APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2007, p. 3924–3935 Vol. 73, No. 12
0099-2240/07/$08.00+0 doi:10.1128/AEM.01502-06

The Early Response to Acid Shock in *Lactobacillus reuteri* Involves the ClpL Chaperone and a Putative Cell Wall-Altering Esterase▽†

Torun Wall,[1] Klara Båth,[1] Robert A. Britton,[2] Hans Jonsson,[1] James Versalovic,[3] and Stefan Roos[1]*

Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-750 07 Uppsala, Sweden[1]; Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824[2]; and Department of Pathology, Baylor College of Medicine and Texas Children's Hospital, 6621 Fannin St., MC 1-2261, Houston, Texas 77030[3]

Received 29 June 2006/Accepted 16 April 2007

To be able to function as a probiotic, bacteria have to survive the passage through the gastrointestinal tract. We have examined survival and gene expression of *Lactobacillus reuteri* ATCC 55730 after a sudden shift in environmental acidity to a pH close to the conditions in the human stomach. More than 80% of the *L. reuteri* cells survived at pH 2.7 for 1 h. A genomewide expression analysis experiment using microarrays displayed 72 differentially expressed genes at this pH. The early response to severe acid shock in *L. reuteri* differed from long-term acid adaptation to milder acid stress studied in other lactic acid bacteria. The genes induced included the following: *clpL*, genes putatively involved in alterations of the cell membrane and the cell wall; genes encoding transcriptional regulators; phage genes; and genes of unknown function. Two genes, *clpL*, encoding an ATPase with chaperone activity, and lr1516, encoding a putative esterase, were selected for mutation analyses. The mutants were significantly more sensitive to acid than the wild type was. Thus, these genes could contribute to the survival of *L. reuteri* in the gastrointestinal tract.

Lactobacillus reuteri is a commensal lactic acid bacterium that commonly inhabits the gastrointestinal tract of humans as well as of animals. No pathogenic properties have been linked to this species; instead strains of *L. reuteri* have been suggested to possess health-promoting, i.e., probiotic, properties (52, 60). To be able to function as a probiotic, bacteria have to survive the passage through the gastrointestinal tract. When entering the stomach, the environment changes rapidly and the bacteria are suddenly exposed to stress factors, such as various enzymes and an extremely acidic pH. Each day, approximately 2.5 liters of gastric juice, containing hydrochloric acid, is produced in the human stomach. Consequently, the fasting gastric pH is approximately 1.5, and the feeding pH is between 3.0 and 5.0 (14).

Several bacterial species are unable to survive the harsh conditions in the stomach, while others can survive the passage by using different defense mechanisms. These mechanisms often involve changes in gene expression and phenotype. A number of strategies to adapt to acidic environments have been reported in gram-positive bacteria; these strategies include pumps removing protons from the cytoplasm, such as the F_1F_o ATPase proton pump or the glutamate decarboxylase system; production of general stress proteins and chaperones to repair and stabilize proteins and DNA; altered metabolism and cell envelope composition; and alkalization of the external environment, for example, with urease or through the arginine deiminase (ADI) pathway (reviewed in references 14, 17, and 57).

* Corresponding author. Mailing address: Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-750 07 Uppsala, Sweden. Phone: 46 (0)18 67 33 82. Fax: 46 (0)18 67 33 92. E-mail: stefan.roos@mikrob.slu.se.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 20 April 2007.

The aim of this study was to examine the response of *L. reuteri* ATCC 55730 after a shift in environmental acidity to a pH close to the conditions in the human stomach. Survival of *L. reuteri* after a transfer from pH 5.1 to pH 2.7 was monitored, and the early changes in gene expression were measured with oligonucleotide DNA microarrays. In this analysis, approximately 70 genes were differentially expressed in the acidic pH. The induced genes included the stress response gene *clpL* and genes encoding putative cell envelope-altering proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Lactobacillus reuteri* ATCC 55730 (earlier named SD2112), a strain isolated from mother's milk was grown in de Man-Rogosa-Sharpe (MRS) broth (Oxoid) at 37°C (unless stated otherwise) in plastic tubes, in which anaerobic conditions were obtained within 1 hour as indicated by an anaerobic indicator (BD). For acid shock experiments, the pH of the MRS broth was adjusted with hydrochloric acid (HCl). When solid growth medium was utilized, *L. reuteri* was grown on MRS agar (Oxoid) and incubated at 37°C (unless stated otherwise) under anaerobic conditions obtained with anaerobic system envelopes (GasPak; BD). When required, antibiotics were added (erythromycin, 5 μg ml^{-1} [Sigma]; chloramphenicol, 7.5 μg ml^{-1} [Sigma]). *Escherichia coli* was grown at 37°C in Luria-Bertani broth (LB) (51). When required, antibiotics were added (erythromycin, 400 μg ml^{-1} [Sigma]; kanamycin, 40 μg ml^{-1} [Sigma]).

Acid shock experiments. For acid shock experiments, MRS at pH 5.8 was inoculated with an overnight culture of *L. reuteri* to an optical density at 600 nm (OD_{600}) of 0.005, and the cells were grown at 37°C to an OD_{600} of 1.0, i.e., late exponential phase. At this OD_{600}, the pH of the culture was 5.1. To lower the pH, the cells were diluted 1:10 in prewarmed MRS at pH 2.3, which yielded a final pH of 2.7 (referred to hereafter as acid-treated cells). For a control, cells were diluted 1:10 in prewarmed MRS at pH 5.1 (referred to hereafter as treated control cells). For a reference for the diluted samples, untreated cells were collected at time zero (referred to hereafter as untreated cells). For expression analyses, transcription was stopped after 5 and 15 min at 37°C by addition of 2/3 volume of ice-cold methanol. The cells were then harvested by centrifugation at 4,000 × *g* for 10 min, the supernatant was decanted, and the pellets were stored at −70°C and later utilized for isolation of RNA. To monitor survival of *L. reuteri*, untreated cells (time zero) and cells diluted in MRS at pH 2.3 or MRS at pH 5.1 were plated on MRS agar after 5, 15, 30, and 60 min ($n = 3$). The plates were incubated under anaerobic conditions for 48 h.

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or primer	Description or primer sequence[a]	Reference or source
Strains		
E. coli EC1000	Strain harboring a copy of the pWV01 *repA* gene in the chromosome; Kan^r	Used by the kind permission of J. Kok, University of Groningen (29a)
L. reuteri ATCC 55730 (earlier named SD2112 or MM53)	Isolated from mother's milk	Used by the kind permission of Biogaia AB, Sweden
L. reuteri ATCC 55730 *clpL* mutant	Variant with a mutated *clpL*	This study
L. reuteri ATCC 55730 lr1516 mutant	Variant with a mutated lr1516	This study
L. reuteri ATCC 55730(pORI28)	Variant harboring both pVE6007 and pORI28	This study
Vectors and constructions		
pVE6007	Cm^r *repA*-positive temperature-sensitive derivative of pWV01	Used by the kind permission of E. Maguin, INRA (34a)
pORI28	Em^r *repA*-negative derivative of pWV01	Used by the kind permission of J. Kok, University of Groningen (29a)
pORI28-lr1864	pORI28 + 738-bp insert from lr1864 (*clpL*)	This study
pORI28-lr1516	pORI28 + 558-bp insert from lr1516	This study
Primers (purpose)		
pORI28-b (detection of pORI28 + insert)	5′-TTGTTGTTTTTATGATTACAAAGTGA-3′	This study
pORI28-e (detection of pORI28 + insert)	5′-TTGGTTGATAATGAACTGTGCTG-3′	This study
pVE6007-p (detection of pVE6007)	5′-GTTTTCCCAGTCACGACGTT-3′	This study
pVE6007-s (detection of pVE6007)	5′-GGCCGCTCTAGAACTAGTGGA-3′	This study
lr1864-f (generation of gene fragment)	5′-TGACTGGATCCTAATCAATTAATGGGTGGCATGA-3′ (starts at position 54)	This study
lr1864-r (generation of gene fragment)	5′-TGACTGAATTCGCCTTGCTTGGAGCATTAAC-3′ (starts at position 791 [rev.])	This study
lr1864-cf (detection of insertion)	5′-ATGGCTCAAAACCCAATGAA-3′ (starts at position 1)	This study
lr1864-cr (detection of insertion)	5′-AGCAGCTTGGAGAACGTCAT-3′ (starts at position 879 [rev.])	This study
lr1516-f (generation of gene fragment)	5′-TGACTGGATCCTAATGATCATCAACATACGATTAAGGAA-3′ (starts at position 84)	This study
lr1516-r (generation of gene fragment)	5′-TGACTGAATTCTCCTCCACATACATTTTCGTA-3′ (starts at position 410 [rev.])	This study
lr1516-cf (detection of insertion)	5′-TGGTTAAAGAGGGAGTTGTTCC-3′ (starts at position 41)	This study
lr1516-cr (detection of insertion)	5′-CATCCGCATATTTGATTTGG-3′ (starts at position 442 [rev.])	This study
469F3 (RT-PCR)	5′-GGTGGATTGGAGAATTACTCTTTATTTG-3′ (starts at position 184)	This study
469R3 (RT-PCR)	5′-CCTACTTCTCCTTGTTTACTCCATTCA-3′ (starts at position 314 [rev.])	This study
RTs0501F (RT-PCR)	5′-ACCACGGCGAACGTTTGA-3′ (starts at position 132)	This study
RTs0501R (RT-PCR)	5′-GTTGAAACACCCCTGATTTTTC-3′ (starts at position 184 [rev.])	This study
RTs0537F (RT-PCR)	5′-AGCAGCGATTACTGGTGCAGTT-3′ (starts at position 33)	This study
RTs0537R (RT-PCR)	5′-ACCTGCTTGCACCGTGTAAATACT-3′ (starts at position 120 [rev.])	This study
RT819F (RT-PCR)	5′-TATTGCTGCTGGGCCTGATC-3′ (starts at position 411)	This study
RT819R (RT-PCR)	5′-AAGCGTCAAGGTTATTTTCAACTTGT-3′ (starts at position 520) [rev.])	This study
RTs0858F (RT-PCR)	5′-CAGCATCATACCTTAACGGTGACTACT-3′ (starts at position 476)	This study
RTs0858R (RT-PCR)	5′-TTTTGCCATGAACCGTAACGA-3′ (starts at position 566) [rev.])	This study
RTs1797F (RT-PCR)	5′-CCCTTGAGCTCGATCGGTTA-3′ (starts at position 212)	This study
RTs1797R (RT-PCR)	5′-CCAAATACACCAGCATCATTTTT-3′ (starts at position 296) [rev.])	This study
1864F (RT-PCR)	5′-AATGGGTGGCATGAATGGTTT-3′ (starts at position 60)	This study
1864R (RT-PCR)	5′-TGGCAATTGACCAGTTCTACGATA-3′ (starts at position 168 [rev.])	This study
RTs1879F (RT-PCR)	5′-CTGAAAAGAAACTTGATTGGTTCCA-3′ (starts at position 155)	This study
RTs1879R (RT-PCR)	5′-TGCGATTACAACTTCATCCTTTGA-3′ (starts at position 231 [rev.])	This study
RTs2076F (RT-PCR)	5′-GCAGCGGCAGATGATTTGTT-3′ (starts at position 244)	This study
RTs2076R (RT-PCR)	5′-ACGCCGGCAATTGTTGAA-3′ (starts at position 308 [rev.])	This study

[a] Underlined nucleotides in the primer sequences indicate inserted restriction enzyme cleaving sites (BamHI and EcoRI), and nucleotides underlined twice indicate inserted stop codons. The positions where the sequences start are indicated in parentheses. rev., reverse.

Isolation of RNA. The cells from the acid shock experiments were suspended in STE (6.7% sucrose, 50 mM Tris [pH 8.0], 1 mM EDTA), harvested by centrifugation, and resuspended in STE. After transfer to tubes with Lysing Matrix B (Qbiogene), the cells were disrupted in a Fast prep instrument (Qbiogene) at the speed setting of 6.0 for 40 seconds. The tubes were centrifuged for 5 min at 16,000 × *g* at 2°C, and the supernatant was used for isolation of RNA with QIAGEN RNeasy kit. DNA was removed on the columns with QIAGEN RNase-free DNase.

Construction of an *L. reuteri* microarray. Long oligonucleotides (60-mers) were designed and constructed for 1,864 open reading frames from a draft genome sequence of *L. reuteri* ATCC 55730 (5) and 15 open reading frames encoding known extracellular proteins from *L. reuteri* DSM 20016 (58) using OligoArray 1.0 software. Six control 60-mer oligonucleotides were also included. These controls are identical to DNA sequences from *E. coli* genes (*yacF*, *ybaS*, *yciC*, *yfiF*, *ygjU*, and *yjcG*) and have no sequence similarity to genes in the *L. reuteri* genome. Once the oligonucleotide was synthesized, oligonucleotide concentrations were normalized to a concentration of 25 μM and spotted onto Corning UltraGAPS-II slides using an OmniGrid robot (GeneMachines). Each gene was represented once on the microarray. All six of the control spots were represented eight times on the array, once in each subgrid. Oligonucleotide design and synthesis and array construction were performed at the Research Technology Support Facility at Michigan State University, East Lansing.

Oligonucleotide microarray experiment. The design of the microarray experiment is presented in Fig. 1. The experiment was performed as direct comparisons between untreated, acid-treated, and treated control cells. Four biological replicates were performed with dye swaps for the 5-min samples and three biological replicates with dye swaps for the 15-min samples. For synthesis of cDNA, 5 to 10 μg of RNA in a volume of 12.8 μl was mixed with 5 μl (0.5 μg μl^{-1}) random hexamers (QIAGEN). After incubation at 70°C for 10 min, 1.5 μl Superscript III (200 U μl^{-1}) (Invitrogen), 0.5 μl RnaseOut (40 U μl^{-1}) (Invitrogen), and 1.2 μl nucleotides (12.5 mM dATP, 12.5 mM dCTP, 12.5 mM dGTP, 7.5 mM dTTP [Invitrogen], 5 mM aminoallyl dUTP [Sigma]) were added.

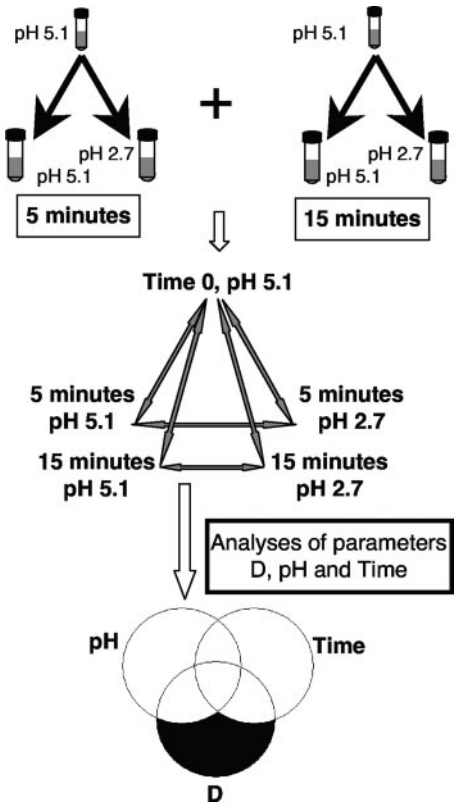

FIG. 1. Experimental design for the gene expression studies. Cells of *L. reuteri* ATCC 55730 were diluted into MRS broth at pH 2.7 or MRS broth at pH 5.1 and incubated for 5 or 15 min. Direct microarray comparisons were performed for both time points between untreated cells at time zero, acid-treated cells at pH 2.7, and the treated control cells at pH 5.1. Four biological replicates were performed for 5-min incubation, and three biological replicates were performed for 15-min incubation. The data were analyzed with an empirical Bayes approach with respect to the three parameters pH, time, and dilution (D). Genes primarily affected by dilution were excluded from further studies.

The reverse transcription reaction was performed at 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. To remove RNA, 15 μl of 0.1 M NaOH was added, and the samples were incubated at 70°C for 10 min. After neutralization by the addition of 15 μl of 0.1 M HCl, the cDNA was purified with the QIAGEN PCR mini kit. Next the cDNA was labeled with Cy3 or Cy5 (CyDye postlabeling reactive dye pack; Amersham) for 1 h in the dark, and the labeled cDNA for each comparison was combined and purified again by using the QIAGEN kit. To the combined sample, 1 μg salmon testes DNA (Sigma) and 1 μg yeast tRNA (Sigma) were added. After incubation at 100°C, formamide buffer (50% formamide, 10× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]) was added to the sample, and the samples were applied to a microarray slide, which had been prehybridized in prehybridization buffer (10 g liter^{-1} bovine serum albumin, 5× SSC, 0.1% SDS) for 45 min, washed in water, and dried by centrifugation at 150 × *g*. The slide was hybridized at 42°C overnight in the dark. The slides were washed first in 2× SSC plus 2.5% SDS and 0.2× SSC plus 2.5% SDS and then repeatedly in 0.2× SSC. Finally, the microarray slides were scanned with a GenePix 4000B scanner (Amersham Pharmacia Biotech).

Microarray and bioinformatic analyses. GenePix Pro 4.0.12 software was utilized for image analysis of the microarrays. The raw data of spots were stored and analyzed using BASE (50), a database system for analyses of microarray data, which had been modified at the Linneaus Centre of Bioinformatics, Uppsala University, Sweden. Statistical analysis was performed in the analysis environment of Linneaus Centre of Bioinformatics Data Warehouse (http://www.lcb.uu.se./lcbdw.php) using LIMMA package in R (http://www.r-project.org) from Bioconductor (24). First, systematic variation was removed by print-tip lowess normalization (63) of the data sets. Genes represented by spots of low quality on more than 15% of the arrays were excluded from further analysis. Using LIMMA, a linear model was fit to the data in order to estimate the effects of pH, time, and dilution (Fig. 1). The genes were then ranked by a parametric empirical Bayes approach (33) to find the genes most likely to be differentially expressed. The cutoff values for the posterior log odds ratio (the B score) were set to 20 for pH, −2.5 for time, and 10 for dilution, since these values isolated distinct subgroups of genes, and for M (i.e., mean $\log_2$ ratio) for pH and dilution, respectively, to a $\|M\|$ of >0.75. In addition, genes that changed less than twofold after 5 or 15 min in the expression ratio between pH 2.7 or pH 5.1 and the untreated cells at time zero were excluded from further analyses. Of the control spots, one gene (*yacF*) indicated expression on all slides and was not regarded in the analyses. The remaining control spots gave signals of low intensity and were used for estimation of background signals. Genes corresponding to spots of low intensity on the majority of the slides were excluded from the analyses. The function of previously uncharacterized proteins, encoded by the selected genes, were predicted with COG (Clusters of Orthologous Groups of proteins) (55, 56) and pfam (4) identified using BlastP (2) at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/). Signal peptides were searched with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (7, 40, 41) for gram-positive bacteria, and transmembrane helices were searched with TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (28).

Real-time RT-PCR experiment. To validate microarray data, the expression of eight genes was analyzed with real-time reverse transcription-PCR (RT-PCR). Genes with various gene expression ratios 15 min after acid shock or genes in the treated control were selected: lr0501 (EF534265; Spo0J-like protein), lr0537 (Apf-like protein), lr0858 (Apf-like protein), *hpk3* (lr0819, encoding a histidine sensor kinase), lr1797 (putative phosphatidylglycerophosphatase), lr1864 (ClpL, ATPase with chaperone activity), lr1879 (PduD, dehydratase medium subunit) and lr2076 (transcriptional regulator). As a reference gene, lr0469 (DQ074824; SecY, protein translocase subunit), a gene exhibiting high signals on the microarrays but not displaying any changes in expression was used. Primers, which are presented in Table 1, were designed with Primer Express Software v2.0 (Applied Biosystems). After an extra DNase treatment, cDNA was constructed from 5 ng RNA and RNA removed with NaOH as described above. The cDNA was stored at −20°C. Real-time RT-PCR mixtures contained 12.5 μl Power SYBR green PCR master mix (Applied Biosystems), 4 μl diluted cDNA, 80 μM of each primer, and distilled H_2O to a final volume of 25 μl. The PCR was performed at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 0.15 min and 60°C for 1 min in an ABI Prism 7000 sequence detection system (Applied Biosystems). Three biological replicates were analyzed using three technical replicates for each sample. For relative quantification, a calibration curve with serial dilutions (0.0626, 0.25, and 1 ng) of *L. reuteri* ATCC 55730 chromosomal DNA for each primer pair was generated. The results were evaluated by the method of Fredlund et al. (22). Briefly, the efficiency of cDNA amplification was calculated by the method of Wilhelm and Pingoud (61). The changes in expression between acid-treated cells or the treated control and untreated cells (time zero) were calculated by comparing the relative gene expression of the target gene to the reference gene by the method of Pfaffl (45).

Construction and analyses of a *clpL* mutant and an esterase mutant. Two genes from the expression analyses were selected for construction of disruption mutants: *clpL*, which is similar to ATPases involved in stress response, and a putative esterase, strongly up-regulated at pH 2.7. Plasmids and primers used are listed in Table 1. The mutants were constructed principally by the method described by Walter et al. (59). The genes were inactivated by site-specific integration of plasmid pORI28 into the *L. reuteri* ATCC 55730 chromosome by using the temperature-sensitive plasmid pVE6007 as a helper plasmid. Internal fragments of the genes were amplified by PCR (using the insertion primers in Table 1) and cloned into pORI28 (using the EcoRI and BamHI sites) using *E. coli* EC1000 as the host. The helper plasmid pVE6007 was electrotransformed into *L. reuteri* ATCC 55730 by the protocol of Ahrné et al. (1). The phenotypic expression and incubation were performed at 35°C. In the next step, pORI28 with the insert was transformed using the same method. Bacteria carrying both plasmids were grown overnight at 35°C in the presence of erythromycin and chloramphenicol. Fifty microliters of this culture was inoculated into 10 ml of prewarmed (44°C) MRS broth without antibiotics. After incubation for 8 h at 44°C, bacteria were plated on prewarmed MRS agar plates containing erythromycin and incubated at 44°C overnight. Clones lacking pVE6007 and possessing an integrated pORI28 were isolated by replica plating on MRS plates containing either erythromycin or chloramphenicol. The integration of the pORI28 plasmids into target genes was checked by PCR using primers flanking the target

region (detection primers in Table 1) and primers flanking the multicloning site of pORI28 (Table 1).

In order to test the survival at low pH, the *L. reuteri* ATCC 55730 *clpL* mutant, esterase mutant, and wild type were grown overnight at 37°C in MRS. The bacteria were diluted to an OD_{600} of 0.1 in 10 ml prewarmed MRS and incubated at 37°C until an OD_{600} of 1.0 was reached. After addition of 10 μl culture to 10 ml synthetic stomach juice (8.3 g liter^{-1} proteose peptone [Oxoid], 3.5 g liter^{-1} glucose, 2.05 g liter^{-1} NaCl, 0.6 g liter^{-1} KH_2PO_4, 0.11 g liter^{-1} $CaCl_2$, 0.37 g liter^{-1} KCl, adjusted to pH 2.0 with HCl, a modification from the synthetic stomach juice of Cotter et al. [13] lacking enzymes and bile), the tubes were incubated at 37°C, and samples were removed after 20 and 50 min. Samples were also taken before addition to the synthetic stomach juice. The samples were diluted in phosphate-buffered saline and spread on MRS plates, which were incubated anaerobically for 24 h at 37°C. The experiment was repeated on three occasions, and duplicate samples were analyzed each time. The differences between the values for the mutant and wild-type strains were tested statistically by Student's *t* test.

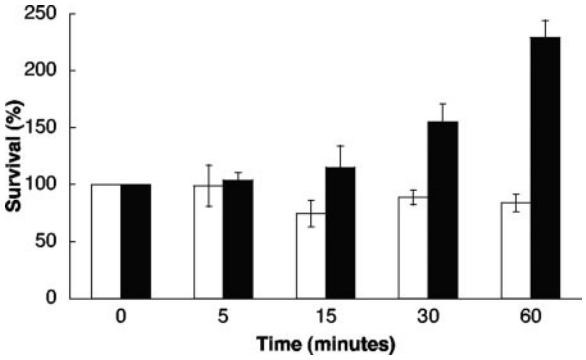

FIG. 2. Survival of *L. reuteri* ATCC 55730 at pH 2.7 (white bars) and at pH 5.1 (black bars). The percentage of cells surviving compared to the number of cells at the start of the experiment ($n = 3$) is shown on the *y* axis. Error bars represent standard deviations.

RESULTS

Survival of *L. reuteri* after acid shock. The survival of *Lactobacillus reuteri* ATCC 55730 after a sudden acid shock is presented in Fig. 2. More than 80% of the cells treated with acid survived at pH 2.7 for 1 h. After 2 h, approximately 60% of the cells were viable (data not shown). In the control, the cells started to grow within 30 min, and after 1 h, the number of cells had more than doubled (Fig. 2).

Gene expression analyses. Since dilution likely would affect the gene expression as well, direct comparisons between untreated cells, treated control cells (diluted into MRS at pH 5.1), and acid-treated cells (diluted into MRS at pH 2.7) were performed. The effect of dilution was estimated using a linear model, and consequently, only genes affected by dilution could be separated. Using the parametric empirical Bayes approach (33), which can account for multiple variables, three parameters, pH, time, and dilution, were considered (Fig. 1). The B score cutoff resulted in 343, 248, and 291 selected genes for the pH, time, and dilution parameters, respectively. Genes affected only by the dilution parameter were not further analyzed. To reduce the number of genes and to isolate strongly affected genes, a cutoff value for *M* was set at a $\|M\|$ of >0.75 for pH. From the statistical ranking of the pH and time parameters, genes with at least a twofold difference in expression after 5 or 15 min compared to the untreated cells were selected. This resulted in 72 differentially expressed genes at pH 2.7 (Table 2). Of these, 34 genes were induced and 38 were repressed.

In addition, 85 of the genes affected by the pH or time parameter, changed expression more than twofold in the treated control (i.e., after dilution in MRS at pH 5.1) compared to the untreated cells, i.e., they were also affected by dilution (see Table S1 in the supplemental material). A selection of these genes is presented in Table 3. Six of these genes were identical to the genes in Table 2. For example, a gene encoding a putative esterase, lr1516, was induced at pH 2.7 but repressed in the treated control. However, the majority of the genes in Table 3 were not affected or only displayed minor changes at pH 2.7 compared to untreated cells. Although affected by both the pH and dilution parameters, for this group of genes, the effect of the lowered pH counteracted the effect of dilution. These genes were consequently higher or alternatively, lower, expressed at pH 2.7 in comparison to the treated control (Table 3). This can be considered an indirect effect of the lowered pH on these genes.

Expression profiles. The predicted cellular functions of the genes in *L. reuteri* ATCC 55730 that directly or indirectly changed expression due to the acidic pH are presented in Fig. 3. Of the genes induced at pH 2.7, several were encoding regulatory, phage-associated, or metabolic proteins. The gene *clpL* (lr1864), which encodes an ATPase with putative chaperone activity, was also up-regulated at pH 2.7. Further, two genes belonged to COG (55, 56) classes related to lipid metabolism. These were encoding a putative deoxyxylulose-5-phosphate synthase (lr0811) and a putative phosphatidylglycerophosphatase (lr1797). The induced genes also included genes potentially involved in cell envelope biogenesis. The protein encoded by lr2064 was a putative endopeptidase, similar (35% identity) to enterolysin A from *Enterococcus faecalis*. Enterolysin A is a bacteriocin with cell wall-degrading ability (42). In addition, lr1516, which was strongly induced (more than sixfold) at pH 2.7, encoded a putative esterase belonging to a class of penicillin-binding proteins (beta-lactamase family class C) according to COG. The functions of several genes induced at the low pH were unknown, although for some of them, a general function was predicted. The gene lr0997 encoded a conserved membrane protein that is widespread in bacteria. This gene also exhibited similarities with the *gdmH*/*epiH* genes found in species such as *Staphylococcus gallinarum* and *Staphylococcus epidermidis*.

In contrast to the genes induced at pH 2.7, the majority of the genes repressed at the same pH could be assigned a function. Interestingly, one of the down-regulated genes, lr0021, was similar to *relA*. In gram-positive bacteria, RelA is involved in both synthesis and hydrolysis of ppGpp, a signal molecule known to be involved in the stringent response and induction of tolerance against different types of stress (37, 48). Furthermore, genes involved in transport and metabolism, energy conversion and production; cell division proteins; genes involved in replication, regulation, translation, ribosome biogenesis or posttranslational modification, and the translocational subunit YajC were down-regulated (Fig. 3).

The genes changing expression at pH 2.7 clearly belonged to other classes than those differentially expressed in the

TABLE 2. *Lactobacillus reuteri* genes induced or repressed during acid shock at pH 2.7

Gene and function	Accession no.[a]	Description of encoded protein[b]	Expression ratio[c]		Mean *M* pH[d]
			5 min	15 min	
Genes up-regulated at pH 2.7					
Stress response					
lr1864	DQ219976	ClpL ATPase with chaperone activity	N	++	1.1
lr1797	DQ219975	Phosphatidylglycerophosphatase A and related proteins	N	++	1.0
Regulation and signal transduction					
lr1474	DQ219969	Rrf2 transcriptional regulator	++	+++	1.3
lr1468	DQ219968	Transcriptional regulator	N	+++	0.83
lr1933	DQ219977	Transcriptional regulator	N	++	0.91
lr1993	DQ219980	Transcriptional regulator	N	+++	*T*[e]
lr2076	DQ219989	Transcriptional regulator	N	++++	*T*
rr7 (lr1804)	DQ219942	Two-component signal transduction response regulator	N	++	0.78
Transport and metabolism					
lr1028	DQ219961	6-Phosphogluconolactonase/glucosamine-6-phosphate isomerase/deaminase	N	++	1.3
lr0811	DQ219956	Deoxyxylulose-5-phosphate synthase	N	++	0.87
Translation and posttranslational modifications					
lr0807	DQ219955	Cysteinyl-tRNA synthetase	N	++	0.90
lr0597	DQ219952	Thioredoxin domain-containing protein	N	++	*T*
Cell envelope biogenesis					
lr2064	DQ219988	Endopeptidases/phage-associated protein	N	++	0.83
lr1516	DQ219970	Putative esterase	++	++++	3.2
Phage-associated genes					
lr1463	DQ074903	Phage terminase-like protein, large subunit	++	+++	1.4
lr2045	DQ219981	Phage-associated protein	N	++	1.1
lr2047	DQ219982	Phage-associated protein	N	++	1.1
lr2051	DQ219983	Phage terminase	N	++	0.82
lr2054	DQ219984	Phage head maturation protease	++	++	1.4
lr2055	DQ219985	Phage ϕC31 gp36 major capsid-like protein	N	++	1.1
lr2057	DQ219986	Phage protein	+	++	1.1
lr2058	DQ219987	Phage protein	N	++	0.93
Unknown function					
lr0922	DQ074860	Extracellular hydrolase	N	++	1.1
lr1158	DQ074883	Extracellular hydrolase	N	++	0.77
lr1794	DQ219974	Flavoprotein	N	++	1.1
lr1937	DQ219979	Conserved intracellular protein of unknown function	N	++	1.1
lr1139	AY970988	Conserved intracellular protein of unknown function	N	++	1.5
lr2103	DQ219991	Conserved intracellular protein of unknown function	+	+++	1.2
lr0997	DQ219958	Conserved membrane protein of unknown function	+	++++	2.1
lr1191	DQ219999	Conserved membrane protein of unknown function	N	++	1.3
lr1515	DQ074905	Unconserved extracellular protein of unknown function	++	++++	2.5
lr2117	DQ220006	Unconserved intracellular protein of unknown function	++	+++	1.8
lr2090	DQ219990	Conserved membrane protein of unknown function	++	++	1.6
lr1934	DQ219978	Conserved intracellular protein of unknown function	N	++	*T*
Genes down-regulated at pH 2.7					
Stress response					
lr0722	DQ219953	Cold shock protein	−	−−	−1.6
Regulation and signal transduction					
lr0021	DQ220000	Guanosine polyphosphate pyrophosphohydrolases/ synthetases	−−−	−−−	−2.4
lr0190	DQ219995	Transcriptional regulator	N	−−−	*T*
lr1578	DQ219971	Transcriptional regulator	−	−−	−0.82

Continued on facing page

TABLE 2—*Continued*

Gene and function	Accession no.[a]	Description of encoded protein[b]	Expression ratio[c]		Mean *M* pH[d]
			5 min	15 min	
Transport and metabolism					
lr0382	AY971000	Branched-chain amino acid transport protein	−−	−−	−1.6
lr0153	DQ219993	Arabinose efflux permease	N	−−	*T*
lr0115	DQ219946	Deoxynucleoside kinase	−	−−	−2.5
lr1001	DQ219959	ADP-ribose pyrophosphatase	−	−−	−0.89
lr1297	DQ219963	Thymidine kinase	−	−−	−1.3
lr1401	DQ219964	Xanthine/uracil permeases	−−	−−−	−1.7
lr1433	DQ219965	Cytidylate kinase	−−	−−	−1.6
lr0320	DQ219998	Transport protein	−−	−−−	−2.0
lr0562	DQ220002	MntH, Mn^{2+} and Fe^{2+} transporter of the NRAMP[f] family	−−	−−	−1.3
Energy production and conversion					
lr0229	DQ219949	Flavodoxin	−−	−−	−1.4
DNA replication, recombination, and repair					
lr0119	DQ219948	DNA polymerase III, delta subunit	N	−−−	−1.6
lr1014	DQ220003	Site-specific recombinase XerD	N	−−	−1.3
lr1240	DQ219962	Recombinational DNA repair ATPase	N	−−	−1.6
Translation and posttranslational modification					
lr0118	DQ219947	Ribosomal protein S20	N	−−−	−1.3
lr0277	DQ219997	Ribosomal protein L10	−−	−−−	−1.8
lr0862	DQ219957	Asp-$tRNA^{Asn/Glu}$-$tRNA^{Gln}$ amidotransferase C subunit	−−	−−	−1.3
lr1432	DQ220005	Ribosomal protein S1	−−	−−−−	−1.8
lr1690	DQ219973	Ribosomal protein L31	N	−−	−1.4
lr0251	DQ219950	RNase P-protein component	N	−−	*T*
lr1002	DQ219960	Peroxiredoxin	−−	−−	−0.95
lr1465	DQ219967	Protease subunit of ATP-dependent Clp proteases	−−	−−−	−1.5
Cell division					
lr1411	DQ220004	Actin-like ATPase involved in cell morphogenesis	−−	N	−1.1
lr0145	DQ219992	Cell division protein	N	−−	−0.89
Cell envelope biogenesis					
lr0537	DQ074830	Apf-like protein	−−−−	−−−	−4.3
lr0858	DQ074851	Apf-like protein	−−	−−	−2.2
Intracellular trafficking and secretion					
lr1103	AY970986	Protein translocase subunit YajC	N	−−	−0.87
Unknown function					
lr0546	DQ220001	Recombination protein	−−	N	−1.1
lr1422	DQ074901	Conserved extracellular protein of unknown function	−	−−	−1.4
lr0318	DQ219951	Conserved intracellular protein of unknown function	−−	−−−	−2.0
lr0733	DQ219954	Conserved intracellular protein of unknown function	N	−−	−1.5
lr1628	DQ219972	Conserved intracellular protein, MarZ, of unknown function	−−	−	−1.0
lr1434	DQ074902	Unconserved extracellular protein of unknown function	−−	−−−	−1.7
lr2118	DQ220007	Unconserved intracellular protein of unknown function	−−	−−	−1.3
lr0195	DQ219996	5-Formyltetrahydrofolate cycloligase or methenyl-tetrahydrofolate synthetase	−−	−−	−1.4

[a] The GenBank accession numbers are given.
[b] Based on COG classes or high similarity to characterized proteins.
[c] Ratio of expression for cells after 5 or 15 min at pH 2.7 and cells at time zero. Comparisons between pH 5.1 and time zero or between pH 2.7 and pH 5.1 are not displayed. The symbols show the degree of up-regulation (+) or down-regulation (−) of the genes as follows: +, 1.75 to 2-fold up-regulation; ++, 2-fold to 3-fold up-regulation; +++, 3-fold to 5-fold up-regulation; ++++, more than 5-fold up-regulation of the gene; −, 1.75 to 2-fold down-regulation; −−, 2-fold to 3-fold down-regulation; −−−, 3-fold to 5-fold down-regulation; −−−−, more than 5-fold down-regulation. N, the expression is up- or down-regulated but the changes were less than 1.75-fold.
[d] Mean *M* (log_2) for parameter pH.
[e] The gene is selected because it is likely to be changed by time (parameter *T*).
[f] NRAMP, natural resistance-associated macrophage protein.

TABLE 3. Expression profiles of selected *Lactobacillus reuteri* genes differently regulated at pH 2.7 and in the treated control

Gene	Accession no.[a]	Description of encoded protein[b]	Expression ratio[c]					
			5 min at the indicated pH			15 min at the indicated pH		
			pH 2.7/T0	pH 5.1/T0	pH 2.7/pH 5.1	pH 2.7/T0	pH 5.1/T0	pH 2.7/pH 5.1
lr0115	DQ219946	Deoxynucleoside kinase	−	+++	−−−	−−	++	−−−−
lr1516	DQ219970	Putative esterase	++	−−−	++	++++	−−	++++
lr0537	DQ074830	Apf-like protein	−−−−	++++	−−−−	−−−	++	−−−−
lr0858	DQ074851	Apf-like protein	−−	++	−−−	−−	N	−−
lr0318	DQ219951	Conserved intracellular protein of unknown function	−−	++	−−−	−−−	N	−−−
lr0733	DQ219954	Conserved intracellular protein of unknown function	N	++	−−	−−	N	−−
hpk3 (lr0819)	DQ219931	Histidine sensor kinase	N	−−	+++	N	−−−	++++
lr0628	DQ233673	LuxS, autoinducer-production protein	N	−−	++	N	−−−−	+++
lr1518	DQ233704	ArgR, arginine repressor	N	−−−	+++	N	−−−	++
lr1020	DQ233707	Ornithine carbamoyltransferase	N	−−	++	N	−−−	++
lr1517	DQ233695	ArcA, arginine deiminase	N	−−−−	++++	N	−−−−	++
lr1731	DQ233708	ArgE, acetylornithine deacetylase	N	−−	++	N	−−	+++
lr1870	DQ233714	PduO	N	N	N	N	−−−	++
lr1871	DQ233715	PduN	N	N	+	N	−−	+++
lr1872	DQ233716	PduM	N	N	++	N	−−−	++++
lr1873	DQ233717	PduL	N	−	+	N	−−−	+++
lr1874	DQ233718	PduJ	N	N	N	N	−−	++
lr1875	DQ233719	PduK	N	−−	++	N	−−−−	++++
lr1876	DQ233720	PduH	N	−	++	N	−−	+++
lr1877	DQ233721	PduG	N	−−	++	N	−−−	+++
lr1879	DQ233723	PduD, dehydratase medium subunit	N	−−	++	N	−−−−	++++
lr1880	DQ233724	PduC, dehydratase large subunit	N	−−	++	N	−−−	++++
lr1881	DQ233725	PduB	N	−−	++	N	−−−	N

[a] The GenBank accession numbers are given.
[b] Based on COG classes or high similarity to characterized proteins.
[c] Ratio of expression for cells after 5 or 15 min at pH 2.7 or 5.1 and cells at time zero (T0). The symbols show the degree of up-regulation (+) or down-regulation (−) of the genes as follows: +, 1.75 to 2-fold up-regulation; ++, 2-fold to 3-fold up-regulation; +++, 3-fold to 5-fold up-regulation; ++++, more than 5-fold up-regulation of the gene; −, 1.75 to 2-fold down-regulation; −−, 2-fold to 3-fold down-regulation; −−−, 3-fold to 5-fold down-regulation; −−−−, more than 5-fold down-regulation. N, the expression is up- or down-regulated but the changes were less than 1.75-fold.

treated control (Fig. 3). In the treated control, the majority of the induced genes were involved in transport and metabolism or energy production. Of the repressed genes, several were involved in transport, lipid metabolism, biosynthesis of secondary metabolites, and catabolic pathways. Among these genes, genes in the *pdu* operon, genes encoding components in arginine deiminase pathway, and *luxS* were found (Table 3).

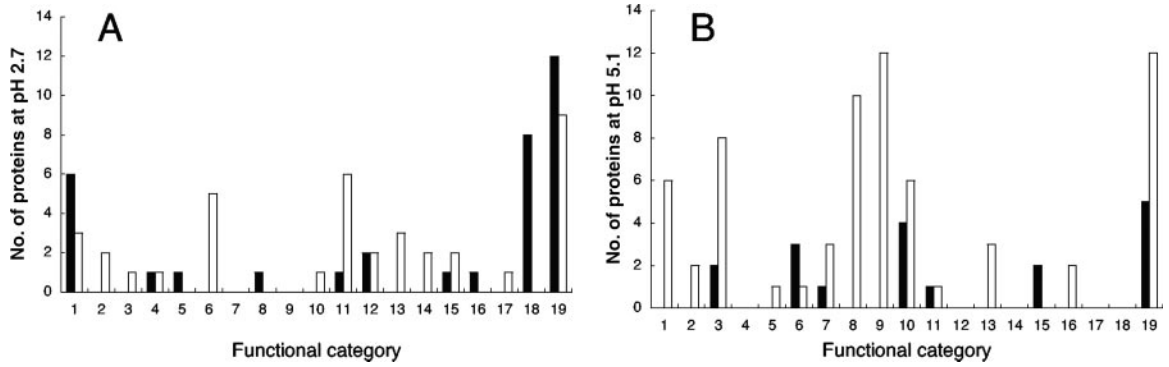

FIG. 3. Predicted functions of the proteins encoded by genes induced or repressed at pH 2.7 (A) and in the treated control (B), based on COG or pfam or high similarity to characterized proteins. The number of proteins is shown on the *y* axis in both panels. Up-regulated genes (black bars) and down-regulated genes (white bars) are shown. Functional categories: 1, regulation and signal transduction; 2, transport; 3, amino acid transport and metabolism; 4, carbohydrate transport and metabolism; 5, coenzyme metabolism/lipid metabolism; 6, nucleotide transport and metabolism; 7, inorganic ion transport and metabolism; 8, lipid metabolism; 9, secondary metabolite biosynthesis, transport, catabolism/energy production, and conversion; 10, energy production and conversion; 11, translation, ribosomal structure, and biogenesis; 12, posttranslational modifications, protein turnover, and chaperones; 13, DNA replication, recombination, and repair; 14, cell division and chromosome partitioning; 15, cell envelope biogenesis; 16, defense mechanisms; 17, intracellular trafficking and secretion; 18, phage proteins; 19, proteins of unknown function.

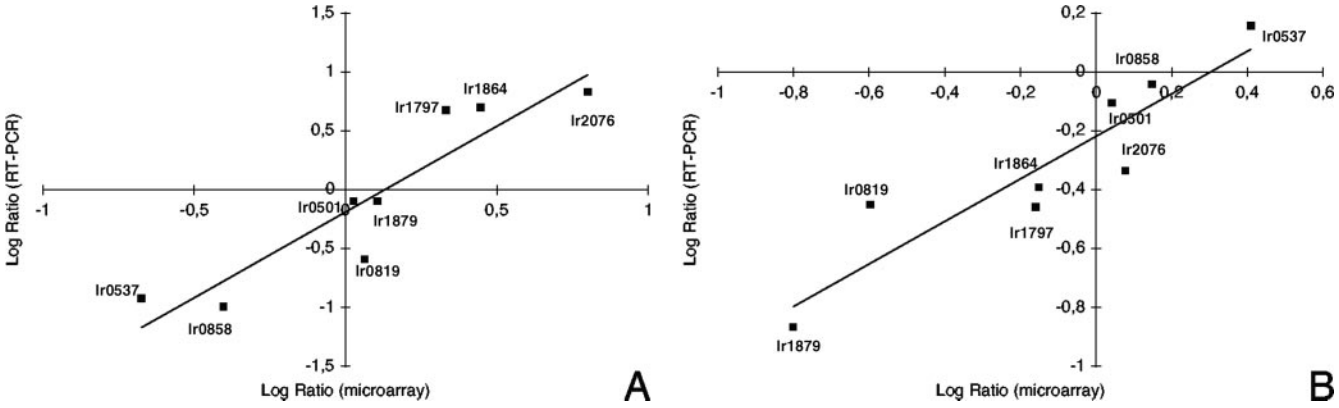

FIG. 4. Comparison of gene expression measurements by microarray and real-time RT-PCR. Changes in gene expression in *L. reuteri* ATCC 55730 after 15 min are presented as ratios (log transformed in base 10) between acid-treated cells (A) or treated control cells (B) and untreated cells. The correlation coefficients (r) were 0.92 and 0.915 for the acid-treated and treated control, respectively.

Validation of microarray data with real-time RT-PCR. In order to confirm the results obtained with microarray hybridization, the expression of eight genes was measured with real-time RT-PCR. The relative expression ratios after 15 min between acid-treated cells or the treated control cells and untreated cells (time zero) were calculated using lr0469 (SecY) as an internal reference. This gene displayed high signals on the microarrays, although the expression did not change. In Fig. 4, the gene expression ratios of the eight genes, determined by microarray hybridizations and real-time RT-PCR, are compared. The results from the two methods displayed good correlation with Pearson correlation coefficients of $r = 0.92$ and $r = 0.915$ for the acid-treated and treated control cells, respectively.

Acid tolerance of the *clpL* and esterase mutants. Because of the resemblance to ATPases involved in general stress response and the strong inducement at pH 2.7, *clpL* and a putative esterase, lr1516, respectively, were selected for functional analyses. Inactivation of the genes did not notably affect the growth of *L. reuteri* in MRS, although the growth rate of the esterase mutant in exponential phase was slightly lower than that of the wild type, i.e., the doubling time increased by approximately 9%. When studied under the microscope, no differences in morphology were detected between the wild type and the mutants (data not shown). Further, the survival of the mutants at low pH was tested and compared to the wild type. There were no clear differences between the wild type and the mutants when diluted in MRS to a final pH of 2.7 (data not shown). The survival was also examined in synthetic gastric juice (modified from that of Cotter et al. [13]; lacking enzymes and bile) at pH 2.0 (Fig. 5). After 20 minutes of incubation, no notable difference between the mutants and the wild type were found. However, after 50 min, the number of surviving cells of the *clpL* mutant and the esterase mutant were lower than that of the wild type. Approximately 47% of the wild type, 30% of the *clpL* mutant, and 18% of the esterase mutant cells were viable after the incubation. The differences between the wild type and mutants were statistically significant ($P < 0.05$ for the *clpL* mutant and $P < 0.001$ for the esterase mutant).

DISCUSSION

Response of *L. reuteri* ATCC 55730 to acid shock. The cell surface of bacteria is a shield against environmental stresses, such as an acidic environment. To increase the acid tolerance, bacteria can change the composition of the cell membrane or cell wall in order to decrease the permeability for protons. In *Streptococcus mutans*, the levels of monosaturated fatty acids and longer-chain fatty acids increase during growth at pH 5 in comparison to growth at pH 7 (47). Inhibition of membrane fatty acid alterations in this species also results in decreased tolerance to acid (21). Other oral bacteria, for example, *Lactobacillus casei*, shift the fatty acid composition of the membrane in response to low pH as well (20). In *L. reuteri*, lr1797 encoding a putative phosphatidylglycerophosphatase was induced at pH 2.7. Phosphatidylglycerophosphatase is a key enzyme in the synthesis of phosphatidylglycerol and cardiolipin, which are major acidic phospholipids of bacterial membranes (18). The up-regulation of lr1797 is thus likely part of a membrane adaptation to acidic conditions. In *L. reuteri* strain CRL 1098, growth with bile salts induces changes in the fraction of glycolipids and phospholipids and in the fatty acid composition

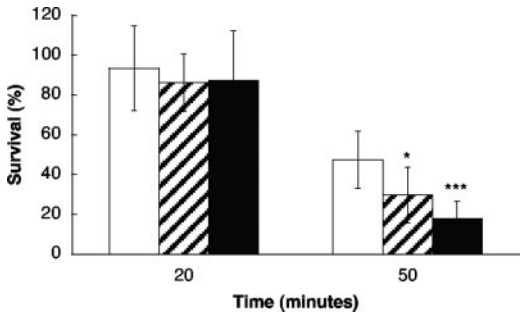

FIG. 5. Survival of *L. reuteri* ATCC 55730 wild type (white bars), *clpL* mutant (shaded bars), and esterase mutant (black bars) in synthetic gastric juice at pH 2.0. The data are presented as a percentage of the number of cells at the start of the experiment ($n = 3$). Asterisks indicate level of statistical significance (*, $P < 0.05$; ***, $P < 0.001$) in comparison to the wild type in Student's t test. Error bars represent standard deviations.

of the cell membrane. The presence of bile also reduces the survival under acid stress and freezing (54). In addition, a putative esterase gene, lr1516, belonging to the COG beta-lactamase family of penicillin-binding proteins was strongly induced in *L. reuteri* under acidic conditions. Penicillin-binding proteins are usually involved in peptidoglycan synthesis (3). In *S. mutans*, the *dltC* gene, which is involved in synthesis and esterification of D-alanyl-lipoteichoic acid, has been studied (8). The *dltC* mutant is highly sensitive to acid treatment and more permeable to protons than the wild type. Furthermore, one of the genes encoding regulatory proteins induced after the acid treatment in *L. reuteri* was *rr7* (lr1804), producing the response regulator of a two-component signal transduction system. This gene is part of an operon homologous to the *yycFG* operon in *Bacillus subtilis* (19), *Streptococcus pneumoniae*, *Staphylococcus aureus* (11), and *Lactobacillus sakei* (39). In *B. subtilis*, this system regulates genes involved in cell wall metabolism, such as components of teichoic acid biosynthesis (27). In addition, the response regulator of this system in *S. pneumoniae* is involved in fatty acid biosynthesis and the fatty acid composition in the cell membrane (38). Up-regulation of lr1797 and lr1516 and of the genes lr2064 and lr0811 putatively involved in cell wall biogenesis and lipid metabolism, respectively, indicates that cell envelope alterations are important for the response to acid shock in *L. reuteri* as well as in other bacteria.

In addition, other cell surface-associated genes were up-regulated after acid shock. The gene lr0997, encoding a conserved membrane protein, had similarities with the *gdmH/epiH* genes found in *Staphylococcus gallinarum* and *Staphylococcus epidermidis* for example. In *S. gallinarum*, GdmH has been reported to be involved in translocation of the lantibiotic as an accessory factor to the ATP-binding cassette transporter GdmT (44) and in immunity to gallidermin (26). Although lacking in several other lantibiotic gene clusters, *gdmH*-like genes are present in various bacterial species, implying that these genes play a role in several microbial processes (26). However, to our knowledge, no connection to acid tolerance of this gene family has been described.

Other well-known mechanisms to adapt to high acidity include inducement of general stress responses. In *L. reuteri* ATCC 55730, the gene *clpL* (lr1864), which encodes an ATPase with putative chaperone activity, was up-regulated at pH 2.7. Many members of the Clp protein family are chaperones that in association with ClpP are also involved in degradation of damaged proteins (23). Several studies have demonstrated up-regulation of the expression of *clp* genes in response to stress. Northern blot analysis and RT-PCR of *clpP* and *clpL* in *Oenococcus oeni* revealed that the both genes are induced by heat stress and in the presence of 10% ethanol (6). In *Lactobacillus rhamnosus*, the *clpL1* and *clpL2* genes were induced by heat stress (53). Further, the ClpL protein in *S. mutans* was produced in larger amounts when cells were grown at pH 5.0 than cells grown at pH 7.0 as detected by two-dimensional gels (31).

Activation of phage-associated genes may be due to activation of the integrated phage as an escape mechanism. Eight of the induced phage-associated genes were located in a cluster on the *L. reuteri* chromosome. Interestingly, one of these genes was lr2064, which may be involved in cell wall degradation. In pathogenic bacteria, phage genes are sometimes associated with genetic islands harboring virulence and colonization factors. Examples of such islands are genes encoding cholera toxin in *Vibrio cholerae* (36) and strain-specific genes of the enterohemorrhagic *E. coli* O157:H7 (25). Horizontal gene transfer is an important mechanism for bacteria to evolve and adapt to new environments. The induction of phage-associated genes at low pH could indicate that *L. reuteri* has obtained genes involved in acid tolerance from phages.

The majority of the genes down-regulated at pH 2.7 in *L. reuteri* ATCC 55730 were involved in basic cellular processes, such as replication, cell division, and translation (Fig. 3). Several species of lactic acid bacteria are able to survive under acidic conditions (57). Survival experiments (Fig. 2) showed that *L. reuteri* survived but did not proliferate at pH 2.7. This probably explains why genes involved in cell division or replication were down-regulated under acidic conditions. Down-regulation of genes encoding ribosomal proteins in response to acidic pH is also observed in the gram-negative bacterium *Shewanella oneidensis* (30). In a study on *Lactobacillus plantarum*, Pieterse et al. (46) demonstrate that growth rate has a large impact on gene expression under lactic acid stress.

Previous studies on acid stress in lactic acid bacteria have reported induction of heat shock proteins. However, the focus of these studies is on long-term acid stress rather than acid shock. In *Lactobacillus acidophilus*, the heat shock proteins DnaK, DnaJ, GrpE, GroES, and GroEL are produced as a response to acid adaptation (34). Further, the production of GroES, GroEL, and DnaK increases in *Lactobacillus delbrueckii* subsp. *bulgaricus* grown at pH 4.75 compared to cells grown at pH 6 (32). However, in *Lactobacillus sanfranciscensis*, only GrpE increases in acid-tolerant mutants and acid-adapted cells, while the amounts of DnaJ, DnaK, and GroES do not change (16), and in *S. oneidensis* (30), the expression of *dnaK*, *dnaJ*, *groES-groEL*, and *grpE* is repressed under acidic conditions. *L. reuteri* ATCC 55730 possesses the heat shock proteins, GroES, GroEL, DnaK, DnaJ, and GrpE (data not shown), but none of the corresponding genes were up- or down-regulated in the early response to acid shock in this study. Also, the F_oF_1 ATPase operon is present in *L. reuteri* (data not shown), but the expression of the genes in this operon did not change significantly. This system is known to mediate the extrusion of protons from the cytoplasm, and other studies on lactobacilli have demonstrated that it is involved in survival at low pH (12, 29). The genes encoding the F_oF_1 ATPase were up-regulated in *L. plantarum* when exposed to bile (10), but not under lactic acid stress (46). In addition, microarray analyses of the acid tolerance response in *S. pneumoniae* display an increase of less than twofold of the F_oF_1 ATPase operon 200 min after acid shock (35).

Indirect effects of the lowered pH. Survival data were considered when designing the experimental setup for expression analysis (Fig. 1). Lowering the pH by direct addition of HCl to a final pH of 2 notably decreased the survival of the cells in comparison to resuspending the cells in MRS at pH 2 (data not shown). Therefore, and in order to mimic the entrance of bacteria into the gastrointestinal tract, the cells were diluted into MRS at a lower pH in the experiments. This experimental design resembles the environmental changes bacteria may experience after oral intake, both regarding the sudden shift in pH and time and the effects of

dilution (Fig. 1). *L. reuteri* produces substantial amounts of lactate and acetate when fermenting food products (43). When the *L. reuteri* bacteria grow, in MRS broth, the bacteria mainly produce lactate (data not shown), but MRS also contains acetate. After the decrease in pH, most of the lactate produced by the bacteria and the acetate from the MRS broth will convert to the more antimicrobial undissociated forms of lactic acid and acetic acid, respectively. This was likely affecting the bacteria in the experiment. The detected response to the lowered pH was thus dependent on the acidity but probably also the formation of undissociated organic acids. However, the contribution of the latter could be regarded an indirect effect of the low pH resembling intake of fermented food products. Properties of the food matrix, such as content of organic acids and buffering capacity, are important to consider when discussing the response of probiotic bacteria to the acidic pH connected to ingestion.

Interestingly, the effect of dilution was not the same at pH 2.7 as in the treated control. Instead, on many genes, the lowered pH had an indirect effect, which counteracted the effect of dilution (Table 3). Most notably, several genes were repressed in the treated control although unchanged at pH 2.7. For example, these genes were involved in transport, lipid metabolism, biosynthesis of secondary metabolites, and catabolic pathways (Fig. 3). In addition, some genes in this group were also known to be important for low-pH tolerance. Four genes encoding the arginine deiminase pathway components, arginine deiminase (lr1517), arginine repressor (lr1518), acetylornithine deacetylase (lr1731), and ornithine carbamoyltransferase (lr1020), were identified. This pathway produces ammonia and contributes to alkalization of the environment. The system has been shown to be regulated by the combined effect of arginine availability, energy depletion, catabolite repression, and oxygenation rather than by low pH (reviewed in reference 57). In the *L. reuteri* strain CRL 1098, the ADI system probably has a role in the acid tolerance response. Adaptation at low pH in the presence of arginine in the exponential phase and energy depletion in the stationary phase seem to activate the pathway (49). The arginine/ornithine antiporter gene, *arcD*, connected to the ADI pathway was also induced in another *L. reuteri* strain, LTH5531, during sourdough fermentation (15). Furthermore, the repressed genes in the treated control contained several genes likely to be changed by decreased cell density. An example of this is the quorum-sensing gene *luxS* (62). The reason why these genes were not down-regulated in the acid-treated cells, as in the treated control, remains to be revealed.

The experimental design in this study provides the ability to estimate how three parameters (pH, time, and dilution) affect gene expression. When entering the gastrointestinal tract, bacteria experience a complete change in environment. The regulation of genes under these conditions will most likely be of an intricate nature. Studying complex interactions leads the focus to the experimental design and the use of relevant controls. If the untreated cells had been excluded from this analysis, the outcome would have been thoroughly different.

Role of ClpL and the putative esterase under acid shock. As the first report on microarray analyses of *Lactobacillus reuteri*, this study describes the response to acid shock after a severe reduction of pH. This response differed from the responses to milder long-term acid stress studied in other species. However, *clpL*, earlier identified to be involved in stress tolerance, was up-regulated at low pH. Several genes putatively involved in cell envelope alterations, such as a putative esterase (lr1516) and a putative phosphatidylglycerophosphatase (lr1797), were also up-regulated after the shift in pH. Disruption of *clpL* or lr1516 significantly increased the sensitivity to acid shock (Fig. 5). Hence, although not essential for surviving at low pH, these genes clearly were of importance for the response of *L. reuteri* to acid shock. The roles of *clp* genes have not been studied in detail in *L. reuteri*, but as a member of this family (23), ClpL is likely to function as a chaperone. In *Lactobacillus plantarum*, in vivo experiments using an in vivo expression technology system demonstrated that *clpC* (lp_1019) is induced in the gastrointestinal tracts of mice (9). The corresponding gene to lr1516 in *L. plantarum* (lp_3312) was also induced in this experiment (9). As mentioned above, lr1516 possibly is involved in changing the cell wall and thus increasing the tolerance of the cells towards acid. Taken together, this implies that the *L. reuteri* *clpL* and lr1516 have roles in managing life in the gastrointestinal tract. The influence of lr1516 on cell wall biogenesis is currently unknown. Therefore, detailed studies of the enzymatic function, molecular mechanisms, and cellular role of this gene are required. Further studies of the composition of the cell membrane and cell wall of *L. reuteri* and the genes altering and regulating these properties would bring the adaptation to acidic environments to light.

ACKNOWLEDGMENTS

This study was supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (50.0288/00 and 20.9/2001-2432) and BioGaia AB, Stockholm, Sweden. R. A. Britton is supported by grants from the Rackham Foundation and the Center for Microbial Pathogenesis at Michigan State University.

We are very grateful to Anette Hagberg, Department for Plant Biology and Forest Genetics, SLU, Uppsala, Sweden, and Hanna Göransson, WCN expression array platform, Rudbeck Laboratory, Uppsala, Sweden, for support with the analyses of the microarray data and for critically reading the manuscript. We also thank the Linneaus Centre of Bioinformatics, Uppsala University, Sweden, for the opportunity to use BASE and the LCB data warehouse. We also thank Kristi Whitehead for assistance in the development of the DNA microarrays.

REFERENCES

1. **Ahrné, S., G. Molin, and L. Axelsson.** 1992. Transformation of *Lactobacillus reuteri* with electroporation: studies on the erythromycin resistance plasmid pLUL631. Curr. Microbiol. **24:**199–205.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Arbeloa, A., J. E. Hugonnet, A. C. Sentilhes, N. Josseaume, L. Dubost, C. Monsempes, D. Blanot, J. P. Brouard, and M. Arthur.** 2004. Synthesis of mosaic peptidoglycan cross-bridges by hybrid peptidoglycan assembly pathways in gram-positive bacteria. J. Biol. Chem. **279:**41546–41556.
4. **Bateman, A., L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E. L. Sonnhammer, D. J. Studholme, C. Yeats, and S. R. Eddy.** 2004. The Pfam protein families database. Nucleic Acids Res. **32:**D138–D141.
5. **Båth, K., S. Roos, T. Wall, and H. Jonsson.** 2005. The cell surface of *Lactobacillus reuteri* ATCC 55730 highlighted by identification of 126 extracellular proteins from the genome sequence. FEMS Microbiol. Lett. **253:**75–82.
6. **Beltramo, C., C. Grandvalet, F. Pierre, and J. Guzzo.** 2004. Evidence for multiple levels of regulation of *Oenococcus oeni clpP-clpL* locus expression in response to stress. J. Bacteriol. **186:**2200–2205.
7. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:**783–795.
8. **Boyd, D. A., D. G. Cvitkovitch, A. S. Bleiweis, M. Y. Kiriukhin, D. V. Debabov, F. C. Neuhaus, and I. R. Hamilton.** 2000. Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. J. Bacteriol. **182:**6055–6065.
9. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. J. Bacteriol. **186:**5721–5729.

10. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2006. DNA micro-array-based identification of bile-responsive genes in *Lactobacillus plantarum*. J. Appl. Microbiol. **100:**728–738.
11. **Clausen, V. A., W. Bae, J. Throup, M. K. Burnham, M. Rosenberg, and N. G. Wallis.** 2003. Biochemical characterization of the first essential two-component signal transduction system from *Staphylococcus aureus* and *Streptococcus pneumoniae*. J. Mol. Microbiol. Biotechnol. **5:**252–260.
12. **Corcoran, B. M., C. Stanton, G. F. Fitzgerald, and R. P. Ross.** 2005. Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Appl. Environ. Microbiol. **71:**3060–3067.
13. **Cotter, P. D., C. G. Gahan, and C. Hill.** 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol. Microbiol. **40:** 465–475.
14. **Cotter, P. D., and C. Hill.** 2003. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol. Mol. Biol. Rev. **67:**429–453.
15. **Dal Bello, F., J. Walter, S. Roos, H. Jonsson, and C. Hertel.** 2005. Inducible gene expression in *Lactobacillus reuteri* LTH5531 during type II sourdough fermentation. Appl. Environ. Microbiol. **71:**5873–5878.
16. **De Angelis, M., L. Bini, V. Pallini, P. S. Cocconcelli, and M. Gobbetti.** 2001. The acid-stress response in *Lactobacillus sanfranciscensis* CB1. Microbiology **147:**1863–1873.
17. **De Angelis, M., and M. Gobbetti.** 2004. Environmental stress responses in *Lactobacillus*: a review. Proteomics **4:**106–122.
18. **Dowhan, W.** 1997. Molecular basis for membrane phospholipid diversity: why are there so many lipids? Annu. Rev. Biochem. **66:**199–232.
19. **Fabret, C., and J. A. Hoch.** 1998. A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. J. Bacteriol. **180:**6375–6383.
20. **Fozo, E. M., J. K. Kajfasz, and R. G. Quivey, Jr.** 2004. Low pH-induced membrane fatty acid alterations in oral bacteria. FEMS Microbiol. Lett. **238:**291–295.
21. **Fozo, E. M., and R. G. Quivey, Jr.** 2004. Shifts in the membrane fatty acid profile of *Streptococcus mutans* enhance survival in acidic environments. Appl. Environ. Microbiol. **70:**929–936.
22. **Fredlund, E., C. Beerlage, P. Melin, J. Schnurer, and V. Passoth.** 2006. Oxygen and carbon source-regulated expression of PDC and ADH genes in the respiratory yeast *Pichia anomala*. Yeast **23:**1137–1149.
23. **Frees, D., K. Savijoki, P. Varmanen, and H. Ingmer.** 2007. Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. Mol. Microbiol. **63:**1285–1295.
24. **Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang, and J. Zhang.** 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. **5:**R80.
25. **Hayashi, T., K. Makino, M. Ohnishi, K. Kurokawa, K. Ishii, K. Yokoyama, C. G. Han, E. Ohtsubo, K. Nakayama, T. Murata, M. Tanaka, T. Tobe, T. Iida, H. Takami, T. Honda, C. Sasakawa, N. Ogasawara, T. Yasunaga, S. Kuhara, T. Shiba, M. Hattori, and H. Shinagawa.** 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res. **8:**11–22.
26. **Hille, M., S. Kies, F. Gotz, and A. Peschel.** 2001. Dual role of GdmH in producer immunity and secretion of the staphylococcal lantibiotics gallidermin and epidermin. Appl. Environ. Microbiol. **67:**1380–1383.
27. **Howell, A., S. Dubrac, K. K. Andersen, D. Noone, J. Fert, T. Msadek, and K. Devine.** 2003. Genes controlled by the essential YycG/YycF two-component system of *Bacillus subtilis* revealed through a novel hybrid regulator approach. Mol. Microbiol. **49:**1639–1655.
28. **Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. **305:**567–580.
29. **Kullen, M. J., and T. R. Klaenhammer.** 1999. Identification of the pH-inducible, proton-translocating F1F0-ATPase (atpBEFHAGDC) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterization. Mol. Microbiol. **33:**1152–1161.

29a. **Law, J., G. Buist, A. Haandrikman, J. KoK, G. Venema, and K. Leenhouts.** 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. **177:**7011–7018.

30. **Leaphart, A. B., D. K. Thompson, K. Huang, E. Alm, X. F. Wan, A. Arkin, S. D. Brown, L. Wu, T. Yan, X. Liu, G. S. Wickham, and J. Zhou.** 2006. Transcriptome profiling of *Shewanella oneidensis* gene expression following exposure to acidic and alkaline pH. J. Bacteriol. **188:**1633–1642.
31. **Len, A. C., D. W. Harty, and N. A. Jacques.** 2004. Stress-responsive proteins are upregulated in *Streptococcus mutans* during acid tolerance. Microbiology **150:**1339–1351.
32. **Lim, E. M., S. D. Ehrlich, and E. Maguin.** 2000. Identification of stress-inducible proteins in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Electrophoresis **21:**2557–2561.
33. **Lönnstedt, I., and T. Speed.** 2002. Replicated microarray data. Stat. Sin. **12:**31–46.
34. **Lorca, G. L., G. Font de Valdez, and A. Ljungh.** 2002. Characterization of the protein-synthesis dependent adaptive acid tolerance response in *Lactobacillus acidophilus*. J. Mol. Microbiol. Biotechnol. **4:**525–532.

34a. **Maguin, E., P. Duwat, T. Hege, D. Ehrlich, and A. Gruss.** 1992. New thermosensitive plasmid for gram-positive bacteria. J. Bacteriol. **174:**5633–5688.

35. **Martin-Galiano, A. J., K. Overweg, M. J. Ferrandiz, M. Reuter, J. M. Wells, and A. G. de la Campa.** 2005. Transcriptional analysis of the acid tolerance response in *Streptococcus pneumoniae*. Microbiology **151:**3935–3946.
36. **McLeod, S. M., H. H. Kimsey, B. M. Davis, and M. K. Waldor.** 2005. CTXphi and *Vibrio cholerae*: exploring a newly recognized type of phage-host cell relationship. Mol. Microbiol. **57:**347–356.
37. **Mechold, U., and H. Malke.** 1997. Characterization of the stringent and relaxed responses of *Streptococcus equisimilis*. J. Bacteriol. **179:**2658–2667.
38. **Mohedano, M. L., K. Overweg, A. de la Fuente, M. Reuter, S. Altabe, F. Mulholland, D. de Mendoza, P. Lopez, and J. M. Wells.** 2005. Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition. J. Bacteriol. **187:**2357–2367.
39. **Morel-Deville, F., F. Fauvel, and P. Morel.** 1998. Two-component signal-transducing systems involved in stress responses and vancomycin susceptibility in *Lactobacillus sakei*. Microbiology **144:**2873–2883.
40. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1–6.
41. **Nielsen, H., and A. Krogh.** 1998. Prediction of signal peptides and signal anchors by a hidden Markov model. Proc. Int. Conf. Intell. Syst. Mol. Biol. **6:**122–130.
42. **Nilsen, T., I. F. Nes, and H. Holo.** 2003. Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. Appl. Environ. Microbiol. **69:**2975–2984.
43. **Østlie, H. M., J. Treimo, and J. A. Narvhus.** 2005. Effect of temperature on growth and metabolism of probiotic bacteria in milk. Int. Dairy J. **15:**989–997.
44. **Peschel, A., N. Schnell, M. Hille, K. D. Entian, and F. Gotz.** 1997. Secretion of the lantibiotics epidermin and gallidermin: sequence analysis of the genes gdmT and gdmH, their influence on epidermin production and their regulation by EpiQ. Mol. Gen. Genet. **254:**312–318.
45. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
46. **Pieterse, B., R. J. Leer, F. H. Schuren, and M. J. van der Werf.** 2005. Unravelling the multiple effects of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. Microbiology **151:**3881–3894.
47. **Quivey, R. G., Jr., R. Faustoferri, K. Monahan, and R. Marquis.** 2000. Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. FEMS Microbiol. Lett. **189:**89–92.
48. **Rallu, F., A. Gruss, S. D. Ehrlich, and E. Maguin.** 2000. Acid- and multi-stress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. Mol. Microbiol. **35:**517–528.
49. **Rollan, G., G. L. Lorca, and G. Font de Valdez.** 2003. Arginine catabolism and acid tolerance response in *Lactobacillus reuteri* isolated from sourdough. Food Microbiol. **20:**313–319.
50. **Saal, L. H., C. Troein, J. Vallon-Christersson, S. Gruvberger, A. Borg, and C. Peterson.** 2002. BioArray Software Environment (BASE): a platform for comprehensive management and analysis of microarray data. Genome Biol. **3:**software0003.1–0003.6.
51. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
52. **Shornikova, A. V., I. A. Casas, E. Isolauri, H. Mykkanen, and T. Vesikari.** 1997. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. J. Pediatr. Gastroenterol. Nutr. **24:**399–404.
53. **Suokko, A., K. Savijoki, E. Malinen, A. Palva, and P. Varmanen.** 2005. Characterization of a mobile *clpL* gene from *Lactobacillus rhamnosus*. Appl. Environ. Microbiol. **71:**2061–2069.
54. **Taranto, M. P., M. L. Fernandez Murga, G. Lorca, and G. F. de Valdez.** 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. J. Appl. Microbiol. **95:**86–91.
55. **Tatusov, R. L., N. D. Fedorova, J. D. Jackson, A. R. Jacobs, B. Kiryutin, E. V. Koonin, D. M. Krylov, R. Mazumder, S. L. Mekhedov, A. N. Nikolskaya, B. S. Rao, S. Smirnov, A. V. Sverdlov, S. Vasudevan, Y. I. Wolf, J. J. Yin, and D. A. Natale.** 2003. The COG database: an updated version includes eukaryotes. BMC Bioinformatics **4:**41.
56. **Tatusov, R. L., E. V. Koonin, and D. J. Lipman.** 1997. A genomic perspective on protein families. Science **278:**631–637.
57. **van de Guchte, M., P. Serror, C. Chervaux, T. Smokvina, S. D. Ehrlich, and E. Maguin.** 2002. Stress responses in lactic acid bacteria. Antonie Leeuwenhoek **82:**187–216.
58. **Wall, T., S. Roos, K. Jacobsson, A. Rosander, and H. Jonsson.** 2003. Phage display reveals 52 novel extracellular and transmembrane proteins from *Lactobacillus reuteri* DSM 20016(T). Microbiology **149:**3493–3505.

59. **Walter, J., P. Chagnaud, G. W. Tannock, D. M. Loach, F. Dal Bello, H. F. Jenkinson, W. P. Hammes, and C. Hertel.** 2005. A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. Appl. Environ. Microbiol. **71:**979–986.
60. **Weizman, Z., G. Asli, and A. Alsheikh.** 2005. Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. Pediatrics **115:**5–9.
61. **Wilhelm, J., and A. Pingoud.** 2003. Real-time polymerase chain reaction. Chembiochem **4:**1120–1128.
62. **Xavier, K. B., and B. L. Bassler.** 2003. LuxS quorum sensing: more than just a numbers game. Curr. Opin. Microbiol. **6:**191–197.
63. **Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed.** 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.