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# Microarray Analysis of a Two-Component Regulatory System Involved in Acid Resistance and Proteolytic Activity in *Lactobacillus acidophilus*

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**Two-component regulatory systems are one primary mechanism for environmental sensing and signal transduction. Annotation of the complete genome sequence of the probiotic bacterium *Lactobacillus acidophilus* NCFM revealed nine two-component regulatory systems. In this study, the histidine protein kinase of a two-component regulatory system (LBA1524HPK-LBA1525RR), similar to the acid-related system *lis*RK from *Listeria monocytogenes* (P. D. Cotter et al., J. Bacteriol. 181:6840–6843, 1999), was insertionally inactivated. A whole-genome microarray containing 97.4% of the annotated genes of *L. acidophilus* was used to compare genome-wide patterns of transcription at various pHs between the control and the histidine protein kinase mutant. The expression pattern of approximately 80 genes was affected by the LBA1524HPK mutation. Putative LBA1525RR target loci included two oligopeptide-transport systems present in the *L. acidophilus* genome, other components of the proteolytic system, and a LuxS homolog, suspected of participating in synthesis of the AI-2 signaling compound. The mutant exhibited lower tolerance to acid and ethanol in logarithmic-phase cells and poor acidification rates in milk. Supplementation of milk with Casamino Acids essentially restored the acid-producing ability of the mutant, providing additional evidence for a role of this two component system in regulating proteolytic activity in *L. acidophilus*.**

Survival of microorganisms during their transit through the gastrointestinal tract requires the ability to sense and respond to the various and changing conditions present in that environment. Two-component regulatory systems (2CRS) are one of the most important mechanisms for environmental sensing and signal transduction. They are found in the majority of gram-positive and gram-negative bacteria and control housekeeping functions as well as regulate proteins important for pathogenesis, stress, and adherence (6, 33, 38). A typical 2CRS consists of a membrane-associated histidine protein kinase (HPK), which detects specific environmental signals, and a cytoplasmic response regulator (RR), which regulates expression of one or more genes in a regulon (29). 2CRS are located in modules with various arrangements of conserved domains (41). HPKs generally consist of a signal input domain and an autokinase domain, which can be divided into two subdomains: a histidine phosphotransferase subdomain and an ATP-binding subdomain. The RR is typically composed of a regulatory (receiver) domain and a DNA-binding (output) domain (12). Detection of an external signal by the input domain of the kinase controls its own activation. The active kinases will autophosphorylate via ATP hydrolysis, on a histidine residue. This phosphoryl group is then transferred to an aspartate residue in the receiver domain of the RR that activates the regulatory protein and promotes the transcriptional response (9).

Genomic sequencing of microorganisms has uncovered the presence of many 2CRS and promoted global analysis of their responses to different environments. For those studies, DNA microarray technology involving high-density arrays of open reading frame (ORF)-specific fragments has been instrumental. Fabret et al. (8) identified and grouped 2CRS in *Bacillus subtilis* in five different groups, and the functions of these 2CRS have been investigated by microarray analysis (15, 28).

In lactic acid bacteria (LAB), production of some class II bacteriocins (plantaricin, sakacin P, sakacin A, and carnobacteriocin B2) is transcriptionally regulated through a signal transduction pathway which consists of three components: an inducer bacteriocin-like peptide, a HPK, and a RR (for a review, see reference 25). In fact, the production of many small antimicrobial peptides appears to be modulated by a cell density response mechanism. In addition, multiple 2CRS have been identified in a number of LAB (22, 23, 24). For example, six 2CRS were detected in *Lactococcus lactis*, with four of them implicated in cellular responses to stress (27).

*Lactobacillus acidophilus* NCFM is a probiotic organism that has been used extensively in yogurt, fermented foods, and dietary supplements (32). The annotated genome sequence of *L. acidophilus* NCFM encodes nine putative 2CRS (2). In the present study, we identified a 2CRS similar to the *lis*RK system described in *Listeria monocytogenes* (6), which participates in both stress response and virulence in *L. monocytogenes*. The HPK gene from the LBA1524HPK-LBA1525RR system was disrupted to investigate its putative role in acid tolerance. A whole-genome array containing 97.4% of *L. acidophilus* annotated genes was constructed and used to compare genome-

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TABLE 1. Primers utilized for probe generation in Northern blot analysis

| ORF | Description | Primer | |
|---|---|---|---|
| | | Orientation[a] | Sequence (5′ to 3′)[b] |
| LBA0197 | ABC transporter, oligopeptide-binding protein OppA1 | F | GCAGCATGTAGTAGTAATAA |
| | | R | CAGAATCACGTAATGTGTAA |
| LBA1300 | Oligopeptide ABC transporter, substrate-binding protein OppA2 | F | ATGCAATAGCTTGACGAAGA |
| | | R | ATGCAATATGGTGCTGAATC |
| LBA1524 | Two-component sensor histidine kinase | F | GATCTCTAGA-CAGCGCTCTAGCA |
| | | R | GATCAGATCT-TCGGCCAATGTG |
| LBA1525 | Two-component system regulator | F | GATCTCTAGA-CACGAACCGTCTT |
| | | R | GATCAGATCT-TTGGCTCGATTTG |
| LBA0698 | Glyceraldehyde-3-phosphate dehydrogenase | F | TCGTAGTTGACGGTAAGAAG |
| | | R | ACCTGCAGTAGTTACCATAG |
| LBA1075 | Malolactic enzyme | F | GTTGTTACAGACGGTGAAGG |
| | | R | TAATGCACGACCATCAGTCC |
| LBA1196 | RNA polymerase sigma factor RpoD | F | GATCTCTAGA-TTCCGCTTCTTACT |
| | | R | GATCAGATCT-ATCTGACGAATACG |

[a] F, forward; R, reverse.
[b] Dashes indicate the introduction of restriction enzyme sites.

wide transcriptional patterns of the control and the HPK mutant, exposed to three different pHs.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in the present study were *Escherichia coli* EC1000 (RepA$^+$ MC1000, kanamycin resistant; host for pORI28-based plasmids) (17) and *L. acidophilus* strains NCFM (human intestinal isolate; (5), NCK1398 (NCFM *lacL*::pTRK685, (31), and NCK1686 (NCFM LBA1524::pTRK807; [the present study]).

*E. coli* strains were propagated at 37°C in Luria-Bertani (LB; Difco Laboratories, Inc., Detroit, MI) broth with shaking. Erythromycin (Em)-resistant clones of *E. coli* were selected on brain heart infusion agar (Difco) supplemented with Em (150 μg/ml). Lactobacilli were propagated statically at 37°C in MRS (Difco) or on MRS supplemented with 1.5% agar. When appropriate, Em (5.0 μg/ml) and/or chloramphenicol (7.0 μg/ml) was added. Reconstituted 10% skim milk (SM), 10% SM supplemented with 0.5% yeast extract (Difco), or 0.25% Casamino Acids (Difco) were used for determination of acidification rates.

**Standard DNA techniques.** Restriction enzymes (Roche Molecular Biochemicals, Indianapolis, IN) and T4 DNA ligase (New England Biolabs, Beverley, MA) were used according to the suppliers' recommendations. Plasmid preparations from *E. coli* were performed by using the QIAprep Spin Plasmid Minipreps kit (QIAGEN, Inc., Valencia, CA). Chromosomal DNA from *L. acidophilus* was extracted according to the method of Walker and Klaenhammer (40). Electrotransformation of *L. acidophilus* was carried out as described by Walker et al. (39). PCR was performed by standard protocols using *Taq* DNA polymerase (Roche Molecular Biochemicals).

**DNA sequence analysis and data submission.** Potential coding sequences were derived from the genomic sequence of *L. acidophilus* NCFM (GenBank accession number CP000033, (2). Protein sequence similarity analysis was conducted by using the BlastP module (3) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). TMHMM (http://www.cbs.dtu.dk/services/TMHMM) was used to predict transmembrane helices in proteins. CD-Search (20) was used to identify conserved domains in protein sequences.

Microarray platform and data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession numbers GPL1401 (platform) and GSE1976 (series).

**RNA isolation and RNA slot blots.** Aliquots (10 ml) of *L. acidophilus* cultures grown on MRS to $A_{600}$ = 0.3 were transferred to MRS (adjusted to desired pH with lactic acid). After 30 min, cells were harvested by centrifugation and frozen immediately in a dry ice-ethanol bath. One ml of TRIzol (Life Technologies, Rockville, MD) was added to the cell pellets; they were then homogenized in a Mini-Beadbeater-8 cell disruptor (Biospec Products, Bartlesville, OK) for five 1-min cycles (and chilled on ice for 1 min between the cycles), and the phases were separated by centrifugation (14,000 rpm, 15 min, 4°C). The aqueous phase was removed to a fresh tube, and 0.4 ml of TRIzol and 0.2 ml of chloroform were added. The mixture was vortexed for 15 s and centrifuged to separate the phases. The TRIzol step was repeated twice, and RNA was precipitated from the final aqueous phase by adding 1 volume of isopropanol, followed by incubation at room temperature for 10 min and centrifugation (12,000 rpm, 10 min, 4°C). The concentration and purity of RNA samples were determined by electrophoresis on agarose gels and standard spectrophotometer measurements.

Total RNA hybridizations using a slot blot apparatus (Bio-Dot SF; Bio-Rad) and Zeta-Probe membrane (Bio-Rad Laboratories, Inc.) were carried out as previously described (7). [α-$^{32}$P]dCTP-labeled probes were generated from PCR fragments by using the Multiprime DNA labeling system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and purified by using the NucTrap Probe purification columns (Stratagene, La Jolla, CA). The primers utilized are listed in Table 1. Radioactive signals were detected by using a Kodak Biomax film, and autoradiographs were analyzed by densitometry using the SpotDenso function with auto-linked background on an AlphaImager 2000 (Innotech Scientific).

**Generation of *L. acidophilus* DNA microarray.** A whole-genome DNA microarray based on the PCR products of predicted ORFs from the *L. acidophilus* genome was used for global gene expression analysis. PCR primers for 1,966 genes were designed by using GAMOLA software (1) and purchased from QIAGEN Operon (Alameda, CA). Total genomic DNA from *L. acidophilus* NCFM was used as a template for 96-well PCR amplifications. To amplify gene-specific PCR products, a 100-μl reaction mixture containing 1 μl of *L. acidophilus* DNA (100 ng/ml), 10 μl of specific primer pairs (10 μM), 0.5 μl of deoxynucleoside triphosphate mix (10 mM), 10 μl of PCR buffer (10×), and 1 μl of *Taq* DNA polymerase (5 U/μl [Roche Molecular Biochemicals]) was used. The following PCR protocol was followed: an initial denaturation step for 5 min at 94°C, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s, and polymerization at 72°C for 45 s. Approximately 95% of the ORFs produced a unique PCR product between 100 and 800 bp. The size of fragments was confirmed by electrophoresis in 1% agarose gels. DNA from 96-well plates were purified by using the QIAGEN purification kit. In general, the total quantity of each PCR product was greater than 1 μg. The purified PCR fragments were spotted three times in a random pattern on glass slides (Corning, Acton, MA) by using the Affymetrix 417 Arrayer at the NCSU Genome Research Laboratory (http://www.cals.ncsu.edu:8050/grl/). To prevent carryover contaminations, pins were washed between uses in different wells. Humidity was controlled at 50 to 55% during printing. DNA was cross-linked to the surface of the slide by UV (300 mJ) and posterior incubation of the slides for 2 h at 80°C. The reliability of the microarray data was assessed by hybridization of two cDNA samples prepared from the same total RNA, labeled with Cy3 and Cy5. Hybridization data revealed a linear correlation in the relative expression level of 98.6% of 5,685 spots (each gene by triplicate) with no more than a twofold change.

**cDNA probe preparation and microarray hybridization.** Identical amounts (25 μg) of DNase-treated (Invitrogen) RNA were aminoallyl-labeled by reverse transcription with random hexamers in the presence of amino-allyl dUTP (Sigma Chemical Co.), using Superscript II reverse transcriptase (Life Technologies) at 42°C overnight, followed by fluorescence-labeling of aminoallylated cDNA with *N*-hydroxysuccinimide-activated Cy3 or Cy5 esters (Amersham Pharmacia Biotech). Labeled cDNA probes were purified by using a PCR Purification Kit (QIAGEN). Coupling of the Cy3 and Cy5 dyes to the AA-dUTP-labeled cDNA and hybridization of samples to microarrays were performed according to the protocols outlined in the TIGR protocols (www.tigr.org/tdb/microarray

/protocolsTGR.shtml). Briefly, combined Cy5- and Cy3-labeled cDNA probes were hybridized to the arrays for 16 h at 42°C. After hybridization, the slides were washed twice in low stringency buffer (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] containing 0.2% sodium dodecyl sulfate) for 5 min each. The first wash was performed at 42°C, and the second one was done at room temperature. Subsequently, the slides were washed in a high-stringency buffer (0.1× SSC containing 0.2% sodium dodecyl sulfate for 5 min at room temperature) and finally in 0.1× SSC (two 2.5-min washes at room temperature).

**Data normalization and gene expression analysis.** Immediately after washing of the arrays, fluorescence intensities were acquired at 10-μm resolution by using a ScanArray 4000 Microarray Scanner (Packard Biochip BioScience; Biochip Technologies LLC) and stored as TIFF images. Signal intensities were quantified, the background was subtracted, and data were normalized by using the QuantArray 3.0 software package (Perkin-Elmer). Two slides (each containing triplicate arrays) were hybridized reciprocally to Cy3- and Cy5-labeled probes per experiment (dye swap). Spots were analyzed by adaptive quantitation. The data were median normalized. When the local background intensity was higher than the spot signal (negative values), no data were considered for those spots. The median of the six ratios per gene was recorded. The ratio between the average absolute pixel values for the replicated spots of each gene with or without treatment represented the fold change in gene expression. All genes belonging to a potential operon were considered for analysis if at least one gene of the operon showed significant expression changes, and the remaining genes showed trends toward that expression. Confidence intervals and *P* values on the fold change were also calculated with the use of a two-sample *t* test. *P* values of ≤0.05 were considered significant (14).

**Construction of the HPK mutant.** A 766-bp internal fragment of ORF LBA1524 was amplified by using *L. acidophilus* NCFM chromosomal DNA as a template and the primers I1524F (5′-GATCTAGACAGCGCTCTAGCA-3′) and I1524R (5′-GATCGATCTTCGGCCAATGTG-3′). The internal fragment was cloned in the integrative vector pORI28 (17) generating pTRK807 and introduced by electroporation in *L. acidophilus* NCFM containing pTRK669 (31). Subsequent steps to facilitate the integration event were carried out according to the method of Russell and Klaenhammer (31). The suspected integrants were confirmed by PCR and Southern hybridization analysis using standard procedures.

**Acid challenge and adaptation assays.** For acid challenge analysis, cells were grown to an absorbance at 600 nm ($A_{600}$) 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 37°C. Survival was determined at 30-min intervals by plating serial dilutions in a 10% MRS broth diluent onto MRS agar using a Whitley Automatic Spiral Plater (Don Whitley Scientific, Ltd., West Yorkshire, England).

For acid adaptation assays, cells were grown to an $A_{600}$ of 0.25 to 0.3 (pH > 5.8). Cells were centrifuged and resuspended in the same volume of MRS (pH 5.5; adjusted with lactate), and incubation continued for 1 h at 37°C as described previously (4). Controls were resuspended in MRS broth at pH 6.8. The cells from the adapted (pH 5.5) and control (pH 6.8) cultures were then centrifuged and resuspended in MRS broth at pH 3.5 (adjusted with lactic acid). Viable-cell counts were performed at 30-min intervals for 2.5 h by plating on MRS agar.

**Ethanol tolerance.** Log-phase cells at an $A_{600}$ of 0.25 to 0.3 (pH > 5.8) from a 2% inoculum in MRS broth were centrifuged and resuspended in the same volume of MRS or in MRS containing 15 or 20% (vol/vol) ethanol. CFU/ml values were determined at 30-min intervals by serial dilutions in 10% MRS and enumeration on MRS agar as described above.

## RESULTS

**2CRS.** Using the CD-Search (20) and BlastP (3) programs, we identified nine signal transduction systems consisting of an HPK and an RR (2). These 2CRS represented almost 1% of *L. acidophilus* NCFM ORFs. In addition, four RRs were identified containing a LytTR DNA-binding motif that were not associated with a histidine kinase. HPKs share a characteristic kinase core composed of a dimerization domain and a catalytic domain for ATP binding and phosphate transfer. The C-terminal half of the HPK proteins showed five conserved amino acid motifs: the H box, containing the His residue that will be phosphorylated, and the N, G1, F, and G2 boxes (35). ORFs LBA0079HPK, LBA0747HPK, LBA1524HPK, LBA1430HPK, LBA1660HPK, and LBA1819HPK were assigned to the group IIIA/OmpR of HPKs in accordance with the region surrounding the histidine that becomes phosphorylated, whereas the HPKs LBA0602HPK and LBA1799HPK were categorized in the class IV (8). The remaining 2CRS (LBA1413 and LBA1414) could not be classified into any known category. LBA1413 showed a domain of unknown function with a GGDEF motif (smart00267, DUF1), which apparently occurs exclusively in eubacteria and might participate in prokaryotic signaling processes. LBA1414 showed also a domain of unknown function (cd01948, EAL), which is found in diverse bacterial signaling proteins. Together with the GGDEF domain, EAL might be involved in regulating cell surface adhesiveness in bacteria (10).

RRs contain two conserved domains. First a regulator, which receives the signal from the sensor partner in bacterial 2CRS. It contains a phosphoacceptor site that is phosphorylated by the histidine kinase. Second, a DNA-binding effector domain in the C terminus of the protein. RRs present in *L. acidophilus* contained these two conserved domains. The RRs ranged from 221 to 274 amino acids (aa) in size. ORFs LBA0078RR, LBA0746RR, LBA1525RR, LBA1431RR, LBA1659RR, and LBA1820RR can be included in the OmpR family of RRs according to the amino acid sequence of their output domains, where the residues involved in the hydrophobic core of the domain are conserved (21). The RRs encoded by LBA603RR and LBA1798RR can be defined as members of the AlgR/AgrA/LytR family of RRs (26).

The 2CRS composed of LBA1524HPK and LBA1525RR formed an operon flanked by two terminators with a free energy of −11.0 and −13.8 kcal/mol, respectively. Also, a typical ribosome binding site sequence and a putative promoter were positioned upstream of LBA1525RR (Fig. 1). The HPK gene showed a 36% identity with the HPK in the *lis*RK system described in *L. monocytogenes* (6). This two-component signal transduction system was shown to participate in the stress response and virulence of *L. monocytogenes*. A *lis*RK-defective mutant, generated by random insertional mutagenesis, grew at higher concentrations of ethanol than the parental strain but was more sensitive to acid stress during logarithmic phase of growth (6). LBA1524HPK also showed homology (32% identity and 55% similarity) to the HPK gene of *csr*RS, a system that represses the expression of the hyaluronic acid capsid and virulence factors of *Streptococcus*, SLS and SpeB (11).

**Insertional inactivation of LBA1524HPK and acid stress assays.** To investigate the physiological function of LBA1524HPK-LBA1525RR 2CRS and to examine its putative association with acid tolerance in *L. acidophilus*, a chromosomally interrupted LBA1524HPK mutant was constructed. For insertional inactivation of the HPK, a 766-bp internal region was amplified by PCR with the primers I1524F and I1524R described in Materials and Methods. This fragment was cloned into pORI28 and the resulting plasmid, pTRK807, was then transferred by electroporation into *L. acidophilus* NCFM, already harboring the helper plasmid pTRK669. Integrants were isolated as described by Russell and Klaenhammer (31) to generate strain NCK1686. PCR experiments and Southern hybridizations were performed to confirm the integration event via junction amplicons and fragments (data not shown). Because this operon was flanked by two putative ter-

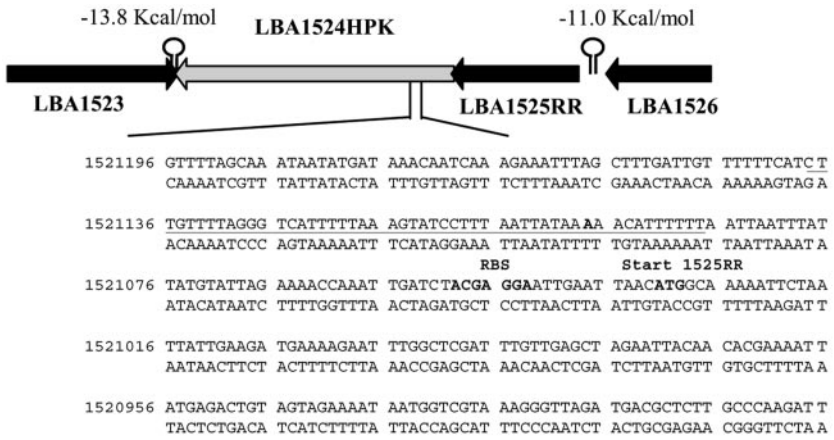


FIG. 1. Organization of LBA1524HPK-LBA1525RR 2CRS in *L. acidophilus* NCFM. The disrupted HPK gene is represented by a gray arrow. Putative terminator regions and their calculated free energy are indicated by hairpin structures. The start, the putative ribosome-binding site, the potential promoter, and the transcription start are indicated.

minators, polar effects from the inactivation of LBA1524HPK were not expected. Phase-contrast microscopy of the HPK mutant revealed a decrease in cell size and chain length compared to the wild-type NCFM cells (Fig. 2).

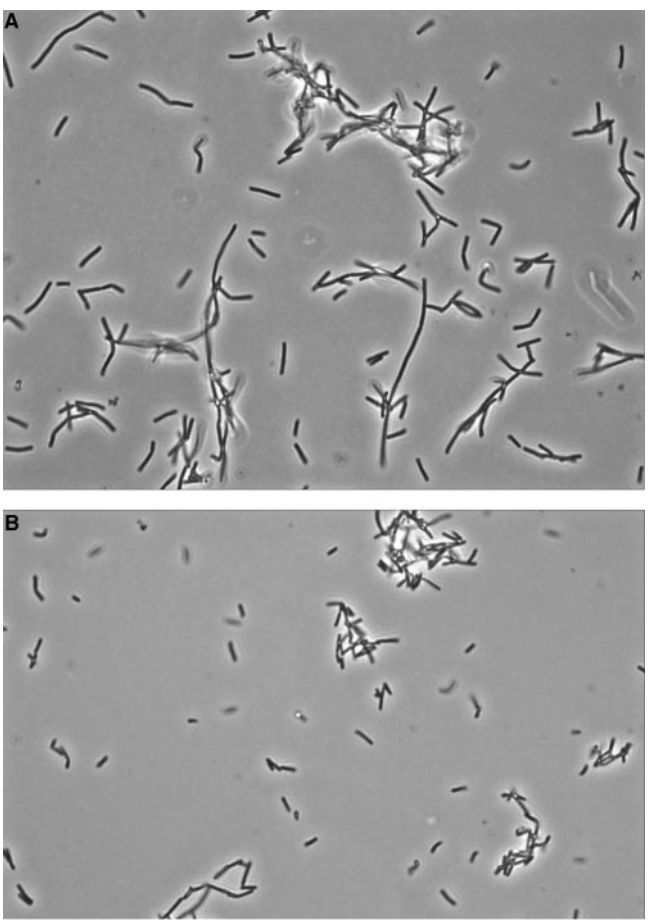


FIG. 2. Morphology of *L. acidophilus* NCFM (A) and NCFM::LBA1524HPK (B) as seen under a phase-contrast microscope. Magnification, ×1,000.

Two strong transmembrane regions can be predicted, by in silico analysis, in the HPK of LBA1524HPK-LBA1525RR 2CRS (from 24 to 42 aa and from 202 to 226 aa). The ATP-binding phosphotransfer (catalytic domain) and the dimerization domain can be located in the carboxy termini of the protein from 396 to 499 aa and from 276 to 341 aa, respectively. The 766-bp internal region of LBA1524HPK, amplified by PCR with the primer pair I1524F-I1524R, used to inactivate the HPK spanned from 51 to 307 aa. As a consequence, insertion of the vector would have affected the second transmembrane and/or the dimerization domain of the HPK.

The response of log-phase cells to pH 3.5 was compared between the HPK mutant strain NCK1686 and control, *L. acidophilus* NCK1398 (NCFM::*lac*L). Strain NCK1398 was used as a control throughout the study so that the effects of antibiotic pressure could be accounted for. When log-phase cells of NCK1686 were exposed to pH 3.5, a >2-log reduction in CFU was observed after 2.5 h compared to a half-log reduction in the control (Fig. 3A). Therefore, similar to *L. monocytogenes* (6), the HPK mutant was more sensitive to acid, indicating that the LBA1524HPK-LBA1525RR 2CRS plays a significant role in acid resistance of *L. acidophilus*.

**Acid adaptation of *L. acidophilus*.** Log-phase cells of *L. acidophilus* NCK1398 and NCK1686 were exposed to pH 5.5 for 1 h, prior to challenge by pH 3.5. Remarkably, both the control and the HPK mutant exhibited a high tolerance to acid challenge (Fig. 3B). Exposure to pH 5.5 appeared to adapt the cells to a higher level of acid tolerance during challenge at pH 3.5. The acid sensitivity incurred by the LBA1524HPK mutant over 150 min was nearly abolished by the adaptation period at pH 5.5, but after 2.5 h at pH 3.5 the mutant still remained more sensitive than the control.

**Global gene expression of the HPK mutant.** In an attempt to identify genes regulated by the 2CRS and potentially affected by inactivation of the LBA1524HPK ORF, parallel cultures of the control strain NCK1398 (NCFM::*lac*L) and the HPK mutant (NCK1686) were grown in MRS broth to an optical density of 0.3 and transferred to MRS adjusted to pH 6.8, 5.5, or 4.5. After 30 min, RNA was isolated and used for hybridization to microarray slides printed with representative sequences of

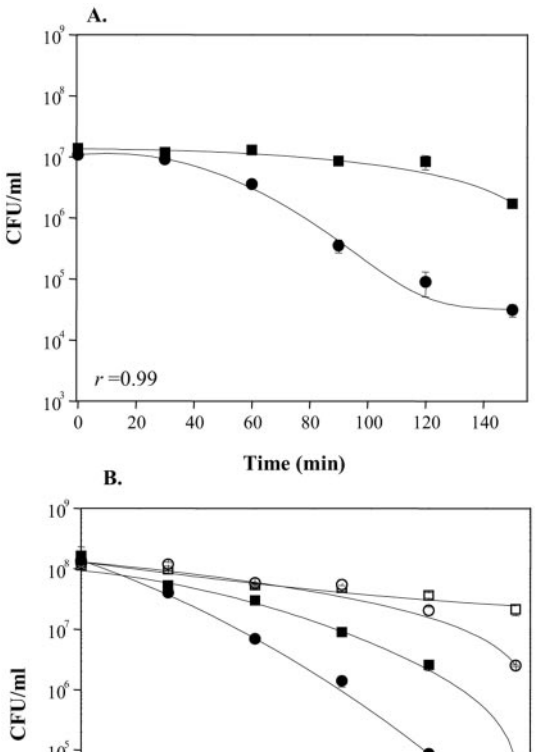


FIG. 3. (A) Survival of *L. acidophilus* NCK1398 (NCFM::*lac*L, squares) and the HPK mutant NCK1686 (circles) in MRS adjusted to pH 3.5 with lactic acid. (B) Cells were exposed to pH 5.5 (open symbols) or pH 6.8 (filled symbols) for 1 h prior to challenge at pH 3.5 (adjusted with lactic acid).

the majority of the identified ORFs on the *L. acidophilus* genome. Statistically significant ($P \leq 0.05$) gene expression changes were considered for ORFs exhibiting at least a twofold change.

Comparison of the expression profiles identified 80 differentially expressed genes showing at least twofold changes in expression patterns (Table 2). As expected, the components of the LBA1524HPK-LBA1525RR 2CRS, as well as the large and small subunits of the β-galactosidase and UDP-glucose 4-epimerase, were differentially expressed, owing to the inactivation of these genes in the compared strains. Surprisingly, the inactivated HPK gene and the RR were overexpressed in the NCK1686 mutant. This might be attributable to amplification of the vector in the chromosome and/or a readthrough event where a longer transcript is generated, but not translated into a functional protein. The same effect was observed for NCFM::*lac*L where the disrupted operon appeared to be highly expressed. Alternatively, a nonfunctional HPK could result in elevated transcriptional expression of the 2CRS, if the phosphorylated form of the RR was involved in the autoregulation of the 2CRS.

The most dramatic changes in expression in the HPK mutant were observed in genes predicted to encode components of the proteolytic enzyme system. Proteolytic systems of LAB are divided in three functional categories: (i) proteinases (that degrade caseins into small peptides), (ii) transport systems (that import those peptides), and (iii) peptidases (16). The expression of ORF LBA1512 encoding the proteinase precursor in *L. acidophilus*, PrtP (39% identical and 53% similar to the cell envelope proteinase PrtR from *L. rhamnosus* GI27527536), increased in the HPK mutant by >7-fold at pH 6.8 and 5.5 (Table 2). However, PrtM (LBA1588), the protein putatively involved in the maturation of the proteinase, showed expression levels comparable to the control strain (ratios between 0.8 and 1.1).

Two operons potentially encoding oligopeptide ABC transporters are present in the *L. acidophilus* genome (Fig. 4): *opp*1 (ORFs LBA0197 to LBA0203) and *opp*2 (ORFs LBA1300 to LBA1306). Each consists of six genes, *opp*1 consists of *opp*D1, *opp*F1, *opp*B1, *opp*C1, *opp*A1, and *opp*A1B, and *opp*2 consists of *opp*D2, *opp*F2, *opp*B2, *opp*C2, *opp*A2, and *opp*A2B, coding for two ATP-binding proteins (OppD and OppF), two membrane proteins (OppB and OppC), and two substrate-binding proteins (OppA and OppA-B). The *opp*A and *opp*A-B genes in both operons are separated by terminators from the downstream genes. The expression of the *opp*1 operon was significantly increased in the HPK mutant at pH 6.8 and 5.5 showing increments of six- to eightfold in most of the genes in the operon. The ORFs encoded by *opp*2 showed an increased expression in the mutant at pH 5.5. Interestingly, *opp*A2 (LBA1300) was downregulated in the mutant under all of the evaluated conditions, but the expression of *opp*A2B (LBA1301) increased significantly at pH 6.8 in the mutant (Table 2). Two other ORFs encoding putative oligopeptide-binding proteins were differentially expressed in the mutant. LBA1665 was consistently under expressed in the HPK mutant at the three pHs. In contrast, LBA1961 was overexpressed at pH 6.8 and 5.5.

Four peptidases were also differentially expressed in the HPK mutant strain. A neutral endopeptidase PepO (LBA0165) was upregulated at all pHs evaluated. The aminopeptidase encoded by LBA0911, and peptidase T (LBA1515) were upregulated at pH 5.5. Finally, a cytosol nonspecific dipeptidase encoded by ORF LBA1837 was significantly upregulated at pH 4.5.

To investigate potential alterations in the proteolytic system of the HPK mutant, we compared the acidification rates of *L. acidophilus* NCFM (wild type; since NCK1398 does not grow in milk) versus the HPK mutant in 10% SM and in 10% SM plus yeast extract (Fig. 5A). The HPK mutant was not able to acidify SM to a pH of <5.0 compared to the control where the pH dropped to nearly pH 4.0. Supplementation of SM with 0.5% yeast extract completely restored a wild-type level of acidification activity in the HPK mutant. In addition, supplementation of SM with 0.25% Casamino Acids also nearly abolished the difference between the wild type and the HPK mutant (Fig. 5B). These data suggest that the mutant was deficient in proteolytic activity. In addition, other component(s) present in yeast extract further stimulated the acidification rates of both the parent and mutant to equal levels in skim milk.

Expression of LBA1080 (a putative methionine synthase)

TABLE 2. ORFs differentially expressed in the HPK mutant (NCK1686) compared to the control *L. acidophilus* NCK1398 (NCFM::*lac*L) under different pH conditions[a]

| COG[b] functional classification/gene | Relative mRNA ratio (HPK/WT)[c] | | |
|---|---|---|---|
| | pH 6.8 | pH 5.5 | pH 4.5 |
| Amino acid transport and metabolism [E] | | | |
| LBA0111 putative ABC transporter (glutamine), ATP-binding protein | **0.36** | **0.47** | **0.57** |
| LBA0112 putative ABC transporter (glutamine), substrate-binding protein | **0.53** | **0.71** | 0.83 |
| LBA0197 ABC transporter, oligopeptide-binding protein *oppA1* | **6.22** | **4.43** | 1.92 |
| LBA0198 ABC transporter, oligopeptide-binding protein *oppA1B* | **7.42** | **1.96** | 1.59 |
| LBA0200 ABC transporter, oligopeptide permease protein *oppB1* | **6.70** | **6.31** | 1.67 |
| LBA0201 ABC transporter, oligopeptide permease protein *oppC1* | **7.27** | **8.09** | **2.55** |
| LBA0202 oligopeptide ABC transporter, ATP-binding protein *oppD1* | **7.44** | **7.09** | **3.89** |
| LBA0203 oligopeptide ABC transporter, ATP-binding protein *oppF1* | **8.01** | **7.10** | **5.57** |
| LBA0849 diaminopimelate epimerase | 1.30 | **2.15** | 0.91 |
| LBA0850 aspartokinase/homoserine dehydrogenase | 1.33 | **1.89** | 0.79 |
| LBA0911 aminopeptidase *pepC* | 1.79 | **1.79** | 0.72 |
| LBA0943 cationic amino acid transporter | 2.94 | **2.90** | 1.51 |
| LBA1042 ABC transporter (glutamine) membrane-spanning permease | 0.93 | **0.44** | 0.71 |
| LBA1044 ABC transporter (glutamine) membrane-spanning permease | 0.91 | 0.47 | 0.72 |
| LBA1045 ABC transporter (glutamine) ATP-binding protein | 0.78 | 0.39 | 0.82 |
| LBA1046 ABC transporter (glutamine) substrate-binding protein | 0.84 | **0.43** | 0.71 |
| LBA1080 putative methionine synthase *metK* | **6.96** | **5.51** | **4.32** |
| LBA1086 amino acid permease | **3.44** | 1.73 | **1.64** |
| LBA1135 macrolide efflux protein | 1.12 | **2.00** | 1.21 |
| LBA1211 homoserine kinase *khsE* | 1.84 | 1.67 | 1.32 |
| LBA1212 homoserine dehydrogenase *hdh* | **2.25** | 1.50 | 1.20 |
| LBA1300 oligopeptide ABC transporter, substrate-binding protein *oppA2* | **0.35** | **0.47** | **0.36** |
| LBA1301 oligopeptide ABC transporter, substrate-binding protein *oppA2B* | **4.92** | 1.74 | 1.37 |
| LBA1302 oligopeptide ABC transporter, permease protein *oppC2* | 1.29 | 2.14 | 1.43 |
| LBA1303 ABC transporter, oligopeptide permease protein *oppB2* | 1.50 | **1.98** | 1.49 |
| LBA1305 oligopeptide ABC transporter, ATP-binding protein *oppF2* | 1.50 | **2.00** | 1.33 |
| LBA1306 oligopeptide ABC transporter, ATP-binding protein *oppD2* | 1.24 | **2.22** | 1.35 |
| LBA1341 branched-chain amino acid aminotransferase ILVE | **2.13** | 1.08 | 1.26 |
| LBA1515 peptidase T *pepT* | 2.26 | **2.05** | 1.32 |
| LBA1665 oligopeptide ABC transporter, substrate-binding protein | 0.38 | **0.15** | 0.58 |
| LBA1837 cytosol nonspecific dipeptidase *pepD/A* | 1.03 | 1.55 | **3.10** |
| LBA1961 oligopeptide ABC transporter, substrate-binding protein | **2.05** | **1.91** | 1.08 |
| Carbohydrate transport and metabolism [G] | | | |
| LBA0600 xylulose-5-phosphate/fructose phosphoketolase | 1.31 | **3.22** | 0.78 |
| LBA1467 beta-galactosidase large subunit (lactase) | **0.07** | **0.17** | **0.27** |
| LBA1468 beta-galactosidase small subunit | **0.17** | **0.43** | 1.05 |
| LBA1777 PTS system, fructose-specific enzyme II | 0.98 | 1.26 | **0.41** |
| LBA1778 fructose-1-phosphate kinase | 1.00 | 1.30 | **0.29** |
| LBA1779 transcriptional repressor (fructose operon) | 0.92 | 1.32 | **0.35** |
| LBA1870 maltose phosphorylase | **0.67** | 0.90 | **0.21** |
| LBA1872 oligo-1,6-glucosidase | 0.97 | 0.88 | **0.46** |
| Inorganic ion transport and metabolism [P] | | | |
| LBA0319 ABC transporter, ATP-binding protein | 1.19 | 1.06 | 1.88 |
| LBA0320 ABC transporter, ATP-binding protein | 1.25 | 0.87 | **1.96** |
| LBA0321 ABC transporter, permease protein | 1.34 | 1.33 | **2.21** |
| LBA0904 outer membrane lipoprotein precursor | 2.11 | **2.11** | 1.19 |
| LBA0905 ABC transporter, ATP-binding protein | 2.08 | **2.07** | 1.40 |
| LBA0906 ABC transporter, permease protein | 1.99 | **2.88** | 2.19 |
| LBA1683 cation-transporting ATPase | **7.95** | 1.92 | 1.83 |
| Signal transduction mechanisms [T] | | | |
| LBA0149 hypothetical protein | 1.28 | 1.01 | **0.56** |
| LBA0403 hypothetical protein | 1.01 | 1.30 | 1.21 |
| LBA1081 autoinducer-2 production protein *luxS* | 1.69 | **2.27** | 1.51 |
| LBA1524 two-component sensor histidine kinase | 1.17 | **2.82** | 0.97 |
| LBA1525 two-component system regulator | **2.09** | **1.54** | 1.03 |
| Defense mechanisms [V] | | | |
| LBA0074 ABC transporter, ATP-binding and permease protein | **2.27** | 0.96 | 1.07 |
| LBA0075 ABC transporter, ATP-binding and permease protein | **3.01** | **3.90** | **2.94** |
| LBA1838 ABC transporter, ATP-binding protein | 1.55 | **4.15** | **7.37** |
| LBA1839 putative permease | 1.48 | **5.16** | **8.72** |
| LBA1876 ABC transporter, ATP-binding/membrane-spanning protein | 1.79 | **1.98** | 1.59 |

*Continued on following page*

TABLE 2—*Continued*

| COG[b] functional classification/gene | Relative mRNA ratio (HPK/WT)[c] | | |
|---|---|---|---|
| | pH 6.8 | pH 5.5 | pH 4.5 |
| Posttranslational modification, protein turnover, chaperones [O] | | | |
| LBA0165 neutral endopeptidase *pepO* | **2.91** | **3.28** | **1.97** |
| LBA1512 proteinase P precursor *prtP* | **7.53** | **7.02** | 1.58 |
| LBA1564 putative membrane protein | 1.42 | **1.47** | **2.08** |
| Cell wall/membrane/envelope biogenesis [M] | | | |
| LBA0018 unknown | 0.90 | 1.00 | **0.55** |
| LBA1469 UDP-glucose 4-epimerase | **0.18** | 0.51 | 0.67 |
| Transcription [K] | | | |
| LBA1840 transcriptional regulator (TetR/AcrR family) | 1.33 | **3.52** | **12.60** |
| General function prediction only [R] | | | |
| LBA0367 putative receptor | 1.04 | **1.74** | 1.26 |
| Energy production and conversion [C] | | | |
| LBA0463 acetate kinase | **2.31** | 1.12 | 1.27 |
| Translation, ribosomal structure, and biogenesis [J] | | | |
| LBA0672 putative phosphate starvation induced protein *yvyD* | 1.12 | 0.93 | **0.43** |
| Intracellular trafficking, secretion, and vesicular transport [U] | | | |
| LBA1496 putative fibrinogen-binding protein | **3.56** | **2.35** | 1.22 |
| Replication, recombination, and repair [L] | | | |
| LBA1565 unknown | 2.02 | 1.43 | 1.37 |
| Function unknown/general function prediction only [S], [R] | | | |
| LBA0555 myosin-crossreactive antigen | 1.05 | 0.98 | **0.43** |
| LBA0872 putative membrane protein | 2.14 | **5.27** | **2.97** |
| LBA1119 putative inner membrane protein | **4.22** | **3.46** | **6.24** |
| LBA1869 beta-phosphoglucomutase | 0.69 | 0.70 | **0.24** |
| LBA1952 hypothetical protein | 1.07 | 0.89 | **2.26** |
| No COG found | | | |
| LBA0352 hypothetical protein | 0.94 | 0.87 | **0.47** |
| LBA0402 unknown | 1.01 | 0.81 | 1.38 |
| LBA0404 hypothetical protein | 0.88 | **0.60** | 0.95 |
| LBA1495 putative fibrinogen-binding protein | 1.62 | 0.97 | 1.15 |
| LBA1611 surface protein *fmtB* | **0.56** | 0.93 | 0.94 |
| LBA1690 putative membrane protein | 1.62 | 1.32 | **2.34** |

[a] Array ratios from two biological replicates and two technical replicates for each condition were averaged.
[b] COG, clusters of orthologous groups (37). Genes were classified according to the COG domain present in the potentially encoded protein sequence; letters in brackets are COG functional category codes.
[c] Values in boldface indicate ratios that meet the *P* criteria ($P < 0.05$).

and LBA1081 (*luxS*) was increased up to 6.9-fold under all conditions in the HPK mutant. At the amino acid level, the LuxS (LBA1081) homolog in the genome sequence of *L. acidophilus* was 77% identical and 84% similar to the *S*-ribosyl-homocysteinase (autoinducer-2 production protein LuxS) from *L. plantarum* (13) and 73% identical and 89% similar to LuxS from *Streptococcus pyogenes* (19). Examination of the surrounding chromosomal region suggested that *luxS* is the sec-

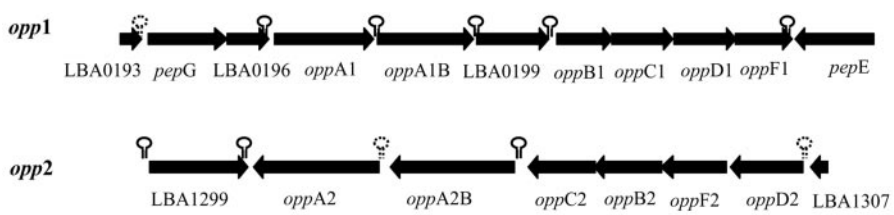


FIG. 4. Organization of the oligopeptide transport (*opp*) operons in *L. acidophilus* NCFM. Predicted rho-independent terminators with a free energy over −10 kcal/mol (continuous line) and under −10 kcal/mol (dotted line) are indicated.

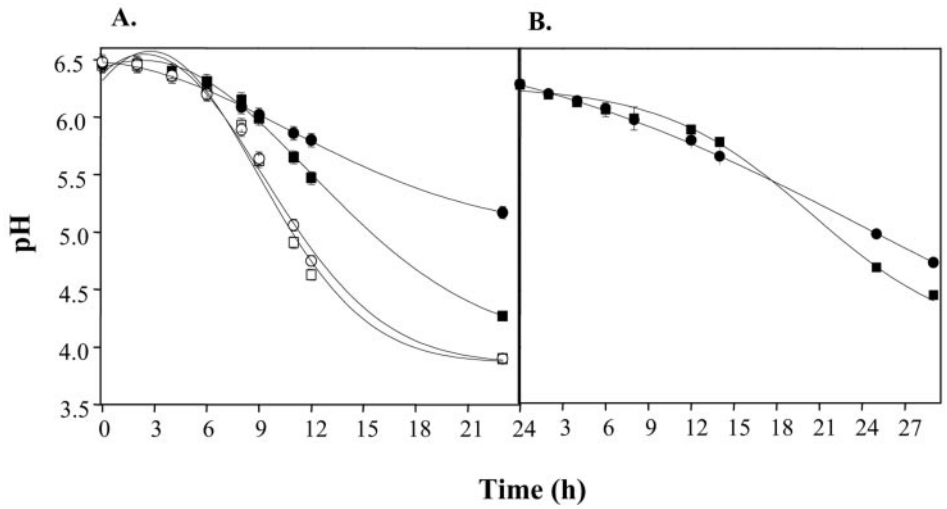


FIG. 5. Growth of *L. acidophilus* NCFM (squares) and NCK1686 (NCFM::LBA1524HPK [circles]) in milk (solid symbols) and milk supplemented with yeast extract (open symbols) (A) and in milk supplemented with 0.25% Casamino Acids (B). $r = 0.99$.

ond member of an operon consisting of five genes whose function is poorly characterized. A putative rho-independent terminator with a low free energy of −8.5 kcal/mol was present downstream of *luxS*.

Among the global transcriptional changes observed in the HPK mutant, two key enzymes involved in lysine biosynthesis—aspartate kinase (EC 2.7.2.4, LBA0850) and diaminopimelate epimerase (EC 5.1.1.7, LBA0849)—were upregulated at pH 5.5. In addition, a putative operon composed of a cytosol nonspecific dipeptidase, an ABC transporter, and a transcriptional regulator from theTetR/AcrR family (ORFs LBA1737 to LBA1840) was highly upregulated at pH 4.5.

Given the similarity of LBA1524HPK with *lisK*, the HPK from *L. monocytogenes* (6) and the fact that a *lisK*-deficient mutant was able to grow at a higher concentration of ethanol than its parent strain, survival of the *L. acidophilus* HPK mutant was investigated in the presence of ethanol. No differences were observed when log-phase cells were exposed to 15% (vol/vol) ethanol, indicating that *L. acidophilus* is naturally highly resistant. However, at 20% ethanol the HPK mutant showed a 4-log reduction in survival after 90 min compared to only a 1-log reduction in the control (data not shown).

**Confirmation of DNA microarray results by Northern blotting.** Cells of the control and the HPK mutant strains were harvested at an $A_{600}$ of 0.3 and exposed to pH 6.8, 5.5, and 4.5 in MRS broth for 30 min. Total RNA was prepared and hybridized with several labeled probes. For analysis of gene expression, DNAs of the ORFs indicated in Table 1 were amplified by PCR and labeled with $\alpha$-$^{32}$P. Selected for analysis by Northern blot were *opp*A1 (LBA0197, upregulated in the HPK mutant), *opp*A2 (LBA1300, downregulated in the HPK mutant cells), and LBA1524HPK and LBA1525RR (components of the inactivated 2CRS) genes. Genes encoding a GAPDH (glyceraldehyde-3-phosphate dehydrogenase; LBA0698), malolactic enzyme (LBA1075), and RNA polymerase sigma factor *rpo*D (LBA1196) were also evaluated as controls because these were not differentially expressed at the different pH conditions when evaluated in the microarrays (data not shown).

The hybridized membranes and comparison between relative expression ratios obtained by microarray and Northern analysis are shown in Fig. 6. The transcription levels of the selected genes, as measured by the DNA microarray method, were consistent with those measured by Northern hybridizations, with one exception. The amounts of RNA detected for the disrupted gene LBA1524HPK showed 10-fold more RNA when measured by Northern blot but only 2-fold more according to microarray. This suggests that Northern analysis was better able to quantitate gene expression at higher levels.

## DISCUSSION

Analysis of the genome sequence of *L. acidophilus* revealed the presence of nine 2CRS (2). All of the identified HPKs showed between two and six transmembrane domains, suggesting their location in the cell membrane. One of the identified 2CRS, LBA1524HPK-LBA1525RR, showed homology to *lis*RK, a signal transduction system previously shown to participate in stress response and virulence in *L. monocytogenes* (6). When we insertionally interrupted LBA1524HPK, log-phase cells became more sensitive to acid pH. We previously reported that *L. acidophilus* induces an adaptive response at pH 5.5 that provides elevated acid tolerance to the cells (4). Both the HPK mutant and the control NCFM::*lac*L exhibited an acid induced tolerance response, although this response was slightly impaired by the LBA1524HPK mutation. This indicates that although LBA1524HPK-LBA1525RR plays some role, additional mechanisms contribute to acid adaptation in *L. acidophilus* that are not regulated by this 2CRS.

A whole-genome array comparing the expression profile between the control and the HPK mutant revealed an altered expression pattern of numerous ORFs encoding genes for major components of the proteolytic enzyme system. Based on its genome sequence, *L. acidophilus* has a limited capacity to synthesize amino acids, with the potential to synthesize only three amino acids (cysteine, serine, and aspartate) de novo. In addition, cysteine and serine could be synthesized from pyru-

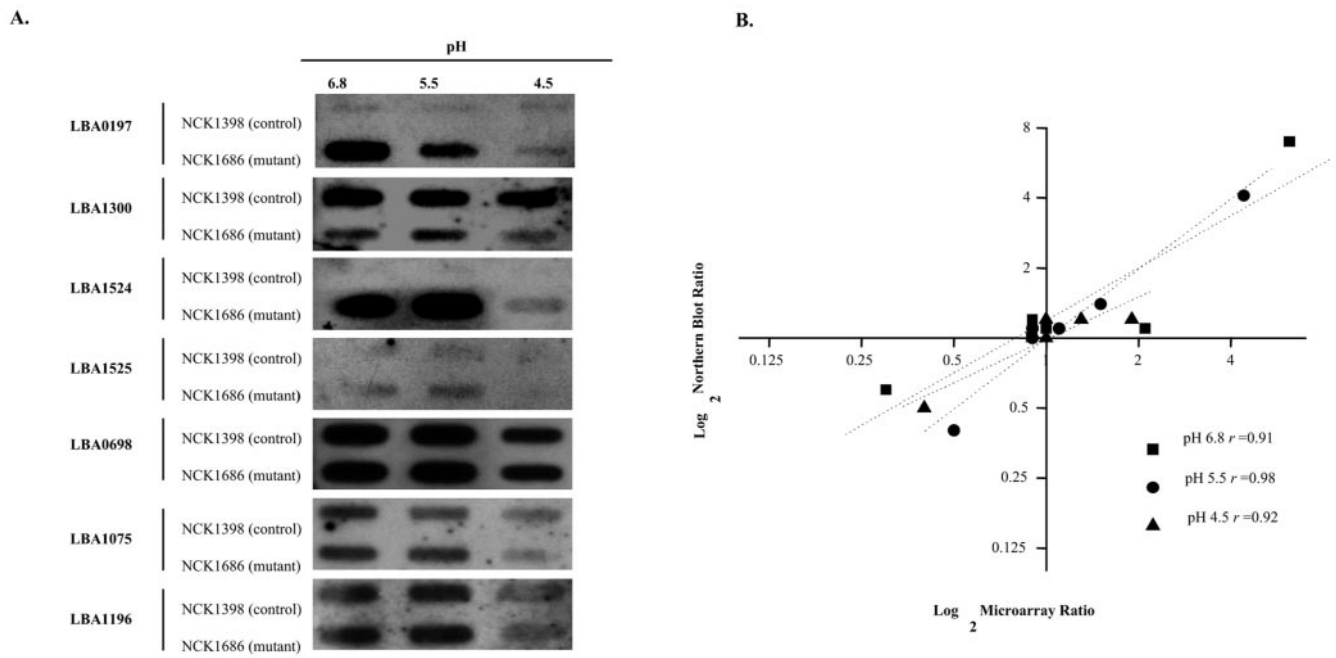


FIG. 6. (A) Northern blot analysis of seven genes was performed with RNA isolated in three independent experiments from *L. acidophilus* NCK1398 (NCFM::*lac*L) and NCK1686 (NCFM::LBA1524HPK) exposed to pH 6.8, 5.5, or 4.5 for 30 min. RNA ratios were calculated by densitometry analysis from data obtained from the Northern blot. (B) Comparison of expression measurements by microarray and Northern blot analysis. The correlation coefficient for each condition is given in the figure.

vate, and aspartate could be synthesized from fumarate. Based on these three amino acids, a series of other derivatives might be generated (asparagine, threonine, glycine, lysine, methionine, glutamine, and glutamate). However, neither de novo nor conversion pathways were predicted for the remaining 13 aa (2). Therefore, amino acid requirements must be satisfied by the uptake of amino acids and oligopeptides. *L. acidophilus* encodes two putative oligopeptide transporting systems (2), *opp*1 (ORFs LBA0197 to LBA0203) and *opp*2 (ORFs LBA1300 to LBA1306). Six additional genes coding for periplasmic substrate-binding proteins (OppA) were also identified (LBA1216, LBA1347, LBA1400, LBA1665, LBA1958, and LBA1961). One major function of oligopeptide transport (Opp) systems for bacterial cells is to internalize peptides to be used as carbon and nitrogen sources. They are also involved in the recycling of the cell wall peptides, which are likely one of the first targets of physiochemical stress. Opp systems are members of the ABC transporters family and usually consist of two ATP-binding proteins, two transmembrane proteins, and an extracellular ligand-specific binding protein. In gram-positive bacteria, the substrate-binding protein aligns with the external face of the cytoplasmic membrane (36) and biochemical evidence suggests that they might have a chaperone-like function in protein folding, protection against thermal denaturation, and interaction with unfolded proteins (30). Since several components of the proteolytic system were overexpressed, we expected that the HPK mutant would be able to grow better in milk than the control. On the contrary, the mutant was not able to acidify 10% SM under pH 5.0. However, when SM was supplemented with yeast extract, both the parent and the mutant were stimulated to the same degree. Yeast extract is the water-soluble portion of autolyzed yeast, containing vitamin B complex. It provides vitamins, nitrogen, amino acids, and carbon in growth media or supplemented milk. Furthermore, supplementation of SM with Casamino Acids essentially abolished differences in acidification rate between the wild type and the mutant strains. These observations provide evidence that the proteolytic system in the HPK mutant was debilitated. An alternative possibility is that inactivation of the 2CRS resulted in the reduced expression of a specific amino acid transporter. The decreased intracellular concentration of that amino acid might trigger the cell to overexpress other options to obtain that amino acid, i.e., through peptide transport and peptidases or through other pathways such as enzymes involved in the biosynthesis of lysine (LBA0849 and LBA0850). Two genes encoding putative Opp binding proteins (LBA1300 and LBA1665) were consistently under expressed in the mutant, suggesting that these transport systems are important for the organism's ability to grow in milk. It is not clear, however, why other Opp transporters present in the genome would not replace any loss of capacity from the limited expression of LBA1300 and LBA1665, especially when a number of these were overexpressed.

Opp systems are also related to mechanisms of signaling since they transport signal peptides that, once inside the cell, will interact with intracellular receptors to regulate cellular functions (18). In gram-positive bacteria, cell density response mechanisms are well studied. A peptide signal precursor locus is translated into a precursor protein that is cleaved to produce an autoinducer signal that is transported out of the cell. When the extracellular concentration of the peptide signal accumulates to the minimal stimulatory level, an HPK of a 2CRS

detects it, and the phosphorylated RR activates the transcription of target genes (22).

Interestingly, the autoinducer-2 production gene, *luxS*, was significantly overexpressed in the HPK mutant. The gene *luxS* is responsible for the production of the autoinducer molecule AI-2 in *Vibrio harveyi* and other gram-positive and gram-negative bacteria (34). LuxS is the autoinducer synthase, responsible for catalysis of the final step in AI-2 biosynthesis. The disruption of *luxS* in *S. pyogenes* had several effects, suggesting that it is an important component of the response machinery that allows this strain to adapt to changing conditions during an infection. These effects include regulation of the SpeB protease and stress response (19). The gene located upstream of *luxS* (LBA1080) was also upregulated in the mutant at both pH 5.5 and 4.5. Further studies are ongoing to elucidate the role of these genes in *L. acidophilus*.

Intriguingly, the expression of the aspartate kinase (EC 2.7.2.4, LBA0850) and diaminopimelate epimerase (EC 5.1.1.7, LBA0849) was increased at pH 5.5 in the HPK mutant. These are key enzymes in the biosynthesis of lysine and are organized in an operon in *L. acidophilus*. However, diaminopimelate decarboxylase (EC 4.1.1.20, LBA0851), the enzyme responsible for the last step in the synthesis of lysine, was not overexpressed in the HPK mutant. We hypothesize that under these conditions D,L-diaminopimelate, instead of being converted to L-lysine, may enter the peptidoglycan biosynthesis pathway. It is unclear whether the HPK mutant produces more peptidoglycan. If so, this may contribute to the changes observed in cell morphology and chain length.

In summary, environmental conditions that included changes in acid concentration and fluctuations of pH were sensed by the 2CRS, LBA1524HPK. It would be expected that this protein then initiates a phosphorylation cascade that regulates expression of a number of genes in the *L. acidophilus* genome. Most of the differentially expressed genes were upregulated in the HPK mutant, suggesting that LBA1525RR may act as a repressor. The inactivation of this 2CRS resulted in alterations in cell morphology, acid sensitivity, ethanol sensitivity, and poor acidification rates in skim milk, indicating a loss of proteolytic activity. Microarray data showed that more than 50% of the genes differentially expressed in the HPK mutant encode putative membrane proteins. In addition, expression of multiple components of the proteolytic enzyme system, i.e., opp transporters, permeases, and peptidases, were dramatically affected by the inactivation of the HPK, but no simple correlation of higher or lower gene expression to proteolytic activity, or the loss thereof, was apparent.

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