). In *L. acidophilus*, the transcriptional regulator is preceded by ORF La866 (51 residues) of unknown function. Upstream of La866 a putative nucleoside hydrolase (EC 3.2.2.1) is present (La865). This enzyme hydrolyzes purine nucleosides to ribose, and a base is then converted to the concomitant nucleotide by phosphoribosyltransferases. Downstream of La867, a protein that showed no similarity to any known proteins is found. The presence of the helix-turn-helix motif and the similarity to a transcriptional regulator involved in acid tolerance in *L. lactis* led us to choose this gene for insertional inactivation.

To investigate the possible involvement of the selected target genes in acid tolerance, mutants were constructed by homologous integration events. A 624-bp internal region of the ornithine decarboxylase gene (La996), a 604-bp internal fragment of the amino acid permease gene (La995), and a 593-bp internal fragment of the transcriptional regulator gene (La867) were amplified by PCR using the primer sets La996, La995, and La867, respectively (Table 2). Integration events were confirmed by both PCR experiments and Southern hybridizations (data not shown). Mutants with disruptions of ORFs La867 (transcriptional regulator), La995 (amino acid permease), and La996 (ornithine decarboxylase) were designated NCK1680, NCK1684, and NCK1682, respectively.

All the integrants were genetically stable when propagated in the presence of antibiotic. However, after 40 generations in the absence of antibiotic selection, instability of the insertions detected by loss of antibiotic resistance occurred at frequencies ranging from 25% for NCFMΔ*lacL* to 90 to 99% for the other four insertional mutants (data not shown).

Acid challenge and adaptation assays. The response of log- and stationary-phase cells to a lethal pH was investigated and was used for comparisons of the mutants and wild-type cultures. A derivative of *L. acidophilus* NCFM designated NCK1398, where the *lacL* gene encoding β-galactosidase was inactivated (25), was used as the wild-type control in the acid tolerance experiments so that antibiotic pressure could be maintained on all strains. In MRS broth cultures, all mutants showed growth rates similar to that of the control at initial pH values of 6.8 or 5.5 (adjusted with either lactic acid or hydrochloric acid; data not shown).

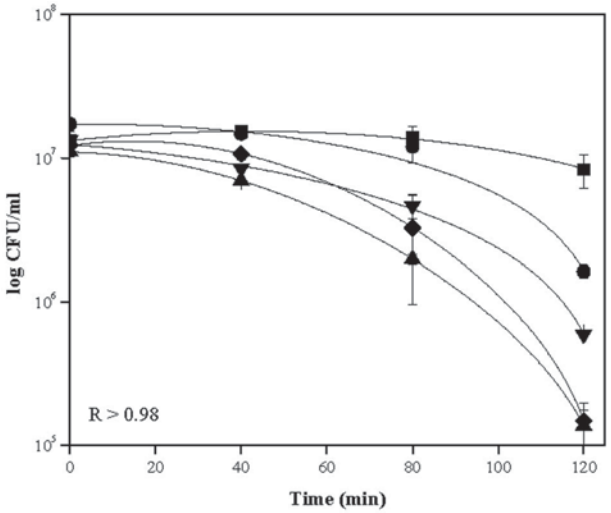

FIG. 4. Survival of log-phase *L. acidophilus* cultures in MRS adjusted to pH 3.5 with lactic acid. Viable cell counts were performed at 40-min intervals. ■, NCK1398 (control); ●, NCK1678 (La57, amino acid antiporter); ▲, NCK1680 (La867, transcriptional regulator); ◆, NCK1682 (La995, amino acid permease); ▼, NCK1684 (La996, ornithine decarboxylase).

Early-log-phase cells were exposed to MRS broth adjusted to pH 3.5 with lactic acid (Fig. 4). All the mutants showed significant differences in specific death rate compared to that of the control. Major differences were observed in NCK1684 (ornithine decarboxylase) and NCK1680 (transcriptional regulator), where the K value for both mutants was 4.8 times higher than that of the control. The K values for NCK1682 and NCK1678 were 3.6 and 2.3 times higher, respectively, than the specific death rate of NCK1398. These data suggested that these genes are involved in acid tolerance in *L. acidophilus*.

Stationary-phase cells sampled from cultures grown for 16 h were exposed to MRS adjusted to pH 3.0, 3.5, or 4.0 with lactate (Fig. 5). No growth or loss of viability was observed for the control or any of the mutants at pH 4.0 over a 2-h incubation period. At pH 3.5, slightly higher K values were observed for NCK1682 and NCK1680. At pH 3.0, a higher specific death rate was observed for the mutants disrupted in the antiporter (NCK1678) and ornithine decarboxylase (NCK1684). The correlation values (R) for the specific death rates were >0.9 at pHs 3.5 and 3.0 and >0.8 at pH 4.0.

Lastly, log-phase cells were resuspended in fresh MRS broth at pH 5.5 for 1 h prior to challenge at pH 3.5 (Fig. 6). The control (parental cells) showed little effect and remained fairly tolerant to treatment at pH 3.5 ($K = -0.24$ in the nonadapted cells versus $K = -0.55$ in the adapted cells). In contrast, all four mutants, which were markedly sensitive at pH 3.5 (see Fig. 4), showed virtually no acid sensitivity following a 1-h treatment at pH 5.5. The results indicate that treatment at pH 5.5 resulted in adapted cells that were more tolerant to acid chal-

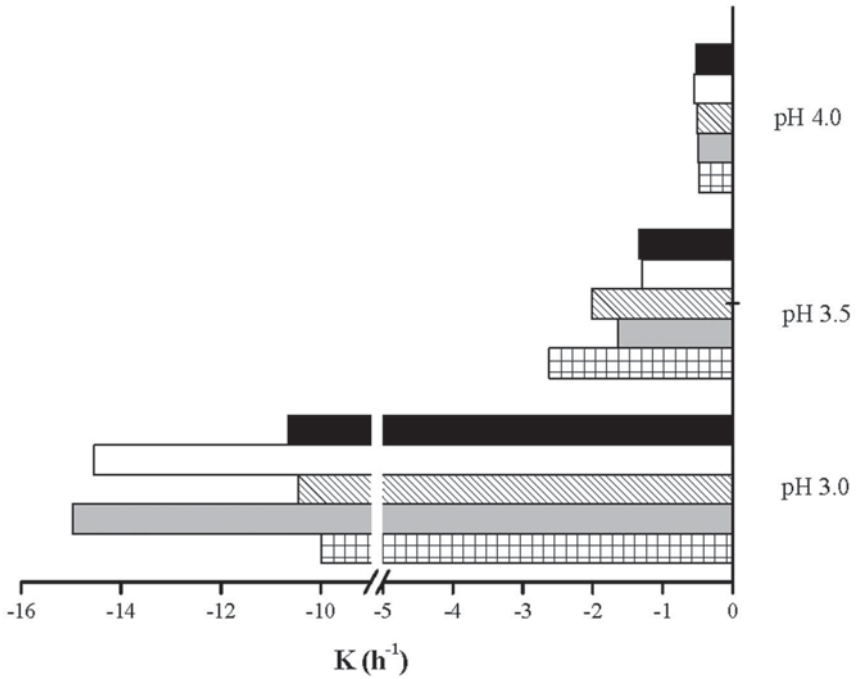

FIG. 5. Specific death rate (*K*) of late-stationary-phase cells of *L. acidophilus* after exposure to pHs 3.0, 3.5, and 4.0, adjusted with lactic acid. Viable cell counts were performed at 30-min intervals. ■, *L. acidophilus* NCK1398 (control); □, NCK1678 (amino acid antiporter); ▨, NCK1680 (transcriptional regulator); ▬, NCK1684 (ornithine decarboxylase); ⊞, NCK1682 (amino acid permease).

lenge. Moreover, the adaptation completely overcame the acid sensitivity of the mutants generated by each of the individual genes inactivated.

Responses to other stress conditions. Many organisms respond to challenging environments by synthesizing a collection of stress proteins that may confer general protection against a variety of stressors. We examined the response of the four acid-sensitive mutants to different stress conditions. The response of log-phase cells to stress was investigated by exposing nonadapted and adapted (in MRS at pH 5.5, adjusted with lactate, for 1 h) cells to bile (5%, wt/vol), sodium chloride (10%, wt/vol), high temperature (55°C), and ethanol (20%, vol/vol). Cells were exposed to the different stress conditions for 2 h and then were plated on MRS.

For nonadapted cultures, cells of *L. acidophilus* NCK1680 (transcriptional regulator) and NCK1682 (amino acid per-

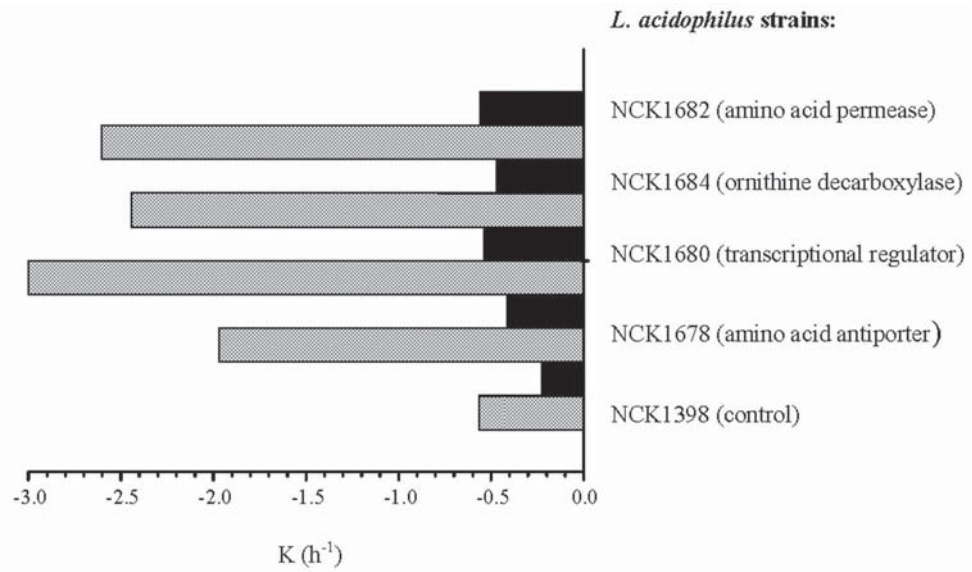

FIG. 6. Specific death rates (*K*) of nonadapted (shaded bars) and adapted (solid bars) log-phase cells of *L. acidophilus* derivatives after challenge at pH 3.5 (adjusted with lactic acid). Cells were adapted at pH 5.5 for 1 h prior to exposure to pH 3.5. Correlation values (*R*) were >0.9. Viable cell counts were performed at 30-min intervals for 2.5 h.

mease) were more sensitive to 5% bile (1.9 and 0.5% survival, respectively) than was the control strain (17.7% survival). Interestingly, nonadapted cells of NCK1684 (ornithine decarboxylase) were more resistant to 5% bile than the control strain (61.6% versus 17.7% survival in the control). No significant differences in survival were observed for nonadapted cells exposed to 10% NaCl, and all survived at levels of 60% after 2 h. Nonadapted mutant strains were more sensitive to heat stress than NCK1398, because no colonies were detected after 2 h at 55°C. Different degrees of sensitivity to ethanol stress were observed, NCK1682 (amino acid permease) being the most sensitive (0.028% versus 52.4% survival in the control).

Preconditioning of strains with acid did not induce ethanol, bile, or heat tolerance. However, acid-adapted strains of *L. acidophilus* NCK1398 (control), NCK1680 (transcriptional regulator), and NCK1684 (ornithine decarboxylase) displayed higher resistance to 10% NaCl (nonadapted cells survived at a level of 70%, and adapted cells survived at levels higher than 99%).

DISCUSSION

In this study, four ORFs with similarities to genes involved in amino acid decarboxylation reactions in other microorganisms were identified from the genomic sequence of *L. acidophilus*. The selected genes were insertionally inactivated, and the acid sensitivities of the derivatives were compared to that of a control strain. The ability of the mutants to mount an adaptation response to acid was also investigated. It was found that all four selected genes, namely, an amino acid antiporter, an ornithine decarboxylase, an amino acid permease, and a transcriptional regulator, contributed to the acid tolerance of *L. acidophilus*. However, over and above the contribution of these individual genes, an acid adaptation response was orchestrated by exposure to pH 5.5 for 1 h. This adaptation provided full acid protection to the organism upon challenge at pH 3.5 and overcame any deficiencies resulting from the loss of each of the four individual genes involved in amino acid decarboxylation reactions.

The selected genes are not essential for growth under the conditions of this study, as no differences in growth rate were observed between the control and the mutant strains in MRS broth or acidified MRS broth (adjusted to pH 5.5 with lactate or hydrochloric acid). However, in acid stress assays the mutant strains showed reduced survival after an acid shock, proving to be more sensitive than the control strain to organic acids. Specifically, the inactivation of the transcriptional regulator encoded by ORF La867 showed the most dramatic effects in the acid tolerance of log-phase cells of *L. acidophilus*. Further experiments are under way to identify putative target genes regulated by La867 during acid stress. Transcriptional arrays comparing the parent to the La867 mutant should point to genes regulated by this protein that may be important to acid tolerance. Due to the lack of a terminator downstream of La867, polar effects cannot be ruled out and any potential contribution by the unknown protein encoded by La868 is unclear. Polar effects are also a potential concern for ORF La996, as no possible terminator was identified for this gene. A putative terminator, however, was located downstream of La997.

While log-phase cells of the transcriptional regulator mutant (NCK1680) were most sensitive to lactate, stationary-phase cells of *L. acidophilus* NCK1678 (antiporter) and NCK1684 (ornithine decarboxylase) showed the most sensitive phenotypes when exposed to high concentrations of lactate (pH 3.0, approximately 870 mM). It has been proposed that lactic acid bacteria maintain an active metabolic state upon entry into stationary phase, with maintenance of the amino acid metabolism appearing essential for survival of *L. lactis* (33). Lactococci are able to survive periods of carbohydrate starvation by transporting certain amino acids (such as arginine, serine, methionine, phenylalanine, lysine, and alanine). They provide an energy source and minimize the breakdown of essential proteins. Moreover, the proton-linked amino acid transporter and the arginine or ornithine antiporter still function under starvation (17).

Among gram-positive bacteria, the GAD acid resistance system is the only amino acid decarboxylation system that has been associated with acid response (6). The GAD system as an acid defense mechanism has been described for *L. lactis* (22) among lactic acid bacteria, although GAD activity was also detected in *Lactobacillus brevis* (35) and *Lactobacillus* sp. (11). Among other amino acid decarboxylases, the pH regulation by a histidine/histamine antiporter was suggested for *Lactobacillus buchneri* (21). Based on our results, we propose a putative role in acid tolerance for the ornithine decarboxylase system in *L. acidophilus*. The role of this system needs to be further investigated to elucidate its physiological function.

L. acidophilus exhibits an acid tolerance response that enables cells pre-exposed to mildly acidic conditions (pH 5.5) to better survive more severe acid challenges (pH < 4.0) than cells that were not pre-exposed (19). Acid tolerance response involves the induction of acid shock genes and depends on several regulatory systems. In this study, we demonstrated that the inactivation of selected genes affecting acid sensitivity did not affect the capability of *L. acidophilus* to adapt to a highly acid-tolerant state.

The ability of microorganisms to withstand other (usually lethal) stresses can be enhanced by exposure to sublethal conditions. Among lactobacilli, cross-protection has been demonstrated in *L. acidophilus* (14). Pre-exposure to bile provided protection against heat stress, and pre-exposure to NaCl provided protection against bile and heat stress. In the present study, acid adaptation did not induce significant bile salt, heat, or ethanol tolerance in *L. acidophilus*. On the other hand, pre-exposure to mild pH induced significant resistance against acid challenge. In addition, acid-adapted cells exhibited higher resistance to NaCl than nonadapted cells, suggesting that partial physiological protection from acid and salt may overlap.

When the amino acid permease encoded by La995 was inactivated, high sensitivity to bile was observed in the mutant strain. Recently, Begley et al. (4) demonstrated that the disruption of lmo0448 (*gadE*) in *L. monocytogenes*, a gene encoding an amino acid transporter with a putative role in pH homeostasis, caused a 1.5-log-unit reduction in survival compared to that of the parent strain after exposure to Oxgall. The inactivation of the ornithine decarboxylase (La996) gene, adjacent to the La995 gene (but on the complementary strand), produces higher resistance to bile. In contrast, interruption of the amino acid permease (La995) results in bile sensitivity.

The survival of *L. acidophilus* in acidic environments has been studied, and this species proved to be highly resistant to acid (30). The main system utilized by *L. acidophilus* to maintain the pH_i, in the presence of organic acids, is the F_0F_1 ATPase. The various subunits of the F_0F_1 ATPase are encoded by the acid-responsive *atp* locus (16). General stress proteins, such as the GroESL operon, are also overexpressed during acid stress (19). However, the roles of other genes or systems putatively involved in acid tolerance have not been further investigated. We have identified four stress-related genes in *L. acidophilus* involved in acid and additional stresses. This study elucidated some of the mechanisms underlying the ability of *L. acidophilus* to survive low-pH environments, an important and desirable characteristic of probiotic cultures.

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