Characterization of a Novel Bile-Inducible Operon Encoding a Two-Component Regulatory System in *Lactobacillus acidophilus*[∇]

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Lactobacillus acidophilus NCFM is an industrially important strain used extensively as a probiotic culture. Tolerance of the presence of bile is an attribute important to microbial survival in the intestinal tract. A whole-genome microarray was employed to examine the effects of bile on the global transcriptional profile of this strain, with the intention of elucidating genes contributing to bile tolerance. Genes involved in carbohydrate metabolism were generally induced, while genes involved in other aspects of cellular growth were mostly repressed. A 7-kb eight-gene operon encoding a two-component regulatory system (2CRS), a transporter, an oxidoreductase, and four hypothetical proteins was significantly upregulated in the presence of bile. Deletion mutations were constructed in six genes of the operon. Transcriptional analysis of the 2CRS mutants showed that mutation of the histidine protein kinase (HPK) had no effect on the induction of the operon, whereas the mutated response regulator (RR) showed enhanced induction when the cells were exposed to bile. These results indicate that the 2CRS plays a role in bile tolerance and that the operon it resides in is negatively controlled by the RR. Mutations in the transporter, the HPK, the RR, and a hypothetical protein each resulted in loss of tolerance of bile. Mutations in genes encoding another hypothetical protein and a putative oxidoreductase resulted in significant increases in bile tolerance. This functional analysis showed that the operon encoded proteins involved in both bile tolerance and bile sensitivity.

Lactobacillus acidophilus is one of several species of bacteria that populate the human gastrointestinal tract. Some species in this group exert properties that confer health benefits on their host. *L. acidophilus* NCFM is an industrially important strain that has been used extensively as a probiotic culture in yogurt and dietary supplements (31). Recently, the complete genome of *L. acidophilus* NCFM was sequenced, leading to the elucidation of groups of genes involved in traits such as acid tolerance and adhesion to intestinal epithelial cells (2, 11, 17). Viability in the intestinal tract depends on the microorganism's ability to survive in this region, where stresses include low pH and the presence of bile.

Bile salts are one of the major components of bile and consist of a cholesterol-derived ring structure that is amide linked to an amino acid, either glycine or taurine. The amphipathic nature of conjugated bile salts allows them to act as emulsifiers that facilitate the dietary absorption of lipids, but this property also gives them the ability to emulsify the lipid membranes of bacterial cells (7). Some microbes in the gastrointestinal tract have the ability to hydrolyze this amide linkage, creating deconjugated bile salts that can damage bacterial membranes and lead to cell death (18). Some bile salts have the ability to cross lipid membranes into the cellular cytoplasm, where they can damage DNA and proteins (20, 25). Bile has been shown to induce transcription of molecular chaperones,

such as GroESL, and other proteins involved in DNA and protein repair (20). While bile-specific defense mechanisms are not wholly understood, a diverse group of them have been elucidated, including those that export bile from the cell and chemically modify bile constituents (7, 27, 32, 34).

Mechanisms to sense the presence of bile and alter transcription are not well characterized but may involve a two-component regulatory system (2CRS). 2CRSs allow bacteria to sense and respond to changes in their environment after receiving an environmental signal through transmembrane sensing domains of the histidine protein kinase (HPK). Once it receives a signal input, the HPK autophosphorylates through ATP hydrolysis. The phosphoryl group is then transferred to the regulatory domain of the response regulator (RR), which in turn promotes a transcriptional response through its DNA binding domain (28). Previous studies have shown the ability of bacteria to alter the transcription of necessary genes in the presence of bile or bile constituents, including potential induction through the RR of a 2CRS (26), and have indicated the increased expression of genes in a 2CRS in response to bile (20, 30). However, to date, no system has been functionally characterized in any gram-positive organism.

The complete genome sequence of *L. acidophilus* NCFM allows examination of the genomic elements that confer probiotic properties on the strain (1). cDNA microarrays have proven a key tool for the examination of bacterial transcriptomes (2, 6). This study employed a whole-genome microarray representing 97.4% of the open reading frames (ORFs) to investigate how *L. acidophilus* NCFM reacts to the presence of bile in its environment and to identify genes that contribute to bile tolerance.

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TABLE 1. Strains and plasmids

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
<i>E. coli</i> EC1000	RepA ⁺ MC1000; Km ^r ; host for pORI28-based plasmids	19
<i>L. acidophilus</i> NCFM	Human intestinal isolate	5
<i>L. acidophilus</i> NCK1392	NCFM containing pTRK669	29
<i>L. acidophilus</i> NCK1871	NCFM with a 301-bp deletion of ORF 1427 (putative oxidoreductase)	This study
<i>L. acidophilus</i> NCK1873	NCFM with a 245-bp deletion of ORF 1428 (hypothetical protein)	This study
<i>L. acidophilus</i> NCK1875	NCFM with a 938-bp deletion of ORF 1429 (transporter)	This study
<i>L. acidophilus</i> NCK1877	NCFM with an 1,153-bp deletion of ORF 1430 (histidine protein kinase)	This study
<i>L. acidophilus</i> NCK1879	NCFM with a 217-bp deletion of ORF 1431 (response regulator)	This study
<i>L. acidophilus</i> NCK1881	NCFM with a 202-bp deletion of ORF 1432 (hypothetical protein)	This study
Plasmids		
pORI28	Em ^r <i>ori</i> (pWV01); replicates only with <i>repA</i> in <i>trans</i>	
pTRK669	<i>ori</i> (pWV01) Cm ^r ; temperature sensitive; provides <i>repA</i> in <i>trans</i>	
pTRK915	3.6 kb; pORI28 with 925- and 868-bp noncontiguous regions flanking LBA1427	This study
pTRK916	3.4 kb; pORI28 with 904- and 862-bp noncontiguous regions flanking LBA1428	This study
pTRK917	3.5 kb; pORI28 with 936- and 901-bp noncontiguous regions flanking LBA1429	This study
pTRK918	3.6 kb; pORI28 with 990- and 975-bp noncontiguous regions flanking LBA1430	This study
pTRK919	3.5 kb; pORI28 with 971- and 846-bp noncontiguous regions flanking LBA1431	This study
pTRK920	3.5 kb; pORI28 with 870- and 943-bp noncontiguous regions flanking LBA1432	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Broth cultures of *Lactobacillus* strains were grown anaerobically at 37°C or 42°C in MRS broth (Difco Laboratories Inc., Detroit, MI). Cells were plated on MRS agar (1.5%) supplemented with chloramphenicol (5 µg/ml) and erythromycin (Em) (5 µg/ml) when appropriate for selection. *Escherichia coli* strains were cultivated aerobically in Luria-Bertani medium (Difco) at 37°C with 150 µg/ml Em. *E. coli* transformants were selected on brain heart infusion medium (Difco) with 150 µg/ml Em. Counts (CFU/ml) were performed by plating cells on agar with a Whitley Automatic Spiral Plater (Don Whitley Scientific Ltd., West Yorkshire, England). Culture growth was monitored at an optical density at 600 nm (OD₆₀₀) in 96-well plates using the BMG Labtech (Offenburg, Germany) FLUOstar Optima plate reader. Cultures were grown at 37°C for 23 h in a volume of 200 µl.

DNA manipulations. Chromosomal *L. acidophilus* DNA was extracted using the method of Walker and Klaenhammer (37). Large-scale plasmid preparations for the purpose of cloning were performed using the Mo Bio UltraClean plasmid preparation kit (Mo Bio Laboratories, Carlsbad, CA). Smaller-scale preparations for screening and transformation were performed using the QIAprep Spin kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. PCR primers (Tables 2 and 3) were synthesized by Integrated DNA Technologies (Coralville, IA), and PCR amplifications were carried out using a *Taq* polymerase PCR system (Roche Molecular Biochemicals, Indianapolis, IN). Endonuclease restriction digests were carried out according to the manufacturer's recommendations (Roche Molecular Biochemicals). Digested fragments were excised from 1% agarose gels and extracted using the ZymoClean Gel DNA Recovery kit (Zymo Research, Orange, CA). Ligations were performed using T4

DNA Ligase (Roche Molecular Biochemicals) according to the manufacturer's directions and transformed into chemically competent *E. coli* cells. Electrocompetent *L. acidophilus* NCFM cells were prepared as described by Walker et al. (36). Southern hybridization of genomic DNA was carried out using Magnacharge nylon transfer membranes (MSI, Westboro, MA) according to standard protocols.

Generation of the *L. acidophilus* NCFM microarray. Microarrays were designed as described previously by Azcarate-Peril et al. (2). Briefly, total genomic DNA was used as a PCR template for the amplification of 1,996 predicted ORFs. PCR products were purified and spotted in triplicate onto glass slides. The arrays were assessed for quality by hybridization of Cy3- and Cy5-labeled cDNA samples prepared from total RNA. Data from these samples revealed a linear correlation in relative expression with no more than a twofold change in expression.

cDNA probe preparation and microarray hybridization. NCFM cells were grown to an OD₆₀₀ between 0.2 and 0.3 (representing early log phase). The cells were then pelleted at room temperature and resuspended in either MRS or MRS with 0.5% (wt/vol) oxgall (Difco) and incubated for 30 min at 37°C. The cells were then cold shocked in an ethanol-dry ice bath, pelleted at 4°C, flash frozen, and stored at -80°C. Total RNA was isolated using Trizol (Invitrogen)-chloroform extraction, and residual genomic DNA was removed using DNase I, amplification grade (Invitrogen), according to the manufacturer's directions. The RNA concentration and quality were determined by spectrophotometry and agarose gel electrophoresis. Twenty-five micrograms of RNA were aminoallyl labeled by reverse transcription with random hexamers in the presence of aminoallyl dUTP (Sigma Chemical Co.) using Superscript II reverse transcriptase (Life Technologies), followed by fluorescent labeling with *N*-hydroxysuccini-

TABLE 2. Primers used to generate gene deletions

ORF	Downstream region	Upstream region
LBA1427	Forward GATCCCATGGTCTACTCTTACCATTCTTAAC Reverse GATCCTCGAGTAGCTGAAATTGCTAGTC	Forward GATCCTCGAGAATCATCACGAGTAATCAC Reverse GATCGAATTCTTGACCAATTGAAGAAC
LBA1428	Forward GATCCCATGGAAAGTTGACCAATTGTAG Reverse GATCCTCGAGAATGGGTTATGGTAAATCAG	Forward GATCCTCGAGAATTCTACCTTTCTTTAATG Reverse GATCGAATTCTTGGTTGCTGTTATTAGAC
LBA1429	Forward GATCCCATGGCTAATTCTTGCCAAATCTG Reverse GATCCTCGAGTTTGGTCCTATGATTAGTG	Forward GATCCTCGAGATCAACGGATTAAACAAACATAG Reverse GATCGAATTCTCGATTCTGGTTGTTATTATG
LBA1430	Forward GATCCTCGAGAATTTCTGAATCATCCTTATC Reverse GATCGAATTCCTAATGGTTATCGTTCTTATC	Forward GATCCCATGGAAGCGCACAGTATCAAAG Reverse GATCCTCGAGTGGATTAGCTATGGCTCAAG
LBA1431	Forward GATCCCATGGTATTGCCTAGCATCTCTTCC Reverse GATCCTCGAGAGCAAGAATTAGCGAGTCAC	Forward GATCCTCGAGTTTGTGTTAAGAGCGGTAATTCC Reverse GATCGAATTCCTATAATGGCGTAAACGAAAG
LBA1432	Forward GATCCCATGGACACGAGTAATACCGACCAATC Reverse GATCCTCGAGACGAGAATTAAAGCGGGTAG	Forward GATCCTCGAGTAATCTTTCTCTTAAACAATAG Reverse GATCGAATTCTTGACTTAACAGGAATATC

TABLE 3. Primers used in RT-QPCR

Target ORF	Forward (5'–3')	Reverse (5'–3')	Product Size (bp)
LBA0383	GCAAATCCTATCAGGTAATC	ACCGTCTACTACTTTCAAC	131
LBA0406	GCCACTTCTTCAAGAAATC	GTTGAAAGTACCACGAATC	115
LBA1247	AGTTAAAGACGCAGTTATTAC	AGTTGGTTCGTTGATAATTC	118
LBA1425	TGAACGATAAGGCTAACC	GCGTATTTCTTGGGAATG	114
LBA1426	AAATTCTGATAAGCCTCAAC	GATGTAGCAGTAATAGCATAG	123
LBA1429	CAATATCTGCTTGGGTAAC	CAGAAATGTGCCAAAGAG	108
LBA1432	TACCCGGTCATTATTTTCGTTG	ATCAGTACCCGCTTTAATTC	140
LBA1460	TTACACAGTCAGGTAAGAG	AATAATAACTGGGCTAGGC	104
LBA1496	GGTCATCTTTCAACGAATC	TTGGAGGTAAGGCAATATC	115
LBA1652	AACGGCAATATTATTGGG	GTTACTTCAATCGTGTATGG	131

mid-activated Cy3 or Cy5 ester (Amersham Biotechnology). The labeled cDNA probes were purified using a PCR purification kit (QIAGEN). Coupling of the Cy3 and Cy5 dyes to the aminoallyl-dUTP-labeled cDNA and hybridization of samples to microarrays were performed according to the protocols outlined by The Institute for Genome Research (<http://www.tigr.org/tdb/microarray/protocols/TIGR.shtml>). 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 high-stringency buffer (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] with 0.2% sodium dodecyl sulfate [SDS]) for 5 min each. The first wash was performed at 42°C and the second at room temperature. The slides were then washed in a low-stringency buffer (0.1× SSC containing 0.2% SDS) for 5 min at room temperature and finally in 0.1× SSC for two 2.5-min washes at room temperature.

Data normalization and gene expression analysis. Immediately after the slides were washed, fluorescence intensities were acquired at 10-μm resolution using a ScanArray 4000 microarray scanner (Packard Biochip BioScience; Biochip Technologies LLC) and stored as TIFF images. Signal intensities were quantified, background was subtracted, and the data were normalized using the QuantArray 3.0 software package (Perkin-Elmer). Two slides per experiment, each containing triplicate arrays, were hybridized reciprocally to Cy3- and Cy5-labeled probes (dye swapping). Spots were analyzed by adaptive quantitation. The data were median normalized. When the local background intensity was higher than the spot signal, no data were recorded for those spots. The mean of the six ratios per gene was recorded, and the ratio between the average absolute pixel values for the replicated spots of each gene, with or without treatment, represented the change in gene expression. Genes in potential operons 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 for the change were also calculated with the use of a two-sample *t* test. *P* values of < 0.05 were considered significant.

Site-specific integration and deletion in *L. acidophilus* NCFM. Two noncontiguous fragments flanking an internal region of the target ORF were amplified using PCR primers listed in Table 2. The fragments were cloned into pORI28 and then transformed into *L. acidophilus* NCK1392 containing the temperature-sensitive helper plasmid pTRK669. Selection of integrants was then carried out as described previously (29). Upon successful integration of the plasmid into the genome, a single integrant colony was propagated in the absence of antibiotic selection and replica plated onto MRS agar and MRS agar containing Em. Em^s cells were screened for a deletion mutation using PCR with primers flanking the targeted region, and deletions were confirmed by Southern blotting.

Bile survival assays. To test deletion mutants for survival in the presence of bile, cells were grown to early log phase (OD₆₀₀ = 0.2 to 0.3), serially diluted, and plated on MRS agar containing 0%, 0.5%, 1.0%, and 2.0% (wt/vol) oxgall (Difco) or bile salts (Sigma-Aldrich, St. Louis, MO) using a Whitley Automatic Spiral Plater. The concentrations of oxgall were chosen to emulate the concentrations found in the human intestinal tract, which range from 0.2 to 2% (16).

MICs of oxgall, glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), and sodium chloride were determined by spotting approximately 10⁴ early-log-phase cells (OD₆₀₀ = 0.2 to 0.3) onto MRS agar plates containing increasing concentrations of these compounds. Inhibitory concentrations of Triton X-100 and SDS (Fisher Scientific, Fair Lawn, NJ) were determined by inoculating 200-μl MRS broth cultures containing increasing amounts of these compounds. Inhibition was reported as the lowest concentration tested that prevented the growth of the strain. The

increments of concentrations tested were as follows: bile salts, 1 μg/ml; salt, 5 μg/ml (1%); TX-100, 0.5%; SDS, 0.02%.

RT-PCR and RT-QPCR. RNA samples were collected and treated as described for microarray experiments. Reverse transcriptase-quantitative PCR (RT-QPCR) primers are shown in Table 3. PCR was performed using the Quanti-Tect Reverse Transcriptase PCR kit (QIAGEN) according to the manufacturer's directions, but the total reaction size was reduced to 20 μl. PCR was performed on a Bio-Rad My-IQ single-color detection system (Bio-Rad Laboratories, Hercules, CA). Absolute mRNA copy numbers were determined using a standard curve of known concentrations, and transcripts were enumerated using the MyIQ software. Copy numbers per cell were determined by normalizing the transcript number of the queried ORF to the transcript number of LBA383 (DNA polymerase III, delta subunit).

RT-PCR was carried out to determine intergenic transcription using the Superscript II kit (Invitrogen) according to the manufacturer's directions. cDNAs were generated using random primers. PCR was performed using primers specific to the intergenic regions of the hypothetical cotranscript.

Northern blotting. Northern blotting of total RNA was carried out using the NorthernMax Gly kit (Ambion). Briefly, RNA was electrophoresed on a 1% agarose gel and transferred to a Magnacharge nylon transfer membrane (MSI). The membrane was probed with a 692-bp fragment of LBA1430 labeled with [γ -³²P]CTP. Transfer, hybridization, washing, and exposure to films (Eastman Kodak Company, Rochester, NY) were carried out according to standard methods and the manufacturer's instructions.

RESULTS

Differentially expressed genes. Global gene expression patterns of bile-exposed NCFM cells were determined using microarray analysis. Table 4 lists the 289 genes that were differentially expressed (≥ 2 -fold upregulated or downregulated) and met the criteria for statistical significance (*P* < 0.05). Overall, 78 genes (3.9% of the genome) were shown to be induced while 168 (8.4%) were repressed.

While the expression of the majority of genes in the genome did not change significantly, patterns of expression emerged across cluster of orthologous groups (COG) classifications (Fig. 1) (33). Most differentially expressed ORFs involved in metabolism of proteins, nucleotides, and lipids were downregulated in the presence of bile, including tRNA synthetases for several amino acids, and the operon involved in purine biosynthesis (LBA1551 to LBA1560). Because of the repression of these groups of genes, growth of NCFM was monitored in the presence and absence of 0.5% oxgall. The maximum specific growth rate of cells in 0.5% oxgall was reduced 25% compared to that of cells grown in MRS (data not shown).

The COG with the most genes upregulated contained those involved in carbohydrate transport and metabolism, including the lactose and galactose operons. Interestingly, the genes in

TABLE 4. *L. acidophilus* NCFM ORFs differentially expressed upon exposure to 0.5% oxgall^a

COG functional classification, gene, and gene product ^b	Ratio ^c	COG functional classification, gene, and gene product ^b	Ratio ^c
C: energy production and conversion		I: lipid metabolism	
LBA0055: D-lactate dehydrogenase ^d	2.1	LBA0948: inner membrane <i>trans</i> -acylase protein.....	0.5
LBA0466: hypothetical protein.....	5.2	LBA1167: mevalonate kinase.....	0.5
LBA0741: acetate kinase AckB.....	0.4	LBA1168: mevalonate diphosphate decarboxylase.....	0.4
LBA1107: glutathione reductase.....	0.4	LBA1169: phosphomevalonate kinase.....	0.5
LBA1109: hypothetical protein.....	0.4	LBA1232: apo-citrate lyase ^d	0.5
LBA1125: manganese-dependent inorganic pyrophosphatase.....	0.5	LBA1308: fatty acid/phospholipid synthesis protein; PlsX.....	0.5
LBA1418: NADH-dependent oxidoreductase.....	0.4	LBA1447: hypothetical protein.....	4.9
LBA1632: NAD-dependent aldehyde dehydrogenase; SsdH.....	2.1		
D: cell division and chromosomal partitioning		J: translation, ribosomal structure, and biogenesis	
LBA0275: hypothetical protein.....	0.3	LBA0272: peptidyl tRNA hydrolase.....	0.3
LBA0277: putative cell cycle protein.....	0.5	LBA0280: transcriptional regulator.....	0.3
LBA1497: unknown.....	2.1	LBA0281: lysine tRNA synthetase; LysRS.....	0.4
LBA1735: EpsC.....	0.5	LBA0347: glutamyl tRNA synthetase; GluRS.....	0.4
LBA1827: chromosome-partitioning protein.....	0.7	LBA0370: 50S ribosomal protein L7/L12.....	0.5
E: amino acid transport and metabolism		LBA0417: alanyl tRNA synthetase; AlaRS.....	0.4
LBA0045: sugar transporter ^d	2.3	LBA0623: methionine aminopeptidase; AmpM.....	0.5
LBA0158: asparagine synthetase.....	0.4	LBA0636: peptide chain release factor 3.....	0.3
LBA0552: multidrug transporter ^d	0.4	LBA0791: 16S pseudouridylyl synthetase.....	0.4
LBA0711: spermidine/putrescine ABC transporter; PotB.....	0.5	LBA0822: tRNA methyltransferase.....	0.4
LBA0789: aminotransferase (NifS family).....	0.2	LBA0830: polypeptide deformylase; Pdf.....	0.4
LBA0850: aspartokinase/homoserine dehydrogenase.....	0.4	LBA1178: [nucleolar protein].....	0.4
LBA0995: amino acid permease.....	0.2	LBA1446: multidrug resistance protein ^d	7.3
LBA1177: iron-sulfur cofactor synthesis protein; YrvO.....	0.3	LBA1448: [alpha-galactosidase].....	2.2
LBA1222: branched-chain amino acid permease.....	0.5	LBA1457: galactose-1-epimerase.....	2.8
LBA1292: amino acid transporter.....	0.3	LBA1518: phenylalanyl tRNA synthetase; SyfB.....	0.4
LBA1429: transporter ^d	3.0	LBA1519: phenylalanyl tRNA synthetase; SyfA.....	0.4
LBA1446: multidrug resistance protein ^d	7.3	LBA1627: [acetyltransferase].....	2.7
LBA1974: pyruvate oxidase.....	2.6		
F: nucleotide transport and metabolism		K: transcription	
LBA0245: GMP synthase.....	0.4	LBA0393: transcriptional regulator (GntR family).....	0.4
LBA0953: 2',3'-cyclic-nucleotide 2'-phosphodiesterase.....	0.4	LBA0516: transcriptional regulator (GntR family).....	0.4
LBA1242: adenine phosphoribosyltransferase; AprT.....	0.3	LBA1148: fibronectin-binding protein.....	0.3
LBA1551: phosphoribosylamine-glycine ligase; PurD.....	0.5	LBA1249: heat-inducible transcription repressor; HrcA.....	3.2
LBA1552: phosphoribosylaminoimidazolecarboxamide, PurH.....	0.4	LBA1431: response regulator ^d	3.0
LBA1553: phosphoribosyl glycineamide; PurN.....	0.5	LBA1461: hypothetical protein.....	2.2
LBA1554: phosphoribosylformylglycinamide cycloligase; PurM.....	0.4	LBA1517: transcription elongation factor; GreA.....	0.5
LBA1555: phosphoribosylpyrophosphate amidotransferase; PurL.....	0.3	LBA1638: [TetR].....	3.4
LBA1556: phosphoribosylpyrophosphate synthetase; PurL.....	0.3	LBA1828: chromosome-partitioning protein.....	0.4
LBA1559: phosphoribosylaminoimidazole-succinocarboxamide synthetase; PurC.....	0.3		
LBA1560: phosphoribosylaminoimidazole carboxylase; PurK.....	0.5	L: DNA replication, recombination, and repair	
LBA1745: guanylate kinase.....	0.4	LBA0273: TrcF.....	0.2
LBA1891: adenylosuccinate lyase.....	0.5	LBA0666: recombination protein; RecA.....	0.3
LBA1892: adenylosuccinate synthase.....	0.4	LBA0688: exinuclease ABC; UvrB.....	0.2
LBA1893: GMP reductase.....	0.3	LBA0689: exinuclease ABC; UvrA.....	0.2
G: carbohydrate transport and metabolism		LBA0740: [modification methylase].....	0.3
LBA0045: sugar transporter ^d	2.3	LBA0946: exinuclease ABC subunit C.....	0.5
LBA0144: N-acetylglucosamine-6-P deacetylase.....	2.0	LBA1077: chromosome segregation helicase.....	0.3
LBA0436: N-acetylglucosamine catabolic protein.....	0.4	LBA1122: DNA topoisomerase IV; subunit B.....	0.4
LBA0552: multidrug transporter ^d	0.4	LBA1123: DNA topoisomerase IV; subunit C.....	0.5
LBA0600: xylulose-5-phosphate/fructose phosphoketolase.....	2.1	LBA1145: phage integrase-recombinase.....	0.4
LBA0618: cellobiose-specific PTS system IIC; PtcD.....	0.5	LBA1243: single-stranded DNA-specific exonuclease; RecJ.....	0.2
LBA0716: phosphoglucomutase.....	0.5	LBA1420: transposase.....	0.4
LBA1376: transmembrane protein.....	0.4		
LBA1429: transporter ^d	3.0	M: cell envelope biogenesis, outer membrane	
LBA1433: dihydroxyacetone kinase.....	2.3	LBA0018: unknown.....	2.6
LBA1848: di/tripeptide transporter.....	0.5	LBA0177: autolysin; amidase.....	0.5
LBA1885: hypothetical protein ^d	0.5	LBA0234: UDP-N-acetylglucosamine 1-carboxyvinyltransferase.....	0.5
LBA1905: amino acid permease; AapA.....	0.4	LBA0462: glucosamine-fructose-6-phosphate aminotransferase.....	0.2
LBA1458: galactose-1-phosphate uridylyltransferase.....	3.1	LBA0514: surface layer protein.....	2.4
LBA1459: galactokinase.....	4.4	LBA0519: UDP-N-acetyl-D-mannosamine transferase.....	2.2
LBA1462: beta-galactosidase.....	2.8	LBA0520: galactosyltransferase.....	2.6
LBA1463: lactose permease.....	3.2	LBA0625: UTP-glucose-1-phosphate uridylyltransferase.....	0.5
LBA1467: beta-galactosidase large subunit.....	2.9	LBA0668: undecaprenyl-phosphate N-acetyl-glucosaminyltransferase.....	0.5
LBA1468: beta-galactosidase small subunit.....	2.1	LBA0708: UDP-N-acetylenolpyruvylglucosamine reductase; MurB.....	0.5
LBA1643: ABC transporter permease.....	0.4	LBA0765: UDP-N-acetylmuramyl tripeptide synthetase.....	0.3
LBA1644: ABC transporter permease.....	0.3	LBA0803: cell division protein; MraW.....	2.2
LBA1645: multiple-sugar ABC transporter.....	0.4	LBA1140: lysin.....	0.1
LBA1812: alpha-glucosidase II.....	3.0	LBA1244: sortase; SrtA.....	0.4
LBA1870: maltose phosphorylase.....	4.2	LBA1351: lysin.....	0.3
LBA1885: hypothetical protein ^d	0.5	LBA1733: phosphoglucosyltransferase; EpsE.....	0.5
H: coenzyme transport and metabolism		LBA1736: EpsB.....	0.5
LBA0055: D-lactate dehydrogenase ^d	2.1	LBA1743: cell wall-associated hydrolase.....	0.3
LBA0790: thiazole biosynthesis protein; ThiL.....	0.4	LBA1744: [glycosidase].....	0.2
LBA1232: apo-citrate lyase ^d	0.5	LBA1757: hypothetical protein.....	0.5
LBA1974: pyruvate oxidase ^d	2.6	LBA1758: hypothetical protein.....	0.5
		LBA1829: glucose-inhibited division protein B.....	0.5
		LBA1883: probable NLP/P60 family secreted protein.....	0.4
		LBA1918: lysin Lj965 phage.....	0.3

Continued on following page

TABLE 4—Continued

COG functional classification, gene, and gene product ^b	Ratio ^c	COG functional classification, gene, and gene product ^b	Ratio ^c
N: cell motility and secretion		LBA1731: hypothetical protein.....	0.5
LBA0176: <i>N</i> -acetylmuramidase ^d	0.4	LBA1759: ABC transporter; ATPase.....	0.5
O: posttranslational modification, protein turnover, chaperones		LBA1869: beta phosphoglucomutase; PgmB.....	3.4
LBA0083: HtrA.....	4.8	LBA1885: hypothetical protein ^d	0.5
LBA0283: ClpP ATPase.....	0.4	LBA1903: glutamine amidotransferase.....	0.5
LBA0278: FtsH cell division protein.....	0.5	LBA1943: [lipoprotein A/antigen precursor].....	0.4
LBA0388: [glycoprotein endopeptidase].....	0.5	LBA1944: sugar ABC transporter ATPase.....	0.4
LBA0390: endopeptidase.....	0.5	LBA1952: hypothetical protein.....	0.5
LBA0405: cochaperonin; GroES.....	6.2	S: function unknown	
LBA0406: chaperonin; GroEL.....	6.1	LBA0084: hypothetical protein.....	3.0
LBA0638: ATP-dependent Clp protease; ClpE.....	5.9	LBA0122: hypothetical protein.....	2.3
LBA0642: negative regulator of genetic competence; MecA.....	2.1	LBA0207: hypothetical protein.....	0.4
LBA0847: ClpX.....	0.3	LBA0435: hypothetical protein.....	0.4
LBA0984: ATP-dependent protease; HlsV.....	0.5	LBA0555: myosin cross-reactive antigen.....	4.6
LBA0985: ATP-dependent protease; HlsU.....	0.5	LBA0624: hypothetical protein.....	0.5
LBA1247: heat shock protein; DnaK.....	2.7	LBA0714: hypothetical protein.....	0.5
LBA1248: cochaperonin; GrpE; Hsp70 cofactor.....	3.2	LBA0715: hypothetical protein.....	0.5
LBA1428: hypothetical protein.....	2.4	LBA1010: hypothetical protein.....	0.3
LBA1910: ATP-dependent protease; ClpE.....	3.9	LBA1119: [inner membrane protein].....	3.1
P: inorganic ion transport and metabolism		LBA1206: hypothetical protein.....	2.2
LBA0045: sugar transporter ^d	2.3	LBA1339: hypothetical protein.....	2.4
LBA0166: K ⁺ uptake protein.....	0.4	LBA1432: hypothetical protein.....	2.8
LBA0552: multidrug transporter ^d	0.4	LBA1628: hypothetical protein.....	2.9
LBA0641: hypothetical protein.....	3.6	LBA1940: hypothetical protein.....	0.3
LBA0904: outer membrane lipoprotein precursor.....	0.5	T: signal transduction mechanisms	
LBA0997: aluminum resistance protein.....	0.4	LBA0722: protein-tyrosine phosphatase.....	0.3
LBA1429: transporter ^d	3.0	LBA0746: response regulator.....	0.5
LBA1446: multidrug resistance protein ^d	7.3	LBA0747: histidine kinase.....	0.3
LBA1683: cation-transporting ATPase.....	3.6	LBA1430: histidine kinase.....	3.0
Q: secondary metabolite biosynthesis, transport and catabolism		LBA1431: response regulator ^d	3.0
LBA0644: hypothetical protein.....	0.5	U: intracellular trafficking, secretion, and vesicular transport	
LBA1422: pyrazinamidase/nicotinamidase; PnC.....	0.3	LBA0176: <i>N</i> -acetylmuramidase ^d	0.4
R: general function prediction only		LBA0673: preprotein translocase; SecA.....	0.4
LBA0045: sugar transporter ^d	2.3	LBA1290: signal recognition protein; Ffh.....	0.3
LBA0055: D-lactate dehydrogenase ^d	2.1	LBA1496: fibrinogen-binding protein.....	2.5
LBA0159: hypothetical protein.....	0.5	LBA1523: SpoIIJ family protein.....	0.4
LBA0336: thiazole monophosphate biosynthesis.....	0.5	LBA1654: surface protein.....	4.6
LBA0342: 2',3'-cyclic nucleotide 3'-phosphodiesterase.....	0.3	V: defense mechanisms	
LBA0346: DNA repair protein; Rada.....	0.2	LBA0751: membrane protein; VanZ.....	2.8
LBA0392: [dehydrogenase].....	0.5	LBA1006: penicillin-binding protein.....	0.4
LBA0552: multidrug transporter ^d	0.4	LBA1132: ABC transporter; ATP binding protein.....	2.0
LBA0612: transport protein.....	0.4	LBA1585: [ABC transporter; ATP-binding].....	0.4
LBA0635: transport protein.....	0.4	LBA1679: ABC transporter permease.....	5.3
LBA0705: hypothetical protein.....	0.5	LBA1680: ABC transporter ATPase.....	3.6
LBA0718: hydrolase (HAD family).....	0.5	LBA1821: ABC transporter ATPase.....	0.4
LBA0828: metallo-β-lactamase superfamily protein.....	0.5	LBA1822: ABC transporter ATPase.....	0.3
LBA0848: GTP-binding protein (ENG family).....	0.3	No COG	
LBA0949: [metallobeta-lactamase superfamily].....	0.5	LBA0019: hypothetical protein.....	2.3
LBA0971: O-linked GlcNAc transferase.....	0.4	LBA0222: unknown.....	2.2
LBA0950: oxidoreductase.....	0.5	LBA0493: aggregation promoting protein.....	0.2
LBA1192: [methyltransferase].....	0.5	LBA0512: hypothetical protein.....	2.2
LBA1310: dihydroxyacetone kinase.....	0.4	LBA0515: hypothetical protein.....	0.2
LBA1429: transporter ^d	2.7	LBA0695: unknown.....	0.4
LBA1446: multidrug resistance protein ^d	7.3	LBA0720: unknown.....	0.3
LBA1637: membrane protein.....	3.8	LBA1426: unknown.....	2.1
LBA1642: hypothetical protein.....	0.4	LBA1460: mucus binding protein precursor.....	2.7
LBA1676: amino acid ABC transporter; permease.....	0.5	LBA1612: fibrinogen-binding protein.....	0.5
		LBA1747: unknown.....	0.5
		LBA1852: [D-Ala ligase].....	2.0

^a Array ratios from three biological replicates and three technical replicates were averaged.^b Genes were classified according to the COG domain present in the encoded protein sequence.^c Ratio indicates relative mRNA ratio (0.5% oxgall/0% oxgall).^d ORF present in more than one COG.

these operons were induced in the presence of bile despite the catabolite repression effects afforded by the 2% (wt/vol) glucose present in MRS (6). Also upregulated were several general stress response genes, in agreement with induction data from other species (20, 27, 30). Finally, microarray analysis indicated the upregulation of some genes potentially involved in bacterial adhesion to intestinal cells, including LBA1460, a mucus binding protein precursor, and LBA1496, a putative

fibrinogen binding protein. Upregulation of several stress response and adhesion genes was confirmed using RT-QPCR. Expression of LBA1652, annotated as a mucus binding protein precursor, was analyzed using RT-QPCR and found to be upregulated almost 11-fold.

Among genes not differentially expressed upon exposure to bile were the two bile salt hydrolases characterized previously. In some species, including *Listeria monocytogenes* and *Lacto-*

FIG. 1. Distribution of ORFs within COG classifications. The black bars represent the numbers of upregulated ORFs (total, 78), and the gray bars represent downregulated ORFs (total, 168).

bacillus plantarum, these genes have been shown to play a role in bile tolerance. In *L. acidophilus* NCFM, knockout mutations of the two *bsh* genes showed no differences in survival in the presence of bile, despite encoding active enzymes that are able to deconjugate the specific bile salts GCA, GDCA, TCA, and TDCA, as well as glycochenodeoxycholic acid and taurochenodeoxycholic acid (24).

Operon configuration and annotations. A cluster of genes seemingly unrelated by function (LBA1425 to LBA1432) was significantly induced in the presence of bile, as indicated by the microarray experiments (ranging from 2.1- to 3.0-fold upregulation). To date, this group of genes has not been shown to be differentially expressed under any test conditions examined (2, 3, 6). This cluster was particularly interesting because it contained genes for a type IIIA HPK (LBA1430) and an RR of the OmpR family (LBA1431), both with uncharacterized functions (1). Its induction in the presence of bile indicated that this 2CRS may potentially regulate transcription under these conditions. LBA1425 and LBA1426 were annotated as a hypothetical protein and an unknown protein, respectively. Neither protein contained a COG that could give a clue to its function, although BLAST analysis showed that LBA1425 had 46% identity to a putative cell surface hydrolase in *L. plantarum* WCFS1. LBA1427 was annotated as a putative oxidoreductase but showed less than 30% similarity to oxidoreductases from other species. It had similarity to COG0656, aldo/keto reductases (E value, $4e-11$). LBA1428 showed homology (58% identity) to a predicted hypothetical protein in *Lactobacillus gasseri*, and it contained similarity to a COG of predicted redox proteins (E value, $3e-5$). LBA1429 was annotated as a transporter based on its similarity to the major facilitator superfamily COG. This protein showed homology to a multidrug transport protein (*L. plantarum* WCFS1; 46% identity). LBA1432, a hypothetical protein, showed similarity to the RelA/SpoT COG (E value, $5e-21$); however, this protein showed stronger similarity to COG2357, an uncharacterized protein domain of unknown function conserved in bacteria.

Because of their upregulation in the presence of oxgall and their physical proximity in the genome, ORFs LBA1425 to LBA1432 were analyzed as a potential cotranscript. Clone Manager Software (Scientific and Educational Software, Cary, NC) revealed a region of dyad symmetry downstream of LBA1425 with a Gibbs free-energy value of -14.9 kcal/mol, followed closely by a T-rich region (Fig. 2). According to de Hoon et al. (13), this free-energy value falls below the average predicted free-energy value for terminators in NCFM (-13.8 kcal/mol), indicating good potential for the area to form a *rho*-independent terminator in an RNA transcript. RT-PCR and Northern analysis were employed to functionally determine the length of transcription in this region. These experiments indicated that the cotranscription of genes in the region extended from LBA1432 through LBA1425, for an overall transcript length of 7 kb.

Mutant phenotypes. In order to investigate the roles of LBA1427 to LBA1432 in bile tolerance, deletion mutant strains were generated by excising internal fragments from each of the ORFs. There were no differences in morphology or growth rate in MRS between these mutant strains and the wild type (data not shown). Survival analysis of the strains in the presence of increasing concentrations of bile showed that the survival of four of the six mutant strains (those with mutations in LBA1429, -1430, -1431, and -1432) was significantly reduced compared to the wild type (Fig. 3). Surprisingly, two of the strains (LBA1427 and LBA1428 mutants) showed increased survival in the presence of bile.

As bile salts are major constituents of bile and are able to inhibit growth in lactobacilli and bifidobacteria (18), the survival of these strains was examined in the presence of 0.2% GDCA and 0.2% TDCA (Fig. 4). Survival was also examined in the presence of 0.2% GCA and TCA, but no differences between strains were seen. Again, strains with deletions in LBA1427 (oxidoreductase) and LBA1428 (hypothetical) showed increased recovery compared to the wild type. The LBA1432 mutant (hypothetical) showed slightly less recovery

FIG. 2. Organization of a bile-induced operon in *L. acidophilus* NCFM. The nucleotide sequence of the upstream promoter is shown, with the ribosomal binding site and -10 and -35 boxes in boldface type. Predicted *rho*-independent terminators are shown with the predicted free energy of folding. ORFs upregulated in the presence of bile are shown in gray. Putative annotations for each ORF are shown. Hypo., hypothetical.

than the wild type in both salts. Strains with mutations in LBA1430 (HPK) or LBA1431 (RR) showed reduced recovery on GDCA, whereas on TDCA, the RR mutant strain showed dramatically reduced recovery. Unexpectedly, the HPK mutant was not sensitive to TDCA.

The growth of the mutant strains was analyzed in a variety of inhibitory compounds, including four bile salts (Table 5). Generally, glycoconjugated bile salts were more inhibitory at lower concentrations than tauroconjugated salts. The RR mutant was the most sensitive overall to bile salts, while among the mutant strains, the LBA1427 (oxidoreductase) and LBA1428 (hypothetical) mutants were the least sensitive. The LBA1429

mutant strain (transporter) was more inhibited by cholic acid-based bile salts, but not deoxycholic acid-based salts, detergents, or NaCl. Growth of the HPK mutant was inhibited at slightly lower concentrations of Triton X-100, SDS, and NaCl than that of the wild-type strain.

Role of the 2CRS in operon transcription. Expression of the LBA1425-LBA1432 operon was analyzed by RT-QPCR in NCFM and both the HPK and RR mutants. Expression of the gene immediately upstream (LBA1432; hypothetical) and the gene immediately downstream (LBA1429; transporter) of the 2CRS was monitored. While induction of the operon in the presence of bile was not affected by the HPK mutation, the RR mutation

FIG. 3. Survival of early-log-phase *L. acidophilus* NCFM (●), NCK 1871 (Δ LBA1427; oxidoreductase) (○), NCK 1873 (Δ LBA1428; hypothetical) (▼), NCK 1875 (Δ LBA1429; transporter) (△), NCK 1877 (Δ LBA1430; HPK) (■), NCK 1879 (Δ LBA1431; RR) (□), and NCK 1881 (Δ LBA1432; hypothetical) (◆) on MRS agar plates with increasing concentrations of oxgall. The error bars represent the standard errors of the mean for triplicate counts.

FIG. 4. Percent recovery of early-log-phase cells on MRS agar supplemented with 0.2% GDCA and TDCA compared to MRS agar. Bars (left to right): *L. acidophilus* NCFM, NCK 1871 (Δ LBA1427; oxidoreductase), NCK 1873 (Δ LBA1428; hypothetical), NCK 1875 (Δ LBA1429; transporter), NCK 1877 (Δ LBA1430; HPK), NCK1879 (Δ LBA1431; RR), and NCK 1881 (Δ LBA1432; hypothetical). The error bars represent the standard errors of the mean for triplicate counts.

led to a significant increase in induction (Fig. 5). In the absence of bile, transcription of LBA1429 and LBA1432 in both mutant strains was not significantly different from that in the wild type, as determined by analysis of variance (SAS, Cary, NC) (data not shown).

DISCUSSION

Bile tolerance is an important aspect of survival for bacteria that habitat the intestinal tract. Examination of the transcriptome of *L. acidophilus* NCFM in the presence of bile showed the induction of 3.9% of the genome, including a previously uncharacterized eight-gene operon containing a 2CRS. Mutations of the HPK and RR genes led to a substantial decrease in recovery on MRS agar containing oxgall. Also within the operon, mutation of a transporter gene and a gene encoding a hypothetical protein led to decreased recovery in the presence of bile. Outside this operon, genes involved in general stress resistance, carbohydrate metabolism, and adhesion were significantly affected by the presence of bile in the cellular environment.

Analysis of a previously uncharacterized operon upregulated

in the presence of bile suggested that the 2CRS within it was concerned with induction of genes involved in response to bile. HPKs and RRs that are in close proximity typically act together in sensing and regulatory capacities. Analysis of these mutant strains on oxgall plates initially indicated that this 2CRS was no exception, with both the HPK and RR deletion mutant strains showing a marked decrease in recovery in the presence of increasing levels of oxgall. The increased survival and increased growth in the presence of the specific bile salt TDCA seen in the HPK mutant strain were surprising.

Some 2CRSs control their own expression, so the transcriptional effects of mutating the HPK and the RR were examined on genes in the operon in the presence and absence of oxgall. Mutation in the RR led to an enhanced level of induction, indicating that the RR acts in the capacity of a repressor of this operon. Since the majority of the differentially regulated genes in the genome (Fig. 1) were repressed in the presence of bile, the role of this RR in the bile transcriptome could be substantial. Mutation in the HPK had no effect on induction of the operon in the presence of bile. This observation, along with the differences in phenotype between the HPK and RR mutant

TABLE 5. Inhibitory concentrations of selected compounds on three independent cultures of *L. acidophilus* NCFM strains

Strain	GCA (μg/ml)	TCA (μg/ml)	GDCA (μg/ml)	TDCA (μg/ml)	TX-100 (% [vol/vol])	SDS (% [wt/vol])	NaCl (μg/ml)
NCFM	>9	>15	6	10	2.5	0.07	30
NCK1871 (oxidoreductase)	9	7	6	>10	2.5	0.07	30
NCK1873 (hypothetical)	9	13	6	>10	2.5	0.07	30
NCK1875 (transporter)	4	7	6	10	2.5	0.07	30
NCK1877 (HPK)	9	>15	4	10	1.0	0.05	25
NCK1879 (RR)	4	11	4	6	2.0	0.07	30
NCK1881 (hypothetical)	4	7	6	10	1.5	0.07	25

FIG. 5. Effects of 2CRS gene mutations on induction of LBA1429 (black bars) and LBA1432 (gray bars) in the presence of 0.5% oxgall. The error bars represent standard errors of the mean for four biological replicates.

strains, raises two possibilities regarding this 2CRS. The first is that these two proteins do not form a cognate 2CRS but that they interact with other 2CRS components in the genome to promote a transcriptional response. Through comparative analysis, Grebe and Stock (15) demonstrated a correlation between the sequence subfamilies of HPKs and RRs that were known to act cognately. Previous analysis of the 2CRS of NCFM (1) showed that LBA1430 is a type IIIA HPK. LBA1431 belongs to the OmpR subfamily of RRs, which type IIIA HPKs have been shown to interact with exclusively. The second possibility regarding this 2CRS is that the RR is activated independently of phosphorylation by its cognate HPK. There are several reports of 2CRSs in which acetyl phosphate acts as a phospho-donor to the RR in the absence of the HPK or where the HPK and acetyl phosphate act synergistically to promote activation (4, 12, 22, 23, 38). The biological purpose for this alternative activation is uncertain, but it has been shown to play a role in transcriptional control during acid stress (4). Since there are no orphan HPK genes present in the genome that might indicate RR cross talk, and sequence analysis indicates the high probability that LBA1430 and LBA1431 form a cognate 2CRS, it is likely that this RR was activated by another phosphate-donating member, such as acetyl phosphate, in the absence of its HPK. Since deletion of the HPK did result in a decrease in survival in the presence of oxgall, it is most likely that this protein does play a role in signal transduction. Also, since the 2CRS within the operon does not act to induce it in the presence of bile, this single system is most likely a player in a more complex regulatory network that controls bile-influenced transcription. Experiments to examine the global transcriptional effects of both the HPK and the RR mutations in the presence of bile are in progress.

In addition to the 2CRS, other genes were functionally shown to play a role in bile tolerance. Decreased survival of the

LBA1429 mutant in bile and growth inhibition by the cholic acid-based bile salts, but not salt or detergents, supports the hypothesis that this transporter interacts with bile salts. Transport proteins, including those belonging to the major facilitator superfamily, have been shown to act in bile salt transport in other species, including *E. coli*, *L. monocytogenes*, *L. plantarum*, and *Eubacterium* sp. (14, 21, 32, 34). Such transporters have been shown to act in various capacities in species, including influx, efflux, and antiport, and are believed to both import salts for modification and export them to prevent their deleterious effects. The differences in the roles of the transporters can lead to varying effects of mutations on bile tolerance. The exact role that LBA1429 plays is unclear from these experiments, although the LBA1429 mutant strain's lowered growth inhibition on GCA and TCA suggest interaction with cholic acid. These experiments introduce the first transporter gene in NCFM that has been implicated in bile tolerance. Sequence analysis of hypothetical protein LBA1432 does not suggest a role that it plays in bile tolerance, although weak similarity to RelA/SpoT domains might suggest that the protein is associated with gene regulation in times of stress or starvation (10). Increased recovery of LBA1427 (oxidoreductase) and LBA1428 (hypothetical) mutant strains in the presence of bile, compared to the wild type, was surprising considering that these genes are members of an operon induced by bile. Since both proteins contain homology to oxidoreductases, they could play a role in bile salt modification, as it is known that some species of intestinal bacteria possess the ability to modify bile salts through oxidation and reduction (7, 9). These proteins may act as previously unrecognized bile salt modifiers, although a role for modifiers of this type in bile tolerance or sensitivity has not been established. Many bacterial interactions with bile, such as those that lead to modification of bile salts, are poorly understood. It is possible that these proteins

play an important role with bile in the gastrointestinal system but that any effects cannot be observed in vitro. Induction of the operon, along with the repression by LBA1431 (RR), could indicate a need for fine control of these genes and suggest that these proteins provide a necessary benefit to the cell.

Other findings from the microarray experiments showed up-regulation of genes involved in carbohydrate metabolism but downregulation of genes involved in many other aspects of growth, indicating that bile slows growth in the cells. The induction of the lactose and galactose operons was an intriguing finding, as this strain is widely utilized in yogurt and other fermented dairy products. It might be speculated that as some lactobacilli evolved in the small intestines of mammals, a frequently encountered energy source was lactose from milk. In the gastrointestinal tract, bile may act as an environmental signal for the cells to increase production of proteins needed to metabolize this energy source. This finding could suggest that consumption of this *Lactobacillus* strain in dairy foods might promote survival in the gastrointestinal tract, as it is better prepared to metabolize those available carbohydrates.

Bile induction of potential adhesion genes was also identified by microarray experiments and confirmed using RT-QPCR. Adhesion of bacterial cells to intestinal cells is considered an important attribute for lactobacilli in the gastrointestinal tract (8, 35). Induction of such adhesion determinants suggests that bile may act to signal arrival in the intestinal tract. While bile's effect on bacterial cells has been indicated as largely detrimental, induction of these genes could indicate a new role for bile as a location indicator in the cellular environment.

Examination of the global transcriptional effects of bile led to the discovery of an inducible operon that encodes proteins involved in both bile tolerance and, surprisingly, sensitivity. This duality illustrates the complexity of transcriptional regulation and reflects how little bile tolerance is currently understood. A 2CRS that aids bile tolerance is a new finding. The fact that *L. acidophilus* NCFM utilizes one of its nine 2CRSs in bile tolerance shows the importance of this characteristic in species that travel through the intestinal tract.

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