Temporal gene expression and probiotic attributes of *Lactobacillus acidophilus* during growth in milk

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ABSTRACT

Lactic acid bacteria have been used as starter strains in the production of fermented dairy products for centuries. *Lactobacillus acidophilus* is a widely recognized probiotic bacteria commonly added to yogurt and used in dietary supplements. In this study, a whole genome microarray was employed to monitor gene expression of *L. acidophilus* NCFM cells propagated in 11% skim milk during early, mid and late logarithmic phase, and stationary phase. Approximately 21% of 1,864 open reading frames were differentially expressed at least in one time point. Genes differentially expressed in skim milk included several members of the proteolytic enzyme system. Expression of *prtP* (proteinase precursor) and *prtM* (maturase) increased over time as well as several peptidases and transport systems. Expression of Opp1 (oligopeptide transport system 1) was highest at 4 h, whereas gene expression of Opp2 increased over time reaching its highest level at 12 h, suggesting that the 2 systems have different specificities. Expression of a 2-component regulatory system, previously shown to regulate acid tolerance and proteolytic activity, also increased during the early log and early stationary phases of growth. Expression of the genes involved in lactose utilization increased immediately (5 min) upon exposure to milk. The acidification activity, survival under storage conditions, and adhesion to mucin and Caco-2 tissue culture cells of selected mutants containing insertionally inactivated genes differentially expressed in the wild-type strain during growth in milk were examined for any potential links between probiotic properties and bacterial growth and survival in milk. Some of the most interesting genes found to be expressed in milk were correlated with signaling (autoinducer-2) and adherence to mucin and intestinal epithelial cells, in vitro.

Key words: *Lactobacillus acidophilus*, probiotic, microarray analysis, dairy

INTRODUCTION

Lactic acid bacteria (LAB) constitute a varied group of microorganisms that occur naturally in dairy, meat, and plants (cabbage, corn, barley, mash, kale, and silage; Carr et al., 2002). The most important application of LAB has been their use as starter cultures in the manufacture of fermented foods, and notably dairy products such as cheese, yogurt, and fermented milk. Today, the dominant vehicles for delivery of probiotic strains in foods are yogurt and fermented milk. *Lactobacillus acidophilus* is a widely recognized probiotic bacteria commonly added to yogurt (Sanders and Klaenhammer, 2001), with approximately 80% of the yogurt in the United States now containing *L. acidophilus* (Hutkins, 2006). Although this microorganism does not participate in the actual yogurt fermentation, it grows slowly in milk during product manufacture (Shah, 2000). Consequently, it is a common practice to incorporate yogurt cultures (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) along with probiotic cultures (*L. acidophilus*, *Lactobacillus casei*, *Lactobacillus reuteri*, and *Bifidobacterium* spp.) in many yogurts and fermented milks (Shah, 2000).

Probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Reid et al., 2003). The International Dairy Federation recommends that dairy products contain at least 10^7 cfu/mL (Champagne et al., 2005). The actual number of colony-forming units that reach the distal portion of the gastrointestinal tract remains debatable, but clearly depends on several aspects that include acid and bile tolerance of the probiotic strain, manufacturing conditions, and survival of the microorganism during the fermentation and postfermentation (cooling and storage) stages. The major factor affecting viability and performance of probiotic strains during processing is the food matrix formulation (Mattila-Sandholm et al., 2002). Additionally, survival of probiotic strains during storage is a key issue in the food industry. Several stud-

Received June 15, 2008.

Accepted November 5, 2008.

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Table 1. Strains and plasmids used in this study

Strain	Characteristics	Inactivated gene	Reference or source
<i>Lactobacillus acidophilus</i>			
NCFM ¹	Human intestinal isolate		Barefoot and Klaenhammer (1983)
NCK1377	NCFM integrant <i>slpA</i> ::pTRK826	LBA0169	Buck et al. (2005)
NCK1375	NCFM integrant <i>cdpA</i> ::pTRK828	LBA0175	Altermann et al. (2004)
NCK1624 ¹	NCFM integrant <i>treE</i> ::pTRK801	LBA1012	Duong et al. (2005)
NCK1746	NCFM integrant <i>mdoB</i> ::pTRK842	LBA0447	Pfeiler (North Carolina State University); unpublished results
NCK1730 ¹	NCFM integrant myosin cross-reactive antigen::pTRK838	LBA0555	Dobson (North Carolina State University); unpublished results
NCK1618	NCFM integrant <i>bshA</i> ::pTRK734	LBA0892	McAuliffe et al. (2005)
NCK1765	NCFM integrant <i>luzS</i> ::pTRK854	LBA1081	Buck et al. (2008); in press
NCK1661	NCFM integrant <i>fbpA</i> ::pTRK833	LBA1148	Buck et al. (2005)
NKC1376	NCFM integrant <i>cfa</i> ::pTRK697	LBA1274	Courtney et al. (North Carolina State University); unpublished results
NCK1660 ¹	NCFM integrant <i>mub</i> ::pTRK834	LBA1392	Buck et al. (2005)
NCK1824	NCFM integrant <i>mub</i> ::pTRK887	LBA1460	Ruse (North Carolina State University); unpublished results
NCK1398 ¹	NCFM integrant <i>lacL</i> ::pTRK685	LBA1467	Russell and Klaenhammer (2001)
NCK1686 ¹	NCFM integrant HPK::pTRK807	LBA1524	Azcarate-Peril et al. (2005)
NCK1662	NCFM integrant R28::pTRK835	LBA1633	Buck et al. (2005)
NCK1822 ¹	NCFM integrant surface exclusion protein::pTRK886	LBA1690	This study
NCK1816	NCFM integrant noncoding region::pTRK883	none	Buck (North Carolina State University); unpublished results
NCK1728	NCFMΔ <i>frc</i>	LBA0395	Azcarate-Peril et al. (2006)
NCK1392	NCFM containing pTRK669	none	Russell and Klaenhammer (2001)
<i>Escherichia coli</i>			
EC1000	RepA ⁺ MC1000, Km ^r ; host for pORI28-based plasmids		Law et al. (1995)

¹Strains selected for adhesion experiments to mucin and Caco-2 tissue culture cells.

ies (reviewed by Champagne et al., 2005) have reported that pH, oxygen, and starter production conditions have significant effects on survival and stability of probiotic cultures during storage in yogurt.

The selection criteria for probiotics include general safety and stability aspects and functional properties (Tuomola et al., 2001). To survive passage through the stomach and small intestine, probiotic strains must tolerate the acidic and digestive protease conditions of the stomach, and must survive and grow in the presence of bile acids. The ability to adhere to the mucosa and intestinal tissues has been related to retention in the gastrointestinal tract and immunomodulation (Grangette et al., 2005; Hisbergues et al., 2007). In this regard, *L. acidophilus* NCFM, delivered widely in yogurt and fermented milks, has been shown to adhere to enterocyte-like Caco-2 cells via cell surface proteins such as a fibronectin-binding protein, a mucin-binding protein, and a surface layer protein (Buck et al., 2005).

Milk is the dominant food vehicle and substrate for delivery of probiotic strains to humans. Our objective was to investigate how growth in milk affected gene expression in *L. acidophilus* NCFM and determine if any potential probiotic traits were affected during exposure to, and fermentation of, milk.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

The bacterial strains used in this study are listed in Table 1. *Escherichia coli* were propagated at 37°C in Luria-Bertani broth (Difco Laboratories Inc., Detroit, MI) with shaking. *Escherichia coli* cultures were propagated on brain heart infusion agar (Difco), supplemented with 150 µg of erythromycin/mL, when appropriate. Lactobacilli were propagated statically at 37°C in de Man, Rogosa, and Sharpe broth (MRS; Difco), or MRS agar supplemented with 1.5% agar. When appropriate, erythromycin (5.0 µg/mL) or chloramphenicol (4.0 µg/mL) was added to MRS broth or agar plates. For growth and microarray experiments, *L. acidophilus* was propagated at 37°C in 11% reconstituted skim milk (SM; MP Biomedicals Inc., Solon, OH). After reconstitution, the milk was steamed at 100°C for 40 min and immediately placed in an ice bath.

Genetic Constructions

Construction of the *L. acidophilus* NCFM::LBA1690 mutant was carried out according to the method described by Russell and Klaenhammer (2001). A 688-

bp internal fragment of open reading frame (**ORF**) LBA1690 was amplified by PCR using *L. acidophilus* NCFM chromosomal DNA as a template and the primers I1690F (5'-GATCTCTAGATCAAGCTGCATCAATTAC-3') and I1690R (5'-GATCAGATCTTGTGAACACTAATCTTAC-3'). The amplified fragment was cloned into the integrative vector pORI28 (Law et al., 1995) generating pTRK886, which was then introduced by electroporation in *L. acidophilus* NCFM (pTRK669). Subsequent steps to facilitate the integration event were carried out as described previously (Russell and Klaenhammer, 2001). Integrants were confirmed by PCR and Southern hybridization analysis using standard procedures (data not shown).

Microarray Experimental Design and RNA Isolation

A loop experimental design (Vinciotti et al., 2005) was employed in a time course experiment to monitor the transcriptional response of *L. acidophilus* NCFM during growth in 11% reconstituted skim milk. The culture was initiated in MRS broth and transferred 3 to 5 times in SM before a 10% inoculation in prewarmed SM. Cells and RNA samples were obtained from 2 biological replicates at times of 5 min (pH = 6.4; $\approx 2.1 \times 10^7$ cfu/mL), 4 h (pH = 5.8; $\approx 3.8 \times 10^7$ cfu/mL), 8 h (pH = 5.4; $\approx 7.1 \times 10^7$ cfu/mL), and 12 h (pH = 4.9; $\approx 1.5 \times 10^8$ cfu/mL; see Supplementary Figure 1 available online: <http://jds.fass.org/content/vol92/issue3/>) and compared in an unbalanced loop-design. Samples were compared as follows (by dye used, Cy3 versus Cy5; Amersham Biosciences, Piscataway, NJ): T1 (5 min) versus T2 (4 h), T2 versus T3 (8 h), T3 versus T4 (12 h), T4 versus T2, and T3 versus T1. One-hundred-milliliter aliquots of *L. acidophilus* cells growing in SM were harvested by centrifugation for 10 min at $3,220 \times g$, and frozen immediately in a dry ice/ethanol bath (the total time of each sampling process was 5 min). Isolation of RNA was performed as described previously (Azcarate-Peril et al., 2005) with the following modifications: 5 mL of Trizol reagent (Invitrogen, Carlsbad, CA) was added to the cell pellets and they were homogenized in a Mini-Beadbeater-8 cell disruptor (Biospec Products, Bartlesville, OK) for seven 40-s cycles (and chilled on ice for 40 s between cycles); the phases were then separated by centrifugation ($3,220 \times g$ for 20 min at 4°C). The aqueous phase was removed to a fresh tube and 2.1 mL of Trizol and 1 mL of chloroform were added. The mixture was vortexed for 15 s and centrifuged ($3,220 \times g$ for 15 min at 4°C) 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 ($11,600 \times g$ for 10 min

at 4°C). Purity and quality of RNA samples were determined by electrophoresis on agarose gels. NanoDrop (ND-3300, NanoDrop Technologies, Wilmington, DE) was used as an ultraviolet/visual spectrophotometer for quantification of the RNA samples.

Microarray Hybridization

A previously developed and validated microarray platform based on the PCR products of predicted ORF from *L. acidophilus* NCFM (Azcarate-Peril et al., 2005) was used to monitor changes in gene expression. Reverse transcription and labeling of identical amounts (20 µg) of DNase-treated (Invitrogen) RNA was performed with the SuperScript Indirect cDNA Labeling System for DNA Microarrays (Invitrogen) according to the manufacturer's directions. Coupling of the Cy3 and Cy5 dyes to the aa-dUTP (2'-deoxyuridine 5'-triphosphate) labeled cDNA and hybridization of samples to microarrays were performed according to the protocols outlined by Hegde et al. (2000) and described previously (Azcarate-Peril et al., 2005).

Data Preprocessing and Analysis

After acquisition of fluorescence intensities at 10-µm resolution using a ScanArray 4000 Microarray Scanner (Packard Biochip BioScience; Biochip Technologies LLC, Billerica, MA), signal intensities (stored as TIFF files) were quantified, and the local background was subtracted using the QuantArray 3.0 software package (Packard BioScience). Afterwards, data were log₂ transformed and imported into JMP Genomics (SAS, Cary, NC). Loess normalization was applied to preprocessed data. Following normalization, gene-specific effects were modeled in terms of the residuals. A mixed model analysis was applied to the prenormalized data (Cui and Churchill, 2003). False discovery rate was selected to adjust for the large number of multiple hypothesis tests across all genes. The default value of α (0.05) was used. The model-based approach, step-down quadratic regression method was applied to 791 significant genes for pattern recognition (Liu et al., 2005).

Microarray Data Submission

Microarray platform and data are available at the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GPL1401 (platform) and GSE12924 (series).

Real-Time Quantitative PCR

Real-time quantitative (qRT) PCR was used to confirm microarray results. The protocols described

previously (Azcarate-Peril et al., 2006) were used for amplification, quantification, and normalization of PCR products. The same RNA samples employed for microarray hybridizations were used for qRT-PCR. The RNA was DNase treated, quantified, and diluted to a concentration of 50 ng/ μ L. The absence of contaminating genomic DNA was confirmed by PCR of the DNase-treated samples. Reverse transcription and PCR were carried out with an iCycler iQ (BioRad Laboratories, Hercules, CA) using the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA). Primers meeting the standard criteria for qRT-PCR for the following genes were designed using CloneManager (Scientific & Educational Software, Cary, NC) software (Supplementary Table 1 available online: <http://jds.fass.org/content/vol92/issue3/>): LBA0367 (encoding a putative receptor), LBA0395 (formyl-CoA transferase), LBA0396 (oxalyl-CoA decarboxylase), LBA0555 (myosin-crossreactive antigen), LBA1234 (cadmium/manganese transport ATPase), LBA1249 (heat inducible transcription repressor HrcA), LBA1512 (Prp precursor), and LBA1588 (Prp precursor).

The induction ratios measured by microarrays were plotted against induction ratios measured by qRT-PCR, for the 8 selected genes and 4 time points, to validate microarray data (Supplementary Table 2 and Supplementary Figure 2 available online: <http://jds.fass.org/content/vol92/issue3/>). Individual *r* values ranged between 0.44 and 0.99 for each of the tested genes with an overall *r* value of 0.86. As reported previously, (Azcarate-Peril et al., 2006), qRT-PCR measurements revealed larger induction levels.

Acidification Activity of *L. acidophilus* Cultures in Milk

The acidification capability of *L. acidophilus* cultures was measured using the Orion EA940 Multi-Channel Benchtop Meter and Multi-Channel Electrode Switchbox (Thermo Fisher Scientific Inc., Waltham, MA). *Lactobacillus acidophilus* cultures grown overnight in MRS broth were centrifuged ($3,220 \times g$ for 10 min at room temperature). The pellet was washed twice with phosphate buffer and resuspended in the same buffer. Forty milliliters of prewarmed SM was inoculated with 0.5 to 2.2×10^7 cells. Acidification curves were performed at 37°C in 11% SM. Four independent experiments were performed for each strain and the acidification rate was calculated as the first time derivative. The time in minutes necessary to reach the maximum acidification rate in milk (*tm*) was calculated as described by Fonseca et al. (2000). The *tm* values were correlated to the natural logarithm of the initial bacterial population (in cfu/mL) as described by Fonseca et al. (2000).

Survival of *L. acidophilus* Cultures in Acidified Milk

Survival of *L. acidophilus* cultures in SM acidified at pH 4.5 (with lactic acid) at 4°C was examined as follows. *Lactobacillus acidophilus* cultures grown overnight in MRS broth at 37°C were centrifuged ($3,220 \times g$ for 10 min at room temperature) and the pellet washed twice with phosphate buffer and resuspended in the same buffer. Fifty milliliters of SM adjusted to pH 4.5 with lactic acid were inoculated with 1.8 to 4.1×10^8 cells. Survival curves were performed at 4°C . Viable-cell counts were determined at 24- to 48-h intervals by plating serial dilutions in a 10% MRS broth diluent onto MRS agar using a Whitley Automatic Spiral Plater (Don Whitley Scientific Limited, West Yorkshire, England).

Adhesion of *L. acidophilus* to Mucin

Mucin and Plate Preparation. The adhesion ability of *L. acidophilus* derivatives was examined by the method of Vishwanath and Ramphal (1984) with minor modifications. One hundred microliters of a 10 mg/mL solution of partially purified type III porcine gastric mucin (Sigma Chemical Co., St. Louis, MO) were immobilized in polystyrene 96-well microtiter plates (Corning Inc., Acton, MA) by overnight incubation at 4°C . Any surplus of mucin was removed by pipetting and wells were washed twice with 200 μ L of PBS. Adhesion experiments were carried out 3 times, each time in triplicate.

Preparation of Microorganisms for Adhesion Assay. *Lactobacillus acidophilus* cultures were grown in SM at 37°C until pH 6.0 was reached. One hundred microliters of the bacterial suspension was added to each well and the plates were incubated 1 h at 37°C . Each well was washed 7 times with 200 μ L of sterile PBS (to remove unbound bacteria), then treated with 200 μ L of a 0.05% (vol/vol) Triton X-100 solution (to desorb bound bacteria). One hundred microliters of the contents of each well were removed, diluted in PBS, and plated on MRS agar plates. The viability of *L. acidophilus* strains was not affected by Triton X-100 under the conditions used (data not shown). Three wells per sample were used and each adherence assay was conducted in triplicate.

Adhesion to Caco-2 Cells

The adherence of *Lactobacillus* strains to epithelial cells was carried out as described by Buck et al. (2005) with the following modifications: Caco-2 cells from passage 24 to 29 were routinely grown in Dulbecco modified Eagle's minimal essential medium (DMEM)

supplemented with 20% inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 mg/mL penicillin G, 100 mg/mL streptomycin sulfate and 0.25 mg/mL amphotericin (Gibco, Invitrogen, Carlsbad, CA). Monolayers of Caco-2 cells were seeded at a concentration of 6.5×10^4 cells per well in 12-well tissue plates, and incubated at 37°C in a 95% air-5% CO₂ atmosphere. Cells were used at late postconfluence culture after 21 d, with a change of medium every 2 d. The monolayer was washed twice with 1 mL of PBS to remove traces of antibiotics, and 1 mL of Dulbecco's modified Eagle's medium without antibiotics was added to each well. The plates were incubated for 1 h at 37°C before the adherence assay.

Lactobacillus acidophilus cultures were grown at 37°C until pH 6.0 in 15 mL of either MRS or SM, inoculated with 750 µL of an overnight culture. To study the influence of the growth medium on adhesion of *L. acidophilus* NCFM, cultures in MRS or SM were centrifuged at $3,220 \times g$ for 10 min, and the pellets were washed once with an equal volume of phosphate buffer (pH 6.0) and resuspended either in phosphate buffer (pH 6.0) or SM acidified to pH 6.0 with lactic acid. Initial cell counts per milliliter were determined in both samples to ensure comparable counts between samples to be used in adherence assays. To compare the adhesion ability of selected *L. acidophilus* mutant strains (listed in Table 1), cultures were grown in SM until reaching pH 6.0, and then the cells were removed, resuspended, counted, and used directly in adherence assays.

One milliliter of each sample was added to each well of the tissue culture plate and incubated at 37°C for 1 h. Monolayers were washed 5 times with 1 mL of PBS and cells were desorbed by addition of 1 mL of 0.05% Triton-X100. The number of viable adhering bacteria was determined by plating serial dilutions on MRS agar plates. Three wells per sample were used and each adherence assay was conducted in triplicate. Bacterial adhesion was expressed as a percentage of the control strain.

RESULTS AND DISCUSSION

Growth and Acidification Activity

To determine the optimal culture conditions for preparation of cells for the microarray studies, the growth and acidification of *L. acidophilus* NCFM 37°C in MRS and 11% skim milk (SM) was examined, in triplicate (Supplementary Figure 1 available online: <http://jds.fass.org/content/vol92/issue3/>). The maximum specific growth rate ($\mu_{\max}$) of *L. acidophilus* in SM was less than half of the $\mu_{\max}$ of the strain growing in MRS (0.17 and 0.47 per h, respectively). The acidification

rate of *L. acidophilus* in MRS and SM was evaluated by continuous measurement of pH. No differences in acidification rates were observed between MRS and SM during the first 3 h of incubation, each decreasing the pH 0.05 units per h). At 9 h, *L. acidophilus* NCFM decreased the culture pH by 0.14 and 0.055 units per h for MRS and SM, respectively.

Gene Expression Profile of *L. acidophilus* During Growth in Milk

A loop experimental design was employed in a time course experiment to monitor the transcriptional response of *L. acidophilus* NCFM during growth in SM. We obtained RNA at times 5 min (pH = 6.4; $\approx 2.1 \times 10^7$ cfu/mL), 4 h (pH = 5.8; $\approx 3.8 \times 10^7$ cfu/mL), 8 h (pH = 5.4; $\approx 7.1 \times 10^7$ cfu/mL), and 12 h (pH = 4.9; $\approx 1.5 \times 10^8$ cfu/mL; see Supplementary Figure 1 available online: <http://jds.fass.org/content/vol92/issue3/>) and the RNA was compared in an unbalanced loop-design. The statistical filter [$-\log_{10}(P\text{-values}) < -1.98$] allowed 791 genes to pass, with 398 that were differentially expressed between paired time points in at least one time point, representing 21% of 1,864 ORF in the complete genome of *L. acidophilus*.

A clusters of orthologous groups (COG) classification of differentially expressed genes during growth in SM (Figure 1) indicated that the majority of genes induced at 4 h belonged to the translation, ribosomal structure and biogenesis [J] category, whereas most of the under-expressed genes belonged to carbohydrate transport and metabolism [G]. At 8 h, amino acid transport and metabolism [E] and [J] were the most up-regulated categories, and the majority of down-regulated genes were either unknown or could not be included in any COG category. Interestingly, at 12 h most of up-regulated genes were in an unknown/no GOG category, followed by genes included in the categories [E], [J], and cell wall, membrane, envelope biogenesis [M]. Again, the majority of genes down-regulated was in the unknown/no GOG category or included in [G]. However, the third most represented category was replication, recombination and repair [L].

Expression of Genes of the Proteolytic Enzyme System

The proteolytic systems of LAB are divided in 3 functional categories: (i) proteinases, which degrade caseins into small peptides; (ii) transport systems, which import peptides and amino acids; and (iii) cytoplasmic peptidases (Savijoki et al., 2006; Table 2). A step-down quadratic regression analysis (Liu et al., 2005) was applied to statistically significant genes to identify bio-

Figure 1. Temporal clusters of orthologous groups (COG; Tatusov et al., 1997) classification of differentially expressed genes at 4, 8, and 12 h. Genes were classified according to the main COG domain present in the potentially encoded protein sequence. The COG functional category codes are indicated on the right.

logically meaningful gene expression patterns. In this analysis, time is considered as a continuous variable. Figure 2 shows the temporal gene expression patterns obtained by this analysis for members of the proteolytic enzyme system. Patterns obtained included: Flat (not shown; no significant differential expression over time), linear up-regulated regression pattern (LU), quadratic concave regulated regression pattern (QC), quadratic convex regulated regression pattern (QV), quadratic linear concave up-regulated regression pattern (QLCU), and quadratic linear convex up-regulated regression pattern (QLVU). Interestingly, no genes fit into the linear down, quadratic linear concave down, or quadratic linear convex down patterns suggesting the absence of down-regulation over time for the studied subset of genes.

Gene expression of both LBA1512 (*prtP*), encoding a putative cell envelope protease, and LBA1588 (*prtM*), encoding a protease maturase protein, increased over

time during growth of *L. acidophilus* in skim milk. Expression of *prtM* increased linearly over time, whereas expression of *prtP* started to decrease at early stationary growth phase, probably coinciding with reduced levels of casein and low pH. The gene LBA1512 (*prtP*) encodes a putative precursor protein of 1,627 amino acids with a deduced molecular mass of 177.5 kDa that shows significant amino acid sequence similarity from residues 189 to 961 (44% identity and 58% similarity) to the PR (catalytic) and A (function unknown) domains of PrtR, the cell wall-bound extracellular proteinase encoded by *prtR* in *Lactobacillus rhamnosus* (Pastar et al., 2003). Additional aa sequence similarity is found from residues 1217 to 1442 (32% identity and 47% similarity), the region of the protein that partially contains the B (cell envelope protease activity/specificity) and W (cell wall spacer) domains.

Previously conducted computer simulation analysis of predicted transporters in the *L. acidophilus* genome

Table 2. Functional components of the proteolytic system of *Lactobacillus acidophilus* NCFM¹

Gene	Open reading frame number	Description/Function	Temporal expression pattern ²
Proteinases			
<i>prtP</i>	LBA1512	Cell envelope located protease	QLCU
<i>prtM</i>	LBA1588	Membrane-bound lipoprotein/Maturation	LU
Transport Systems			
<i>gadC</i>	LBA0057	Glutamate:gamma-aminobutyrate antiporter	
	LBA0111	Putative ABC transporter (glutamine), ATP binding protein	
	LBA0112	Putative ABC transporter (glutamine), substrate binding protein	
<i>glnP</i>	LBA0134	ABC transporter (glutamine), permease protein	
<i>glnQ</i>	LBA0135	ABC transporter (glutamine), ATP-binding protein	
<i>oppA1</i>	LBA0197	Oligopeptide ABC transporter, binding protein	QC
<i>oppA1B</i>	LBA0198	Oligopeptide ABC transporter, binding protein	QC
<i>oppB1</i>	LBA0200	Oligopeptide ABC transporter, permease protein	QLCU
<i>oppC1</i>	LBA0201	Oligopeptide ABC transporter, permease protein	QLCU
<i>oppD1</i>	LBA0202	Oligopeptide ABC transporter, ATP binding protein	QLCU
<i>oppF1</i>	LBA0203	Oligopeptide ABC transporter, ATP binding protein	
	LBA0262	Amino acid transporter	
	LBA0460	Amino acid transporter	
<i>glnH</i>	LBA0572	ABC transporter (glutamine), glutamine-binding protein	
<i>glnQ</i>	LBA0578	ABC transporter (glutamine), ATP-binding protein	
<i>glnH</i>	LBA0579	ABC transporter (glutamine), glutamine-binding protein	
<i>glnM</i>	LBA0581	ABC transporter (glutamine), membrane protein	
<i>glnP</i>	LBA0582	ABC transporter (glutamine), permease protein	
	LBA0615	Amino acid transporter	
	LBA0696	Amino acid transporter	
<i>potA</i>	LBA0709	Spermidine/putrescine ABC transporter	
<i>potB</i>	LBA0711	Spermidine/putrescine ABC transporter	
<i>potC</i>	LBA0712	Spermidine/putrescine ABC transporter	
<i>potD</i>	LBA0713	Spermidine/putrescine ABC transporter	
	LBA0729	Amino acid transporter	
	LBA0899	Amino acid transporter	
	LBA0943	Cationic amino acid transporter	QC
	LBA0995	Amino acid transporter	
	LBA1036	Branched-chain amino acid permease	
	LBA1042	ABC transporter (glutamine) membrane-spanning permease	QLVU
	LBA1044	ABC transporter (glutamine) membrane-spanning permease	
	LBA1045	ABC transporter (glutamine) ATP-binding protein	
	LBA1046	ABC transporter (glutamine) substrate-binding protein	QLVU
	LBA1086	Amino acid transporter	LU
	LBA1115	Amino acid transporter	QV
<i>oppA3</i>	LBA1216	Oligopeptide ABC transporter, substrate binding protein	
	LBA1222	Branched-chain amino acid permeases	
	LBA1224	Branched-chain amino acid permease	
	LBA1292	Amino acid transporter	LU
<i>oppA2</i>	LBA1300	Oligopeptide ABC transporter, substrate binding protein	
<i>oppA2B</i>	LBA1301	Oligopeptide ABC transporter, substrate binding protein	QC
<i>oppC2</i>	LBA1302	Oligopeptide ABC transporter, permease protein	
<i>oppB2</i>	LBA1303	Oligopeptide ABC transporter, permease protein	LU
<i>oppF2</i>	LBA1305	Oligopeptide ABC transporter, ATP binding protein	LU
<i>oppD2</i>	LBA1306	Oligopeptide ABC transporter, ATP binding protein	LU
<i>oppA4</i>	LBA1345	Oligopeptide ABC transporter, substrate binding protein	
<i>oppA5</i>	LBA1347	Oligopeptide ABC transporter, substrate binding protein	
<i>oppA6</i>	LBA1400	Oligopeptide ABC transporter, substrate binding protein	
	LBA1423	Putative amino acid transporter	
	LBA1424	Amino acid transporter	
<i>oppA7</i>	LBA1665	Oligopeptide ABC transporter, substrate binding protein	LU
	LBA1672	Amino acid ABC transporter	
<i>glnQ</i>	LBA1673	ABC transporter (glutamine), ATP-binding protein	
<i>glnP</i>	LBA1674	ABC transporter (glutamine), permease protein	
<i>dtpT</i>	LBA1848	Di- and tri-peptides permease protein	
	LBA1902	Amino acid transporter	QLVU
	LBA1904	Amino acid transporter	
<i>aapA</i>	LBA1905	Amino acid permease	LU
<i>oppA8</i>	LBA1958	Oligopeptide ABC transporter, substrate binding protein	
<i>oppA9</i>	LBA1961	Oligopeptide ABC transporter, substrate binding protein	

Continued

Table 2 (Continued). Functional components of the proteolytic system of *Lactobacillus acidophilus* NCFM¹

Gene	Open reading frame number	Description/Function	Temporal expression pattern ²
Peptidases			
<i>pepO</i>	LBA0035	Dipeptidase	LU QLVU
	LBA0092	Prolyl aminopeptidase	
	LBA0165	Metallopeptidase	
	LBA0186	Pyrrolidone carboxyl peptidase	
<i>pepG</i>	LBA0195	Dipeptidase	QLVU
<i>pepE</i>	LBA0204	Dipeptidase	
<i>pepC</i>	LBA0236	Dipeptidase	LU
	LBA0286	Prepilin peptidase	
	LBA0343	Aminopeptidase C	
	LBA0388	Glycoprotein endopeptidase	
	LBA0390	Endopeptidase	
	LBA0430	Xaa-pro dipeptidase	LU
<i>ampM</i>	LBA0623	Methionine aminopeptidase	QLVU
<i>pepC</i>	LBA0911	Aminopeptidase C	LU
<i>pepD</i>	LBA0994	Metallopeptidase	LU
<i>lspA</i>	LBA1152	Lipoprotein signal peptidase A	QV
	LBA1182	Signal peptidase	
<i>pepT</i>	LBA1190	Tripeptide aminopeptidase	
<i>pepO</i>	LBA1275	Metallopeptidase	QLVU
	LBA1294	Dipeptidase	
<i>pepP</i>	LBA1336	Xaa-Pro aminopeptidase	QLVU QLVU
<i>pepQ</i>	LBA1343	Metallopeptidase	
<i>pepQ</i>	LBA1344	Metallopeptidase	
<i>pepX</i>	LBA1373	X-prolyl dipeptidyl aminopeptidase	
<i>pepT</i>	LBA1515	Tripeptide aminopeptidase	
	LBA1567	Aminopeptidase	QLVU
<i>vanY</i>	LBA1603	d-alanyl-d-alanine carboxypeptidase	QV QLVU QC
	LBA1646	Dipeptidase	
	LBA1658	Prolyl aminopeptidase	
<i>pepF</i>	LBA1763	Oligoendopeptidase F	
	LBA1837	Dipeptidase	QLVU
<i>pepN</i>	LBA1849	Membrane alanyl aminopeptidase	
	LBA1909	Signal peptidase I	
<i>pepL</i>	LBA1957	Leucyl aminopeptidase	

¹Genes differentially expressed during growth in 11% skim milk are in boldface.

²LU = linear upregulated regression pattern; QC = quadratic concave regulated regression pattern; QLCU = quadratic linear concave upregulated regression pattern; QLVU = quadratic linear convex upregulated regression pattern; QV = quadratic convex regulated regression pattern.

revealed the presence of 2 complete oligopeptide ABC transporters (Altermann et al., 2005; Azcarate-Peril et al., 2005): Opp1 (ORF LBA0197 to LBA0203) and Opp2 (ORF LBA1300 to LBA1306). Each consists of 6 genes coding for 2 ATP-binding proteins (OppD and OppF), 2 membrane proteins (OppB and OppC), and 2 substrate-binding proteins (OppA and OppA-B). Expression of Opp1 fell into the QLCU temporal expression pattern. In fact, Opp1 reached its maximum expression level at 4 h. Expression of Opp2 increased over time falling into the LU pattern (Figure 3). These results suggest that Opp1 and Opp2 might have different specificities. In addition to the 4 periplasmic substrate-binding proteins (OppA) encoded within Opp operons, 6 additional putative *oppA* genes were identified in *L. acidophilus* (LBA1216, LBA1347, LBA1400, LBA1665, LBA1958, and LBA1961). Only 4 *oppA* genes were differentially expressed over time: LBA0197, LBA0198

(adjacent to Opp1), and LBA1301 (adjacent to Opp2) fell in the QC regression pattern, and LBA1665 was included in the LU pattern.

According to its genome sequence, *L. acidophilus* has a restricted capacity to synthesize amino acids (Altermann et al., 2005). *Lactobacillus acidophilus* NCFM has the potential to synthesize cysteine, serine, and aspartate de novo. Additionally, cysteine and serine could be synthesized from pyruvate, and aspartate from fumarate. Based on these 3 amino acids, a series of other derivatives might be generated (asparagine, threonine, glycine, lysine, methionine, glutamine, and glutamate). Because neither de novo nor conversion pathways could be predicted for the remaining 13 amino acids, amino acid requirements must be satisfied by the uptake of amino acids and oligopeptides. Expression of several predicted amino acid transporters was also increased during growth in SM (Table 2), including a cationic

Figure 2. Temporal expression patterns of genes of the proteolytic system identified by the quadratic regression method (Liu et al., 2005). Patterns obtained included: Flat (not shown; no significant differential expression over time), linear up-regulated regression pattern (LU), quadratic concave regulated regression pattern (QC), quadratic convex regulated regression pattern (QV), quadratic linear concave up-regulated regression pattern (QLCU), and quadratic linear convex up-regulated regression pattern (QLVU).

amino acid transporter and members of the glutamine ABC transporter operon. Additionally, 16 peptidases were differentially expressed over time. Expression of 5 peptidases increased over time in an LU pattern.

Expression of Carbohydrate Utilization Genes

Lactose is the principal carbohydrate in the milks of all mammals. Milk also contains trace amounts of

other sugars, including glucose, fructose, glucosamine, galactosamine, neuraminic acid, and small amounts of oligosaccharides, with sialyllactose being the most represented in bovine milk (Kunz et al., 2000). *Lactobacillus acidophilus* NCFM can transport and utilize glucose, sucrose, fructose, trehalose, melibiose, gentiobiose, cellobiose, maltose, mannose, fructooligosaccharides, raffinose, lactose, galactose, amygdalin, salicin, arbutin, and N-acetyl-glucosamine (Altermann et al.,

Table 3. Ratios and patterns of expression of genes involved in stress and autoinducer-2-mediated quorum sensing

Gene	Cluster trend ²	Overall <i>P</i> -value	Description	Log2 Ratio ¹		
				4 h	8 h	12 h
LBA0081	QV	2.94E-06	YycI	-1.0	-1.1	-0.5
LBA0082	QV	0.001	Hypothetical protein	-1.5	-1.1	-0.8
LBA0083	QV	2.1E-11	HtrA	-1.7	-1.2	0.1
LBA0367	QV	4.9E-07	Putative receptor	-1.5	-1.2	0.1
LBA0405	QLVD	3.1E-09	Cochaperonin GroES	-2.5	-2.6	-1.4
LBA0406	QV	9.7E-12	Chaperonin GroEL	-3.0	-2.0	-0.5
LBA0411	QV	6.5E-06	RuvB helicase Holliday junction	-0.7	-0.8	0.1
LBA0414	QLVD	6.6E-06	Protein DNA-damage-inducible	-0.6	-1.0	-1.0
LBA0422	QLVU	0.0003	Thioredoxin reductase	0.4	0.5	2.0
LBA0638	QLVD	2.7E-09	ATP-dependent ClpE	-2.3	-2.2	-1.3
LBA0672	QLVU	4.8E-12	Putative phosphate starvation induced protein YvyD	-0.9	-1.1	0.9
LBA0694	QLVU	2.1E-13	ATP-dependent Clp protease P	-2.1	-1.3	0.7
LBA0779	QLVU	0.0008	ATP synthase epsilon subunit	0.0	0.4	0.6
LBA1023	QLVU	4.9E-05	Oxidoreductase	0.5	0.4	2.2
LBA1027	QV	1.8E-06	Oxidoreductase	-0.5	-1.3	0.2
LBA1081	QLVU	9.7E-07	Autoinducer-2 (AI2) production protein LuxS	0.3	0.2	1.6
LBA1107	QLVU	2.2E-05	Glutathione reductase	0.0	0.1	0.8
LBA1246	QV	4.5E-07	Heat shock protein dnaJ	-2.3	-1.1	-0.7
LBA1247	QV	8.3E-07	Heat shock protein DnaK	-2.7	-1.7	-0.6
LBA1248	QV	1.58E-06	Cochaperonin GrpE, Hsp70 cofactor	-1.9	-1.3	-0.5
LBA1249	QV	2.7E-10	Heat inducible transcription repressor HrcA	-2.1	-1.7	-0.6
LBA1401	QLVD	4.0E-06	NadH Peroxidase (Npx)	-1.2	-1.5	-0.8
LBA1525	LU	7.6E-05	Two-component system regulator	1.3	0.9	1.5
LBA1910	QV	3.5E-09	ATP-dependent protease ClpE	-2.6	-2.4	-1.0

¹Genes differentially expressed (>1.5-fold; $-\log_{10}(P\text{-values}) < -1.98$) compared with time 0 h.

²LU = linear up-regulated regression pattern; QLVD = quadratic linear convex down; QLVU = quadratic linear convex up-regulated regression pattern; QV = quadratic convex regulated regression pattern.

2005; Barrangou et al., 2006). Several sugar transporters were differentially expressed in *L. acidophilus* growing in SM. Among the phosphotransferase transport systems, a lactose-specific transporter (LBA0989) was overexpressed at 12 h, 2 cellobiose-specific transporters (LBA0491 and LBA0606) were overexpressed at 4 h, and an arbutin-specific transporter was repressed at times 4 and 12 h. The ABC transporters responsible for the uptake of maltose (LBA1865–LBA1866), lactose/galactose (LBA1463) and one uncharacterized sugar transporter (LBA0045) were highly expressed at 5 min and under expressed at times 4, 8, and 12 h. Interestingly, an uncharacterized sugar transport system encoded by LBA1944 to LBA1946 was induced mainly during logarithmic growth phase, at 8 h. The genes/systems involved in carbohydrate utilization differentially expressed upon exposure to milk included lactose/galactose, fructose, maltose and N-acetylglucosamine. In contrast with the expression patterns of genes involved in proteolysis, expression of genes involved in lactose utilization were increased at time 5 min, suggesting that this region is rapidly induced upon exposure of *L. acidophilus* to the dairy environment, especially when cultures have previously been transferred in SM. The same pattern was observed for genes involved in maltose and fructose utilization. On

the other hand, N-acetylglucosamine-6-P deacetylase (LBA0144) and glucosamine-6-phosphate isomerase (LBA1948), involved in the utilization of glucosamine, were among the highest genes induced at 4 h. In fact, microarray data indicated that *L. acidophilus* uses the glucosamine-6-phosphate isomerase (LBA1948; EC 3.5.99.6) and not the D-fructose-6-phosphate amidotransferase (LBA0462; EC 2.6.1.16) to process glucosamine 6-phosphate generating fructose 6-phosphate (and ammonia).

Components of the general carbohydrate utilization regulon were differentially expressed in *L. acidophilus* during growth in milk; specifically, *ptsH* (HPr, LBA0639) *ptsI* (EI, LBA0640), and *ccpA* (CcpA, LBA0431) showed an LU trend indicating increased expression over time

Stress, Quorum Sensing, and 2-Component Regulatory Systems

Step-down quadratic regression analysis (Liu et al., 2005) applied to statistically significant genes for pattern recognition indicated that 24 genes, potentially involved in the stress response of *L. acidophilus*, showed biologically meaningful temporal expression patterns (Table 3). Interestingly the gene responsible for the

generation of the autoinducer-2 (AI2) molecule, which mediates cell-density responses, followed a QLVU pattern of expression. The genome sequence of *L. acidophilus* NCFM contains the complete pathway for the production of AI2 (Buck et al., 2008); however, only *luxS* (LBA1081) was differentially expressed.

Of nine 2-component regulatory systems (2CRS) encoded by *L. acidophilus*, only 2 showed high levels of expression and were differentially expressed. The transcriptional regulator 1525RR, involved in the regulation of acid resistance and proteolytic activity in *L. acidophilus* (Azcarate-Peril et al., 2005) showed a linear up trend of expression in milk, mirroring the expression of the oligopeptide transport systems (Figure 3). In addition, the 2CRS similar to the one encoded by the *yycF* and *yycG* genes in *Bacillus subtilis* (Fabret and Hoch, 1998) showed a quadratic convex regulated regression pattern, with high levels of expression at 5 min and 12 h. In this organism, as well as in *L. acidophilus* NCFM, the 2CRS is part of an operon containing 3 genes with no known function and a gene coding for an HtrA-like protease. In *B. subtilis*, this operon was transcribed during growth, and its transcription decreased as the cells approached stationary phase. Most of the stress-related genes differentially expressed showed either a QV or a QLVU pattern, suggesting high levels of expression toward stationary phase.

Expression of Adhesion Genes in SM

Adhesion to the intestinal mucosa is considered an important trait for bacterial retention in the gastrointestinal tract and interaction with immunomodulatory cells of the mucosal immune system. Consequently, the capacity to adhere to the intestinal mucosa is a very important selection criterion for probiotics (Tuomola et al., 2001). The genome sequence of *L. acidophilus* indicated the presence of several genes encoding putative adhesion factors (Altermann et al., 2005), and some of these have already been shown to play a role in adherence to Caco-2 epithelial cells, in vitro (Buck et al., 2005). Genes putatively involved in adhesion, containing a cell wall anchoring motif LPxTG or with similarities to previously characterized adhesion genes, differentially expressed during growth of *L. acidophilus* in SM are listed in Table 4. Eight genes encoding proteins containing the LPxTG and/or LPQTxE motif were differentially expressed; however, only 3 of them (2 *mub* genes, LBA1392 and LBA1652, and *thrS*) showed recognizable patterns of expression over time. Expression of ORF LBA0493 and LBA1850, encoding putative aggregation-promoting proteins, increased over time falling into QLCU and QLVU temporal patterns, respectively. LBA0493 was one of the most induced

Figure 3. Mean gene expression of oligopeptide transport 1 (*opp1*, continuous line), *opp2* (dashed line), and LBA1524HPK-LBA1525RR 2-component regulatory system (dotted line) over time during growth of *L. acidophilus* NCFM in milk.

genes over time [up to 5.6-fold (2.5-fold in log2 scale) at 4 h].

Growth and Survival of Selected Mutant Strains in Milk and Acidified Milk

Microarray analysis allowed us to identify genes that were differentially expressed during growth of *L. acidophilus* in reconstituted 11% SM. To further examine for any relationships between milk, growth, and potential probiotic properties we selected 14 *L. acidophilus* mutant strains present in our culture collection (Table 1), that were inactivated for genes that were differentially expressed in milk.

The method described by Fonseca et al. (2000) was used to correlate acidification rates with the initial cell concentration to determine if any of the inactivated genes were involved in acidification activity and, hence, growth of *L. acidophilus* in milk (Table 5). Four mutant strains showed significantly ($P < 0.1$) different *tm* values than their respective control strain. As expected, the histidine protein kinase (LBA1524HPK) mutant previously characterized as involved in the regulation of genes involved with acid resistance and proteolysis showed an increased *tm* value in SM (Azcarate-Peril et al., 2005). The other mutant strains showing significantly different *tm* values were *cdpA* (Altermann et al., 2004), mucin-binding protein (LBA1392; Buck et al., 2005), and an uncharacterized putative myosin cross-reactive antigen (LBA0555; A. Dobson, North Carolina State University; unpublished results).

Table 4. Cell wall anchoring proteins (LPxTG and LPQTxE motifs) and putative adhesion factors in *L. acidophilus* NCFM (modified from Altermann et al., 2005)¹

Gene	Open reading form designation	Motif type	COG category	Temporal expression pattern ²
LBA0036	Conserved hypothetical	LPxTG	[D] COG1196 Chromosome segregation ATPases	Not significant
LBA0493	Aggregation promoting protein		SP0107	QLCU
LBA0514	Surface layer protein		[M] Cell wall_membrane_envelope biogenesis	QV
LBA0530	Conserved hypothetical	LPxTG	[R] COG4851: prot. involved in sex pheromone biosynthesis	Not significant
LBA0701	Unknown	LPxTG	[M] COG4932 Predicted outer membrane protein	Flat
LBA0782	RodA	LPxTG	[D] COG0772: Bacterial cell division membrane protein	Flat
LBA0833	FtsW	LPxTG	[D] COG0772: Bacterial cell division membrane protein	Not significant
LBA0919	Conserved hypothetical	LPxTG	[S] Function unknown	Not significant
LBA0927	Membrane protein	LPxTG	[S] Function unknown	Flat
LBA1018	Mub	LPxTG	SA1552	Not significant
LBA1019	Mub	LPxTG, LPQTxE	[U] COG3210: Large prot. involved in heme utilization or adhesion	Not significant
LBA1148	FbpA		[K] COG1293 Predicted RNA-binding protein	Flat
LBA1155	Conserved hypothetical	LPxTG	[L] COG0116: Predicted N6-adenine-specific DNA methylase	Not significant
LBA1244	Sortase		[M] COG3764 Sortase	Flat
LBA1287	tRNA Methyltransferase	LPxTG	[J] COG0336 tRNA-(guanine-N1)-methyltransferase	Flat
LBA1377	Mucus binding protein precursor	LPxTG	[U] COG3210: Large prot. involved in heme utilization or adhesion	Not significant
LBA1392	Mub	LPxTG	L39650	LD
LBA1496	Fibrinogen-binding protein	LPxTG, LPQTxE	[U] COG3210: Large prot. involved in heme utilization or adhesion	Not significant
LBA1543	ThrS	LPxTG	[J] COG0441: Threonyl-tRNA synthetase	QLVU
LBA1611	FmtB	LPxTG	SA126	Not significant
LBA1633	Surface protein	LPxTG, LPQTxE	MYPU_5250	Flat
LBA1634	Surface protein	LPxTG	SA0520	Not significant
LBA1652	Mub	LPxTG	L39650	QC
LBA1654	PspC	LPxTG	[U] COG3210: Large prot. involved in heme utilization or adhesion	Not significant
LBA1690	Putative surface exclusion protein		SPy0269	QLVU
LBA1740	Putative membrane protein	LPxTG, LPQTxE	lin0803	Not significant
LBA1793	Conserved hypothetical	LPxTG	YDR439w	Not significant
LBA1850	Aggregation promoting protein		SP0107	QLVU

¹Significantly differentially expressed genes during growth in 11% skim milk and their temporal expression pattern are in boldface.

²Flat = no significant differential expression over time; LD = linear down; QC = quadratic concave regulated regression pattern; QLCU = quadratic linear concave up-regulated regression pattern; QLVU = quadratic linear convex up-regulated regression pattern; QV = quadratic convex regulated regression pattern.

Table 5. Acidification activity of *Lactobacillus acidophilus* strains in 11% skim milk¹

NCK number	Strain	pH (16 h)	Acidification parameter ²			
			x_0 (cfu/mL)	a (min)	b (min)	tm (min)
NCK0056 ³	NCFM, wt	4.2 (±0.01)	$1.2 (\pm 0.4) \times 10^7$	400.0 (±2.0)	−9.2 (±0.7)	249.4 (±14.5)
NCK1624 ⁴	NCFM:: <i>treB</i>	4.4 (±0.2)	$1.2 (\pm 0.2) \times 10^7$	396.5 (±6.5)	−8.0 (±1.4)	265.4 (±15.7)
NCK1398 ⁵	NCFM:: <i>lacL</i>	6.1 (±0.2)	$1.5 (\pm 0.2) \times 10^7$	381.7 (±3.7)	−0.5 (±0.03)	373.4 (±4.1)*
NCK1375	NCFM:: <i>cdpA</i>	4.6 (±0.2)	$0.6 (\pm 0.2) \times 10^7$	400.3 (±13.9)	−6.9 (±0.8)	294.2 (±9.0)*
NCK1376	NCFM:: <i>cfa</i>	4.2 (±0.1)	$1.0 (\pm 0.4) \times 10^7$	396.4 (±3.2)	−8.6 (±0.4)	258.0 (±4.3)
NCK1377	NCFM:: <i>slpA</i>	4.1 (±0.1)	$1.5 (\pm 0.4) \times 10^7$	402.7 (±10.4)	−7.8 (±0.9)	274.9 (±20.5)
NCK1618	NCFM:: <i>bshA</i>	4.3 (±0.01)	$1.3 (\pm 0.6) \times 10^7$	401.0 (±5.6)	−7.9 (±1.2)	271.2 (±29.1)
NCK1660	NCFM::LBA1392 [<i>mub</i>]	4.5 (±0.2)	$1.4 (\pm 0.5) \times 10^7$	426.6 (±34.6)	−7.6 (±0.9)	301.8 (±18.8)*
NCK1661	NCFM:: <i>fbpA</i>	4.4 (±0.02)	$1.5 (\pm 0.8) \times 10^7$	396.0 (±4.3)	−7.7 (±0.8)	269.0 (±7.2)
NCK1662	NCFM::R28	4.2 (±0.2)	$1.4 (\pm 0.7) \times 10^7$	396.6 (±3.0)	−7.9 (±1.0)	267.9 (±11.2)
NCK1686	NCFM::1524HPK	5.4 (±0.1)	$2.2 (\pm 0.5) \times 10^7$	391.3 (±4.4)	−4.0 (±0.3)	323.9 (±4.2)*
NCK1728	NCFMΔ <i>fre</i>	4.3 (±0.1)	$1.5 (\pm 0.2) \times 10^7$	400.7 (±2.5)	−9.6 (±0.3)	242.1 (±2.6)
NCK1730	NCFM::LBA0555	4.5 (±0.1)	$1.3 (\pm 0.6) \times 10^7$	400.9 (±10.4)	−7.2 (±0.8)	282.9 (±1.1)*
NCK1746	NCFM:: <i>mdoB</i>	4.6 (±0.2)	$2.0 (\pm 0.3) \times 10^7$	439.9 (±5.0)	−10.1 (±0.4)	269.7 (±12.1)
NCK1765	NCFM:: <i>luxS</i>	4.4 (±0.1)	$1.5 (\pm 0.4) \times 10^7$	421.1 (±30.4)	−8.1 (±0.2)	288.0 (±30.3)
NCK1816	NCFM::noncoding	4.4 (±0.1)	$1.5 (\pm 0.5) \times 10^7$	396.6 (±3.7)	−7.6 (±1.1)	271.6 (±14.9)
NCK1822	NCFM::LBA1690	4.3 (±0.2)	$1.0 (\pm 0.3) \times 10^7$	403.0 (±1.5)	−9.3 (±0.8)	254.8 (±13.8)

*Asterisks indicate tm significantly different ($P < 0.1$) from the corresponding control strain.

¹Each value represents the mean of 4 independent experiments. Standard deviations are indicated in parentheses.

² x_0 = initial bacterial concentration; a and b = equation parameters for each strain; tm = time in minutes necessary to reach the maximum acidification rate in milk (Fonseca et al., 2000).

³Used as a reference strain and control for NCK1728.

⁴Used as a control for the knockout strains.

⁵Negative control (lactose negative strain).

To evaluate the effect of the inactivated genes on cell survival under conditions emulating yogurt storage, *L. acidophilus* NCFM (wild type), NCK1376 (NCFM::*cfa*; used as Em^R control), NCK1398 (NCFM::*lacL*), NCK1660 (NCFM::*mub*), NCK1686 (NCFM::LBA1524HPK), NCK1730 (NCFM::LBA0555), and NCK1822 (NCFM::LBA1690) were held at 4°C over a 2-week period in SM acidified at pH 4.5 with lactic acid (Table 6). All the selected mutant strains showed a >2-fold reduction in survival rates after a 14-d period compared with the control. Mutants containing the 1524HPK, *lacL*, and *mub* inactivated genes showed increased death rates (K , per h) suggesting that the inactivated genes have a role in survival during

storage at 4°C in acidified SM. A strong correlation was observed between tm and K values for all the studied strains, indicating that strains showing high tm values and, consequently, lower acidification activities are also less able to survive at low pH and 4°C (Figure 4).

Effect of Milk on Adhesion of *L. acidophilus* Strains to Mucin and Caco-2 Cells

To determine the overall effect of SM on the adhesion capability of selected *L. acidophilus* strains, we examined the effect of SM as a growth medium and as a suspension-adhesion medium on the ability of *L. acidophilus* NCFM to adhere to Caco-2 cells. *Lactobacillus*

Table 6. Survival of selected *L. acidophilus* strains in acidified skim milk (pH 4.5 adjusted with lactic acid) at 4°C over a 14-d period

Strain	Genotype	% survival after		K (h ^{−1})	R
		6 d	14 d		
NCFM	Wild-type	45	8	−0.06	−0.99
NCK1376 ¹	NCFM::LBA1274 <i>cfa</i>	33	3	ND ²	ND
NCK1398	NCFM::LBA1398 <i>lacL</i>	7	0	−0.22	−0.99
NCK1660	NCFM::LBA1392 <i>mub</i>	11	0.5	−0.11	−0.95
NCK1686	NCFM::LBA1524 <i>hpk</i>	10	0.01	−0.19	−0.99
NCK1730	NCFM::LBA0555	19	0.7	−0.08	−0.90
NCK1822	NCFM::LBA1690	21	0.1	−0.06	−0.93

¹Em^R control strain.

²Not determined.

Figure 4. Correlation between *tm* (time in minutes necessary to reach the maximum acidification rate in milk) and *K* (death rate) values corresponding to acidification rates in milk and survival in acidified milk, respectively.

acidophilus NCFM was grown either in MRS or SM until pH 6.0, after one wash with phosphate buffer (pH 6.0); cells were resuspended either in the same buffer or in SM at pH 6.0 before exposure to Caco-2 cells. Resuspension of bacterial cells in SM after growth in MRS strongly reduced adhesive abilities of the bacterium (37% adhesion loss). No significant differences in adhesion were observed when cell cultures were resuspended in phosphate buffer at pH 6.0 or SM at pH 6.0 after growth in SM, suggesting that the resuspension medium did not have an effect on the adhesion properties of cultures grown in SM. The adherence abilities of

L. acidophilus NCFM was not affected by the growth medium, because the levels of adherence of cells grown in SM or MRS and resuspended in phosphate buffer were similar (data not shown).

To identify potential associations between genes differentially expressed in SM and adhesion properties, we compared the ability of *L. acidophilus* NCFM versus 6 selected mutants (see Table 1) to adhere to mucin and to Caco2-tissue culture cells. Two mutant strains, NCK1686 (NCFM::LBA1524HPK) and NCK1822 (NCFM::LBA1690) showed a 30% decrease in adhesion to Caco-2 cells (Figure 5), suggesting that these proteins may play a role in adhesion of *L. acidophilus* when grown in milk. On mucin, NCK1686 showed a 31% reduction and NCK1822 more than a 68% reduction in adhesion. None of the other mutant strains were affected in adhesion on the 2 models tested, indicating that the insertionally inactivated genes in these strains; that is, myosin-crossreactive antigen (LBA0555), mucus binding protein precursor (LBA1392), and the β -galactosidase large subunit (LBA1398), are not involved in adherence of *L. acidophilus* when grown in milk.

Discussion

The overall transcriptional response of *L. acidophilus* NCFM, a widely used probiotic strain, was examined during growth in reconstituted SM. Temporal overall gene expression profiles in milk indicated that the expression of genes involved in carbohydrate utilization were rapidly induced at early stages of growth and decreased over time. Decreased levels of expression of carbohydrate utilization genes coincided with trends

Figure 5. Adherence abilities of 6 *L. acidophilus* NCFM derivatives grown in skim milk, to mucin (■), and to Caco-2 cells (□). The results are expressed as a percentage of the wild-type strain. Values represent the mean of 3 independent experiments and error bars represent the standard deviation. The asterisks represent statistically significant ($P < 0.05$) differences between mutant and control strains.

of increasing expression of key components involved in catabolite repression (*ptsH*, HPr, LBA0639; *ptsI*, EI, LBA0640; and *ccpA*, CcpA, LBA0431). The carbon catabolite repression (CCR) system controls the transcription of genes involved in the transport and catabolism of carbohydrates. The mechanism is widely distributed in bacteria (Gorke and Stulke, 2008), including lactic acid bacteria. In the model firmicute, the transcription factor CcpA (catabolite control protein A), HPr protein, and the bifunctional HPr kinase/phosphorylase (HPrK) are the main players in CCR (Gorke and Stulke, 2008). Catabolite control protein A is one key component in the regulation of the sugar transport and utilization in LAB. Although some studies indicate a role as an activator, most of the published data indicate that CcpA acts as a repressor (reviewed by Titgemeyer and Hillen, 2002). Therefore, the overexpression of different sugar transport systems might be the reflection of gene derepression rather than induction of gene expression.

Conversely, proteolytic genes showed increasing patterns of expression over time. Interestingly, the 2 oligopeptide operons (*opp1* and *opp2*) encoded by *L. acidophilus*, which are involved in the transport of oligopeptides into the cell, showed different expression patterns. The maximum expression level of *opp1* was at 4 h (early logarithmic phase of growth), but the expression of *opp2* reached its maximum level at 12 h (early stationary phase). Expression of the 2-component regulatory LBA1524HPK-LBA1525RR in milk correlated with the expression of the oligopeptide transport systems as it showed increased levels of expression at 4 and 12 h (Figure 3). These results support previous data that indicated that LBA1524HPK-LBA1525RR was involved in regulation of gene expression of members of the proteolytic enzyme system (Azcarate-Peril et al., 2005). Studies of oligopeptide gene expression in lactobacilli are scarce. In *L. delbrueckii* ssp. *bulgaricus* no significant differences were detected in the amount of oligopeptide mRNA per total RNA in preparations from the early- and late-exponential phase cultures grown in whey broth culture (Peltoniemi et al., 2002). A recent study indicated that, in *L. helveticus*, growth in milk also significantly induced various peptidases, proteinases, and the oligopeptide transporters (Smeianov et al., 2007).

Sixteen peptidases showed noteworthy temporal patterns of expression in *L. acidophilus*. In contrast, only 9 peptidases were induced in *L. helveticus* (Smeianov et al., 2007) during growth in milk; however, in that study, gene expression was analyzed at one time point (9 h, pH 5.5) and not over the various growth phases. In *L. acidophilus*, only expression of oligoendopeptidase F (*pepF*, LBA1763) peaked at 4 h displaying a QC

pattern of gene expression because most of the differentially expressed peptidases were mainly expressed at 12 h (early stationary phase). The *pepF* gene is well conserved among lactobacilli, and other members of the LAB group. The *pepF* gene encoded by LBA1763 shows a 49% identity with *pepF* from *Lactococcus lactis*. In this bacterium, PepF hydrolyzes peptides containing more than 7 and less than 17 amino acids (Monnet et al., 1994). These expression profiles suggest an early role of PepF breaking larger peptides into smaller ones that will be then processed by other peptidases of the proteolytic system. Additionally, several aa transporters showed increased levels of transcription over time (Table 2). Interestingly, the differentially expressed genes LBA1115 and LBA1902 coding for aa transporters appear to be unique to the acidophilus group (including *L. acidophilus*, *L. gasseri*, and *L. johnsonii*) and *L. reuteri*.

Most of the stress-related genes differentially expressed showed either a QV or a QLVU pattern, suggesting high levels of expression toward stationary phase during growth of *L. acidophilus* in milk. These trends are in accordance with the accumulation of lactic acid (and consequent decrease in pH) and other secondary metabolites. Several mutant strains were evaluated for their acidification activity, survival during storage conditions, and adhesive properties to mucin and Caco-2 cells. These derivatives contained mutations in genes found to be differentially expressed when *L. acidophilus* was grown in milk. As expected, the derivative with a mutation in *lacL* showed decreased acidification ability (as indicated by higher *tm* values) and decreased survival in acidified skim milk at 4°C, indicating that β -galactosidase activity is required not only for growth but also for survival under storage conditions. We observed increased *tm* values and reduced survival in acidified milk at 4°C for strains carrying mutations in LBA1524HPK, LBA0555 (annotated as a myosin-crossreactive antigen) and LBA1392, a mucus-binding protein precursor (*mub*) demonstrated to mediate adherence to intestinal epithelial cells (Buck et al., 2005).

Interestingly, *luxS*, responsible for the transformation of S-ribosylhomocysteine into homocysteine in the methyl activated cycle and the production of AI2 from methionine, showed similar trends of expression in milk as in MRS (Buck et al., 2008), with increased expression toward stationary phase. It is noteworthy that the *luxS* mutant strain showed increased *tm* values (although not significant at a $P > 0.1$ level) suggesting an important role for LuxS during growth in milk.

The adhesion capabilities of probiotic strains in the presence of milk have not been extensively analyzed. Ouwehand et al. (2001) reported reduced adhesion

levels of *L. brevis*, *L. reuteri*, and *L. rhamnosus* upon exposure and growth in skim milk, in milk containing 1.5% fat, and in nonhomogenized milk with fat content of 1.6 to 1.9%. In our study, we observed comparable levels of adhesion when *L. acidophilus* NCFM cells were grown in skim milk or MRS. This is a technologically relevant fact because probiotic cultures are often consumed via milk-based food products. Additionally, milk, through its buffering action, is likely to provide a protective medium to probiotics that will increase the number of microorganisms surviving the passage through the stomach to reach the small intestine. Some genes putatively involved in adhesion were expressed differentially during growth of *L. acidophilus* in milk. Especially relevant is the overexpression of the genes encoding aggregation-promoting proteins, specifically the protein encoded by LBA0493, which is similar to the aggregation-promoting factor (APF) described in *L. crispatus* M247 (Marcotte et al., 2004). It has been shown that a nonaggregating isogenic spontaneous mutant derived from M247 was unable to colonize the intestinal tract suggesting a role of aggregation in colonization of the gastrointestinal tract (Cesena et al., 2001).

In the present study, the comparison of adhesion abilities of 6 mutants indicated that an unknown membrane protein (LBA1690) was involved in adhesion of *L. acidophilus* NCFM grown in milk to mucin. Detectable differences were also observed for the HPK mutant and LBA1690 to Caco-2 cells. Some histidine protein kinases from 2CRS have already been shown to be involved in regulation of adherence of gram-positive pathogens such as *Streptococcus agalactiae* to immobilized fibrinogen via the regulation of the expression of bacterial cell surface components (Spellerberg et al., 2002), and adhesion of *L. plantarum* WCFS1 to a glass surface via the overexpression of genes involved in cell surface polysaccharides synthesis (Sturme et al., 2005). The specific role of LBA1524HPK-LBA1525RR in adhesion might be related to the function of the 2CRS in the regulation of the transport of oligopeptides (Azcarate-Peril et al., 2005) as it was demonstrated in *S. gordonii* and *S. parasanguis*, where a mutation of the oligopeptide permease systems led to a decrease in bacterial cell adhesion [reviewed by Leonard and Podbielski, (1999)]. However, it is not clear whether the oligopeptide-binding proteins can act as adhesins themselves or if they are required for the transport of signaling peptides that impact adherence factors.

The data available on the hypothetical protein LBA1690 revealed the presence of a signal peptide and a transmembrane domain, suggesting that this protein is secreted and anchored to the membrane. Further studies are needed to determine the function of this pro-

tein and its potential role in adhesion. Surprisingly, the putative mucus-binding protein encoded by LBA1392, although differentially expressed during growth in milk, did not appear to have a role in adhesion during growth in milk, whereas this protein was previously shown to be involved in adherence on Caco-2 cells after the strain was grown in MRS (Buck et al., 2005). It might be speculated that growth in milk alters the cell surface display to promote attachment in spite of the absence of the LBA1392:*mub* protein.

Our study presents, for the first time, the analysis of the effect of milk, as a delivery vehicle, on the global gene expression of a probiotic strain, *L. acidophilus*. Additionally, genes involved with growth and survival in milk have been identified, some of which may be potentially important for probiotic traits.

ACKNOWLEDGMENTS

This study was supported by the Southeast Dairy Foods Research Center; Dairy Management, Inc.; and the North Carolina Dairy Foundation. We thank J. Bruno-Barcena (Department of Microbiology, North Carolina State University) and E. Durmaz (Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University) for scientific advice and helpful suggestions, and Jason Osborne, at the Department of Statistics (North Carolina State University), for his advice on microarray data analysis. We are grateful to the Genome Research Laboratory at North Carolina State University for technical assistance and staff that supported printing of the microarray slides.

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