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# Genetic Characterization of the Bile Salt Response in *Lactobacillus plantarum* and Analysis of Responsive Promoters In Vitro and In Situ in the Gastrointestinal Tract

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**In this paper we describe the growth, morphological, and genetic responses of *Lactobacillus plantarum* WCFS1 to bile. Growth experiments revealed that a stepwise increase in the porcine bile concentration led to a gradual decrease in the maximal growth rate. Moreover, the final density reached by an *L. plantarum* culture growing in MRS containing 0.1% bile was approximately threefold lower than that in MRS lacking bile. The morphology of the cells grown in MRS containing 0.1% bile was investigated by scanning electron microscopy, which revealed that cells clumped together and had rough surfaces and that some of the cells had a shrunken and empty appearance, which clearly contrasted with the characteristic rod-shaped, smooth-surface morphology of *L. plantarum* cells grown in MRS without bile. An *alr* complementation-based genome-wide promoter screening analysis was performed with *L. plantarum*, which led to identification of 31 genes whose expression was potentially induced by 0.1% porcine bile. Remarkably, 11 membrane- and cell wall-associated functions appeared to be induced by bile, as were five functions involved in redox reactions and five regulatory factors. Moreover, the lp_0237 and lp_0775 genes, identified here as genes that are inducible by bile in vitro, were previously identified in our laboratory as important for *L. plantarum* in vivo during passage in the mouse gastrointestinal tract (P. A. Bron, C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem, J. Bacteriol. 186:5721-5729, 2004). A quantitative reverse transcription-PCR approach focusing on these two genes confirmed that the expression level of lp_0237 and lp_0775 was significantly higher in cells grown in the presence of bile and cells isolated from the mouse duodenum than in cells grown on laboratory medium without bile.**

After ingestion bacteria meet several biological barriers, the first of which is the gastric acidity encountered in the stomach of the host. Bacteria able to survive these harsh conditions transit to the intestine, where they encounter stresses associated with low oxygen availability, bile salts, and competition with the microbiota. Bile salts are synthesized in the liver by conjugation of a heterocyclic steroid derived from cholesterol (17). The resulting conjugated bile salts are stored and concentrated in the gall bladder during the fasting state, and after consumption of a fat-containing meal these compounds are released into the duodenum, where they play a major role in the dispersion and absorption of fats, including bacterial phospholipids and cell membranes (34). Bile salts are reintroduced in the liver following their reabsorption in the distal small intestine and colon after deconjugation by the microbiota (16). This deconjugation reaction is performed by bacterial bile salt hydrolases, which are encoded in the genomes of several intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species (7, 10, 19, 33).

Studies of gram-positive, food-associated bacteria and their tolerance to digestive stress have focused mainly on physiological aspects, such as determination of the levels of acid and bile salt tolerance (6, 18), as well as the development of complex media in order to selectively enrich the bacteria that are digestive stress tolerant (30). Additionally, in several studies workers have described defense mechanisms of gram-negative bacteria against bile acids, which include the synthesis of porins, transport proteins, efflux pumps, and lipopolysaccharides (15). A few genome-wide approaches with gram-positive bacteria aimed at identification of proteins important for bile salt resistance have been described. In *Propionibacterium freudenreichii*, *Listeria monocytogenes*, and *Enterococcus faecalis* two-dimensional gel electrophoresis led to identification of several proteins that were expressed more highly in the presence of bile salts than under control conditions (12, 20, 26). In *P. freudenreichii* these induced proteins were characterized further, which led to the identification of 11 proteins that are induced by bile stress. These proteins include general stress proteins, such as ClpB and the chaperones DnaK and Hsp20 (20). Analogously, a subset of the proteins identified in *E. faecalis* appeared to be inducible by multiple sublethal stresses, including heat, ethanol, and alkaline pH (27). The fact that general stress proteins are induced by bile is in agreement with the cross-protection against bile after thermal or detergent pretreatment that has been observed in several bacteria, including *E. faecalis*, *L. monocytogenes*, and *Bifidobacterium adolescentis* (2, 12, 29). Furthermore, random gene disruption strategies with *L. monocytogenes* and *E. faecalis* resulted in strains that were more susceptible to bile salts than the wild-type strains. Subsequent genetic analysis of the mutants revealed that the disrupted genes encode diverse functions, including an efflux pump ho-

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mologue (2), and genes that may be involved in the biosynthesis of cell walls and fatty acids (3).

Lactic acid bacteria (LAB) are used extensively in the production of fermented food products. Because of the frequent consumption of dairy, vegetable, meat, and other fermented food products, large amounts of LAB are ingested. Moreover, LAB have the potential to serve as delivery vehicles for health-promoting or therapeutic compounds to the gastrointestinal tract (GI tract) (13, 31). One of the LAB, *Lactobacillus plantarum*, is encountered in many environmental niches, including dairy fermentations, meat fermentations, and a variety of vegetable fermentations (9, 11, 28). The complete 3.3-Mbp genome sequence of *L. plantarum* WCFS1 was recently determined (19). This strain is a single-colony isolate of strain NCIMB8826, which effectively survives passage through the stomach in an active form, reaches the ileum in high numbers compared to other strains, and is detectable in the colon (35). The availability of its genome sequence allows effective investigation of the genes and regulation mechanisms underlying the observed persistence of *L. plantarum* in the GI tract. In the research described here we focused on the genetic response of *L. plantarum* after exposure to a toxic concentration of bile acid molecules. Previously, utilization of alanine racemase as a promoter probe for the genome-wide identification of *L. plantarum* genes whose expression is induced by high salt conditions was described (5). Here the same system was utilized, which led to the identification of 31 genes induced by bile salts. Strikingly, two of the genes identified have previously been demonstrated to be induced in vivo in *L. plantarum* during passage through the mouse GI tract (4). In a quantitative reverse transcription-PCR analysis we focused on these two genes, demonstrating both their in vitro chromosomal induction by porcine bile and their in vivo expression in the duodenum in a mouse model.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** The bacterial strains, plasmids, and primers that were used in this study are listed in Table 1. *L. plantarum* WCFS1 (19) and its *alr* mutant derivative MD007 (23) were grown at 37°C in MRS (Difco, Molesey, Surrey, United Kingdom) without aeration. Various concentrations of D-cycloserine (from freshly prepared, filter-sterilized stock solutions), 5 μg of erythromycin per ml, and 200 μg of D-alanine per ml were added to MRS medium as indicated below.

**Scanning electron microscopy.** Nuclepore polycarbonate membranes (Costar, Cambridge, Mass.) with 1-μm pores were incubated for 30 min in a 0.01% poly-L-lysine solution in 0.1 M Tris-HCl buffer. Several drops of a logarithmically growing *L. plantarum* culture were positioned on the poly-L-lysine-coated membranes and incubated for 5 min at 100% humidity. After this the bacteria were fixed on the membranes for 30 min in 3% glutaraldehyde. Subsequently, the membranes were washed three times with Milli Q water (MQ), dehydrated with ethanol by using 30, 50, 70, and 90% ethanol and finally 100% ethanol three times, and critical point dried by the $CO_2$ method (Balzers CPD 020; Balzers Union, Liechtenstein). The dried membranes were mounted on sample holders by using carbon adhesive tabs (Electron Microscopy Sciences, Washington, D.C.). The sample holders were positioned inside a preparation chamber (CT 1500 HF; Oxford Instruments, Abingdon, Oxon, England). The samples were sputter coated with 10 nm of platinum and analyzed with a field emission scanning electron microscope (JEOL 6300F; JEOL, Tokyo, Japan) at 2.5 kV. Images were recorded digitally, and image processing was performed with Adobe PhotoShop 5.5.

**Identification of bile salt-inducible loci by using the *alr* complementation library.** Construction and utilization of an *L. plantarum* promoter probe library for effective identification of conditionally active promoters were described previously (5). Here this library was exploited for identification of clones that contain bile salt-inducible *L. plantarum* chromosomal fragments. Appropriate dilutions of a −80°C stock of the library were immediately plated on MRS plates containing erythromycin and 0.1% porcine bile (B-8631; Sigma, Zwijndrecht, The Netherlands). After 2 days of growth approximately 4,000 individual colonies on these plates were used for replica plating on plates containing erythromycin, one with 0.1% porcine bile and one without porcine bile. Growth on the two plates was periodically compared, which led to the primary identification of 96 colonies that could grow only in the presence of porcine bile (Table 1). The colonies displaying bile-dependent growth were cultured overnight in a microtiter plate containing MRS with erythromycin and D-alanine. The full-grown cultures were used to reconfirm the initially observed conditional growth phenotype and to assess the conditional promoter strength by observing the conditional growth phenotype in the presence of different concentrations of the Alr inhibitor D-cycloserine (5). The microtiter plate was replica plated by using a 96-pin replicator and MRS plates containing erythromycin and 0, 2.5, 5.0, 10, 25, 50, 100, or 200 μg of D-cycloserine per ml, with or without 0.1% bile salts. The bile-dependent, differential growth phenotype in the presence of different concentrations of D-cycloserine was determined by periodically comparing the growth on the plates with bile salts and the growth on the plates without bile salts. For the clones for which bile-dependent growth could be confirmed in this experiment, the chromosomal *L. plantarum* inserts harbored by their pNZ7120 derivatives were amplified by PCR by using primers PB1 and PB2 (5). The resulting amplicons were used for partial DNA sequence analysis with primer PB3 (5), and the insert sequences determined were assigned to chromosomal loci by using BlastN (1) and the *L. plantarum* genome sequence as the database (19).

**RNA isolation.** Appropriate dilutions of an overnight culture of *L. plantarum* WCFS1 were plated on MRS with or without 0.1% porcine bile salts. After 3 days of growth approximately 100 colonies were rapidly collected from the plates in 3 ml of MRS, which was added to 12 ml of quench buffer (60% methanol, 66.7 mM HEPES; pH 6.5; −40°C) (B. Pieterse, unpublished data). Following quenching, the cells were immediately pelleted by centrifugation at 5,000 × *g* for 10 min, and the cell pellets were resuspended in 0.4 ml of ice-cold MRS. The cell suspensions were added to ice-cold tubes containing 1 g of zirconium glass beads, 0.4 ml of phenol, 100 μl of chloroform, 30 μl of 10% sodium dodecyl sulfate, and 30 μl of 3 M sodium acetate (pH 5.2). The cells were disrupted with two 40-s treatments in a Fastprep (Qbiogene Inc., Illkirch, France) separated by 1 min on ice. After centrifugation, 200 μl of the aqueous phase was used for RNA isolation with a High Pure kit, which included 1 h of treatment with DNase I (Roche Diagnostics, Mannheim, Germany).

TABLE 1. Strains, plasmids, and primers used in this study and their relevant characteristics

| Strain, plasmid, or primer | Relevant features | Reference |
|---|---|---|
| *L. plantarum* strains | | |
| WCFS1 | Wild type whose genome sequence is available | 19 |
| MD007 | *L. plantarum* WCFS1 Δ*alr*, D-alanine auxotroph | 23 |
| Plasmids | | |
| pNZ7120 | Em$^r$, pIL252 derivative containing *Lactococcus lactis alr* promoter probe | 5 |
| BI1 to BI96 | PNZ7120 derivatives containing chromosomal *L. plantarum* fragments that were initially identified as inducible by 0.1% porcine bile | This study |
| Primers | | |
| PB1 | 5′-ACCGCTACGGATCACATC-3′ | 5 |
| PB2 | 5′-CTCGGGGCAGTAAGACTA-3′ | 5 |
| PB3 | 5′-GTGGTGAAGTTTTCATGG-3′ | 5 |
| 16S-fo2 | 5′-TGATCCTGGCTCAGGACGAA-3′ | This study |
| 16S-re2 | 5′-TGCAAGCACCAATCAATACCA-3′ | This study |
| 237-fo | 5′-CTACTGATATGGTTGTCGGGAATTA-3′ | This study |
| 237-re | 5′-ACGGGTGCGTAGAAGAAGC-3′ | This study |
| 775-fo | 5′-GCTCTTGCACCGGATATCAA-3′ | This study |
| 775-re | 5′-TTTCTTCTTCCCGTGACCAGT-3′ | This study |
| 1898-fo | 5′-GTGGCGACGGTTCTTACCAT-3′ | This study |
| 1898-re | 5′-CCCTGGAAGACCAATCGTGT-3′ | This study |
| 1027-fo | 5′-CCATGATGGTGCTTCACAA-3′ | This study |
| 1027-re | 5′-TCGTGGCAGCAGAGGTAATG-3′ | This study |

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For detection of in vivo mRNA levels in an animal model, a mouse experiment was performed in an accredited establishment (no. A59107) according to the N°86/609/CEE guidelines of the French government. Seven-week-old female BALB/c mice were purchased from Iffa Credo (St. Germain sur l'Arbresle, France) and had free access to tap water and standard mouse chow during the experiments. After overnight culturing, bacterial cells were pelleted by centrifugation and resuspended at a concentration of $10^{10}$ CFU per ml in MRS. A mouse received a 100-μl ($10^9$-CFU) dose of a freshly prepared bacterial suspension by intragastric administration, and the next day the mouse received a dose of $10^{10}$ CFU. Four hours later, the mouse was sacrificed, and a section of the mouse small intestine representing the duodenum (0.48 g) was quickly collected and frozen in liquid nitrogen until it was processed with a liquid $N_2$-cooled BioPulverizer (BioSpec Products, Bartlesville, Okla.). The powdered sample was immediately used for cell disruption and RNA isolation essentially as described above for pure bacterial cell pellets.

**cDNA synthesis and quantitative reverse transcription-PCR.** The expression levels of *L. plantarum* WCFS1 genes derived from cells grown on MRS plates were compared to the expression levels of genes derived from cells grown on MRS plates containing 0.1% porcine bile and to the expression levels in the mouse duodenum. First, cDNA was synthesized by using Superscript III reverse transcriptase (Invitrogen, Breda, The Netherlands), 2 pmol of a gene-specific primer (Table 1), 40 U of RNaseOUT RNase inhibitor, each deoxynucleoside triphosphate at a concentration of 0.5 mM, and either 0.11 μg of total RNA from laboratory medium-grown *L. plantarum* WCFS1 or 2.1 μg of total RNA from a mouse GI tract sample. Reverse transcription was performed at 55°C for 60 min, and this was followed by inactivation of the reverse transcriptase by incubation at 70°C for 15 min. Primers were designed by using Primer 3 (www.genome.wi.mit .edu) and the software package Primer Express (PE Applied Biosystems, Nieuwekerk a/d Ijssel, The Netherlands). All primers were designed to have a melting temperature of 58 to 60°C, and the amplicon sizes ranged from 70 to 81 bp. Quantitative PCR was performed with the synthesized cDNAs by using an ABI Prism 7700 with SYBR Green technology (PE Applied Biosystems). Each 50-μl reaction mixture contained 1× SYBR Green master mixture (Applied Biosystems), each primer at a concentration of 400 nM (Table 1), and 0.1 or 200 ng of reverse-transcribed RNA from either plate-grown WCFS1 cells or a mouse GI tract sample. Amplification was initiated at 95°C for 10 min, and this was followed by 40 cycles of 95°C for 15 s and 55°C for 60 s. The identities of the amplicons resulting from the reactions with cDNA originating from culture- and mouse-derived templates were checked after amplification by melting curve analysis and amplicon DNA sequence analysis. Reaction mixtures containing no template and reaction mixtures containing DNase-treated RNA were included in each real-time PCR experiment to assess contamination and residual chromosomal DNA, respectively. Cycle threshold ($C_t$) values were obtained by manually setting the baseline and threshold values at which fluorescence was appreciably above the background fluorescence for each reaction in the exponential phase of amplification for all reactions. Relative transcript levels were calculated by using the comparative $\Delta\Delta C_t$ method described by Pfaffl et al. (24, 25). By using this method, PCR efficiencies were calculated with the equation $E = 10^{(-1/\text{slope})}$, where the slope is calculated from a standard curve of $C_t$ values obtained for a dilution range of template cDNA. The average $C_t$ values observed for the target gene transcripts (lp_0237, lp_0775, lp_1027, and lp_1898) were normalized to the average $C_t$ values obtained for the reference gene transcripts (16S rRNA) from the same RNA sample. Three or four replicates of all samples and primer pairs were included in each quantitative PCR experiment, and all experiments were performed in triplicate. Statistical analyses of the differences in expression between samples were performed by using group means for statistical significance by the pairwise fixed reallocation randomization test, which was performed with the relative expression software tool (REST) (24, 25).

## RESULTS

**Growth characteristics and morphology of *L. plantarum* in the presence of bile salts.** The effects of different concentrations of bile salts on the morphology and growth of *L. plantarum* were investigated. After pregrowth in liquid MRS, *L. plantarum* WCFS1 was subcultured in MRS containing 0, 0.01, 0.05, 0.1, and 0.15% porcine bile salts. Growth was monitored for 24 h by measuring the optical density at 600 nm ($OD_{600}$), and the data were used to calculate the maximal growth rate of *L. plantarum* in the presence of different concentrations of bile salts (Fig. 1). The maximal growth rate was found to decrease significantly as the bile concentration increased. Moreover, the final $OD_{600}$ reached by *L. plantarum* grown in medium containing 0.1% porcine bile was approximately threefold lower than the final $OD_{600}$ reached by *L. plantarum* grown in standard MRS (data not shown). Four hours after inoculation logarithmically growing cells were collected from the cultures with different bile salt concentrations (Fig. 1), and the *L. plantarum* cell morphology was investigated by scanning electron microscopy (Fig. 2). Cells grown under standard conditions exhibited the characteristic rod-shaped, smooth-surface morphology of *L. plantarum*. When 0.05% bile was present in the medium, the cells had a slight tendency to clump together, their surfaces appeared to be less smooth, and membrane vesicle structures were visible. At bile concentrations of 0.1 and 0.15% these changes in morphology were more pronounced. Moreover, at these higher concentrations of bile a proportion of the bacterial cells had a shrunken and empty appearance. Similar observations were made when the growth rate and morphology on solid medium were investigated. Growth of colonies on MRS plates containing 0.1% bile salts was slightly retarded, and the colonies appeared to be very flat compared to the colonies on plates without added bile salts (data not shown). Since growth was the primary selection criterion in the conditional *alr* complementation screening analysis described below, it was essential to perform this screening analysis by comparing conditions at which the growth rates did not differ greatly. Therefore, a porcine bile concentration of 0.1% was chosen for identification of bile-inducible promoter elements in *L. plantarum*. Notably, 0.1% bile is in the physiological concentration range that occurs in the GI tract (2, 8).

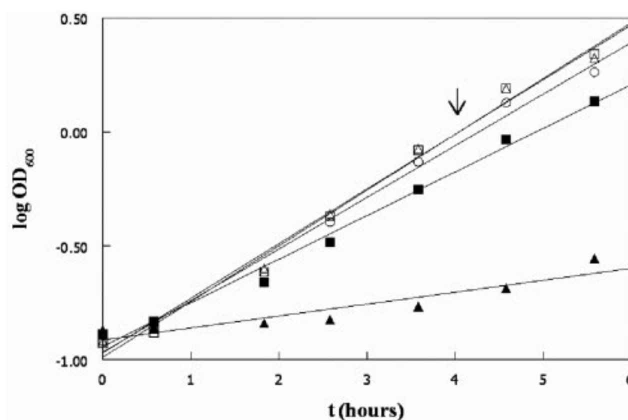


FIG. 1. Maximal growth rateS of *L. plantarum* in the presence of increasing concentrations of bile salts. A full-grown culture was diluted 50-fold in MRS containing no porcine bile (□), 0.01% porcine bile (△), 0.05% porcine bile (○), 0.10% porcine bile (■), or 0.15% porcine bile (▲), and growth was monitored by measuring the $OD_{600}$. The resulting data were used to calculate the maximal logarithmic growth rateS (0.56, 0.54, 0.52, 0.44, and 0.14 $h^{-1}$, respectively). The arrow indicates the time at which the morphology of the cells was investigated (Fig. 2).

**Identification of bile-inducible genes by using the *alr* complementation library.** Use of the essential alanine racemase-encoding *alr* gene as a promoter probe in *L. plantarum* WCFS1 was described recently (5); this included analysis of a chromosomal *L. plantarum* WCFS1 library in the *alr* promoter probe vector pNZ7120. Here, the same library was screened for

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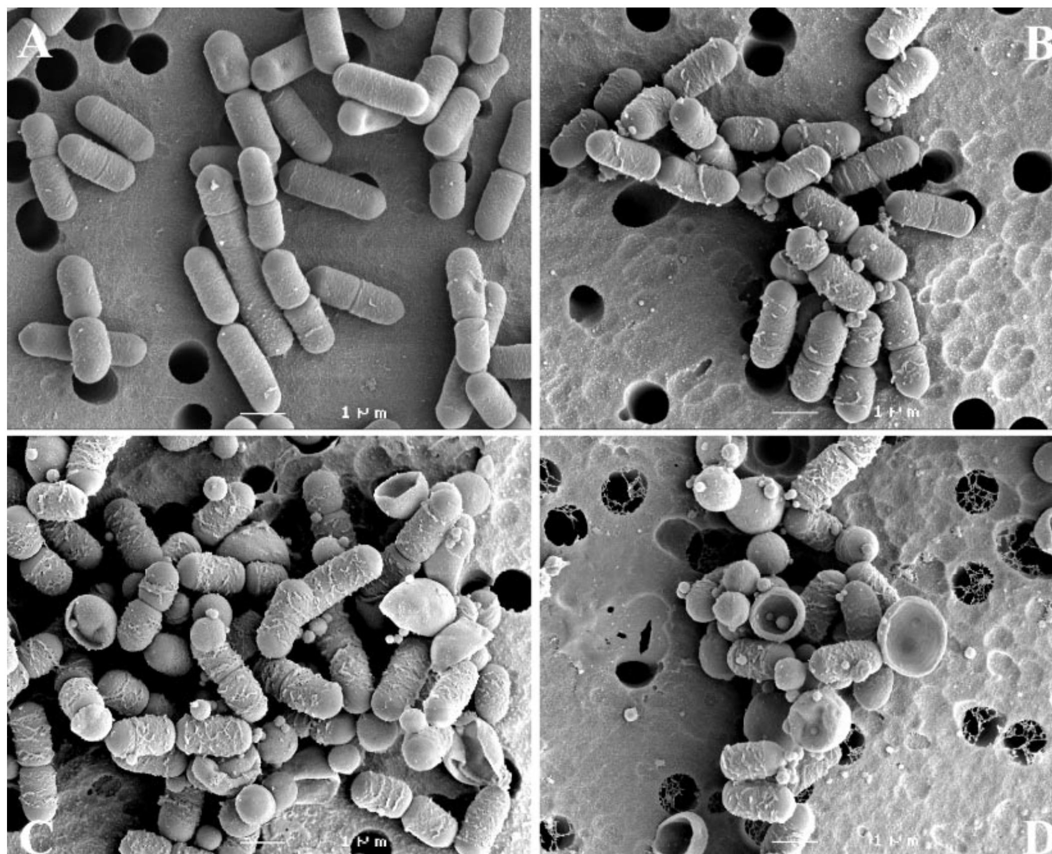


FIG. 2. Morphological changes in *L. plantarum* during bile salt stress. After 4 h of exposure to no bile salts (A), 0.05% bile salts (B), 0.10% bile salts (C), or 0.15% bile salts (D) cells were investigated by scanning electron microscopy.

pNZ7120 derivatives harboring chromosomal *L. plantarum* WCFS1 fragments that contained promoter elements conditionally activated by 0.1% porcine bile. For 72 h the growth characteristics of approximately 4,000 colonies from the *alr* complementation library were compared on plates with and without bile, which resulted in the initial identification of 96 (2.4%) colonies displaying conditional growth only in the presence of bile. This conditional growth phenotype could be confirmed for 46 of these colonies when growth with and without bile was monitored for 72 h on plates containing D-cycloserine at concentrations ranging from 0 to 200 μg/ml. The partial sequences of the chromosomal inserts present in the pNZ7120 derivatives originating from 41 of the 46 clones were successfully determined. These sequences corresponded to 30 unique loci of the *L. plantarum* genome, since one locus was found six times, one locus was found three times, and four loci were found twice. Two independent clones (BI21 and BI49) both contained a different chromosomal fragment corresponding to lp_3415, encoding a transcription regulator homologue, and its upstream sequence (Table 2). Hence, the bile induction of lp_3415 was independently confirmed twice during the screening procedure. According to the current genome annotation database for *L. plantarum* WCFS1 (19), the loci harbor 31 unique genes and their upstream sequences in the proper orientation, which explains the observed induction of *alr* expression (Table 2). Notably, eight loci contained more than one putative 5′ end of an annotated open reading frame (ORF) and the upstream region. All ORFs that were identified in this *alr* complementation screening analysis were functionally categorized in groups involved in cell membrane function (eight ORFs), cell wall function (three ORFs), redox reactions (five ORFs), regulation (five ORFs), and other functions (four ORFs). The remaining six genes encoded (conserved) hypothetical proteins with unknown functions (Table 2).

Clone BI41 harbors three genes, including a gene encoding a homologue of the bile acid 7α-dehydratase of *Eubacterium* sp. strain VPI 12708 and *Clostridium* sp. strain TO-931 (30 and 28% identity at the protein level, respectively) (21, 36). In these intestinal organisms the *baiE* gene, encoding bile acid 7α-dehydratase, is localized in an operon. In *Eubacterium* the expression of this operon appeared to be induced in the presence of bile (21). A similar operon structure is not found in *L. plantarum*. Nevertheless, the fact that this *baiE* homologue was identified in the *alr* complementation screening analysis strongly suggests that the expression of this single gene is also regulated by bile in *L. plantarum* WCFS1. Clone BI87 harbors a 3′-truncated fragment of lp_1435, encoding an integral membrane protein, which was previously identified in an *alr* complementation screening analysis for high-salt-inducible promoters of *L. plantarum* (5). These findings suggest that there is a partial overlap in the responses of this organism to high NaCl concentrations and bile salts, possibly caused by the membrane stress induced under both these conditions. A very striking observation was the identification of lp_0237 and lp_0775, en-

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TABLE 2. Identification of clones in the *alr* complementation library that display conditional growth only in the presence of 0.1% porcine bile

| Clone(s) | Insert start coordinate | Estimated insert size (kb) | lp no. (gene)[a] | Gene product | Growth with D-cycloserine at the following concn (μg/ml)[b]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0 | 2.5 | 5 | 10 | 25 | 50 | 100 | 200 |
| BI1 | 79159 | 1.0 | lp_0082[c] | Oxidoreductase | | | X | X | | | | |
| BI84 | 80981 | 2.3 | lp_0085[d] | Cation efflux protein | | | | | | | X | |
| BI19 | 215774 | 1.5 | lp_0237[d] | Integral membrane protein | | | | | | X | X | X |
| | | | lp_0240[e] | Hypothetical protein | | | | | | | | |
| BI79 | 711316 | 2.0 | lp_0775 (*argG*)[f] | Argininosuccinate synthase | | | | | | X | X | |
| | | | lp_0774 (*luxS*)[f] | Autoinducer production protein | | | | | | | | |
| BI26, BI81 | 800854 | 2.0 | lp_0858[c] | Redox protein, regulation of disulfide bond formation | | | | | X | X | X | |
| BI65 | 1057803 | 1.5 | lp_1158 (*lys*)[g] | Lysozyme | | | | | X | X | X | |
| | | | lp_1157[h] | Transcription regulator (RpiR family) | | | | | | | | |
| BI87 | 1315054 | 0.9 | lp_1435[d] | Integral membrane protein | | | | | | X | X | X |
| BI14, BI95 | 1335147 | 1.5 | lp_1459[e] | Conserved hypothetical protein | | | | X | X | | | |
| BI55 | 2013511 | 1.5 | lp_2230[e] | Conserved hypothetical protein | X | X | X | X | X | | | |
| BI9 | 2218907 | 1.5 | lp_2484[h] | Transcription regulator (MarR family) | | X | X | X | X | X | | |
| BI96 | 2284309 | 1.5 | lp_2564[d] | Integral membrane protein | | | | | X | X | X | X |
| BI31 | 2528088 | 1.5 | lp_2835[f] | 2-Haloacid dehalogenase | | | | | X | X | | |
| BI64 | 2814574 | 2.0 | lp_3145[c] | Oxidoreductase, N-terminal fragment | | | | | X | X | | |
| BI40 | 2819823 | 1.5 | lp_3155[d] | Cell surface protein, ErfK family | | | | | | | | |
| | | | lp_3154 (*acm3-C*)[g] | Muramidase, C-terminal fragment | | | | | | X | X | |
| | | | lp_3153 (*acm3-M*)[g] | Muramidase, middle fragment | | | | | | | | |
| BI41 | 2821528 | 1.7 | lp_3158[c] | Oxidoreductase | | | | | | | | |
| | | | lp_3159 (*baiE*)[f] | Bile acid 7α-dehydratase | | | | | X | X | | |
| | | | lp_3160[d] | Multidrug transport protein | | | | | | | | |
| BI28 | 2832336 | 2.0 | lp_3175[d] | Integral membrane protein | | X | X | X | X | X | | |
| BI45 | 2962988 | 2.0 | lp_3330[e] | Conserved hypothetical protein | | | | | X | X | | |
| BI50 | 2976142 | 2.0 | lp_3343[e] | Conserved hypothetical protein | | | | | | | | |
| | | | lp_3344[h] | Transcription regulator (MarR family) | | | | | X | X | X | |
| | | | lp_3345 (*nrpR4*)[h] | Negative regulator of proteolysis | | | | | | | | |
| BI21, BI49 | 3032470 | 1.5 | lp_3415[e] | Conserved hypothetical protein | | | | | | | X | X |
| BI29 | 3100458 | 1.5 | lp_3488 (*galR2*)[h] | Galactose operon repressor (LacI family) | | | | | | | X | X |
| | | | lp_3489[c] | Oxidoreductase | | | | | | | | |
| BI2, BI7, BI8, BI14, BI63, BI69 | 3240643 | 1.5 | lp_3626[d] | Sugar transport protein | | | | X | X | X | X | |

[a] When multiple ORFs in the correct orientation to drive *alr* expression are present in a clone, the ORF that is located closest to the *alr* gene is presented first.
[b] An X indicates that growth was observed only in the presence of bile.
[c] Redox reaction gene.
[d] Gene with a membrane-associated function.
[e] Gene encoding a hypothetical protein.
[f] Gene with another function.
[g] Gene with a cell-wall-associated function.
[h] Regulation gene.

coding an integral membrane protein and an argininosuccinate synthase, in clones BI19 and BI79, respectively (Table 2). Both of these genes were previously identified in our laboratory by a resolvase-based in vivo expression technology approach as being important for *L. plantarum* during passage through the GI tract of mice (4).

**Expression analysis of lp_0237 and lp_0775.** The bile-inducible characteristics of lp_0237 and lp_0775 were investigated further by quantitative reverse transcription-PCR. RNA was isolated from *L. plantarum* cells grown on plates with and without bile. The isolated RNA samples were used for gene-specific synthesis of cDNA, which was used as a template for quantitative real-time PCR with specific primers for the bile-induced genes lp_0237 and lp_0775 and the 16S rRNA gene. The latter RNA was used to correct for the total amount of *L. plantarum*-specific RNA added to the different reaction mixtures. Negative control reaction mixtures containing the *L. plantarum*-specific16S rRNA primers and DNase-treated RNA were included in each PCR. These reactions never produced any detectable amplicon, indicating that there was no DNA contamination in the RNA samples. Moreover, the specificity of the PCRs was confirmed by a combination of melting curve analysis and DNA sequence analysis of the amplicons (data not shown). All signals were correlated to the 16S rRNA signal derived from the corresponding cDNA samples. The in vitro induction of lp_0237 and lp_0775 by the presence of porcine bile was investigated. This experiment showed that the expression levels of lp_0237 and lp_0775 were significantly induced (24- and 4-fold, respectively) in cells grown on plates containing 0.1% porcine bile compared to control plates lacking bile (Table 3). These data demonstrate that the bile-inducible regulatory characteristics obtained for these two genes by using the plasmid-based *alr* promoter probe can be extrapolated to the native, single-copy situation on the chromosome. Moreover, the observed induction by bile suggests that the previously observed in vivo induction (4) of lp_0237 and lp_0775 occurs in the duodenum, as this is the site of bile release in the host. Therefore, RNA was isolated from duodenum samples from a mouse fed *L. plantarum*, and a second quantitative reverse transcriptase PCR experiment was performed to assess the in vivo expression levels of lp_0237 and lp_0775 in the duodenum. This experiment revealed signifi-

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TABLE 3. Levels of induction of *L. plantarum* genes in vitro by 0.1% bile and in situ in the duodenum of a mouse model compared to the level in MRS without bile

| lp no. (gene) | Function | Fold induction in[a]: | |
|---|---|---|---|
| | | MRS + 0.1% bile | Duodenum |
| lp_0237 | Integral membrane protein | 23.7[b] | 12.5[b] |
| lp_0775 (*argG*) | Argininosuccinate synthase | 4.3[b] | 28.9[b] |
| lp_1027 (*fusA2*) | Elongation factor | ND[c] | 2.5 |
| lp_1898 (*pfk*) | 6-Phosphofructokinase | ND | 1.4 |

[a] The coefficient of variation among replicates ($n = 3$) was <25%.
[b] The relative expression level of the target gene is significantly different from the level observed in MRS lacking bile according to the pairwise fixed reallocation randomization test ($P < 0.05$).
[c] ND, not determined.

cantly higher expression levels (13- and 29-fold, respectively) for these genes during passage through the mouse duodenum than in *L. plantarum* grown on MRS, while in an identical experiment the expression levels of two *L. plantarum* household genes (lp_1027 [*fusA2*] encoding an elongation factor and lp_1898 [*pfk*] encoding 6-phosphofructokinase) were not significantly increased in vivo compared to the expression levels in MRS (Table 3). These data demonstrate that the in vitro regulatory characteristics observed for lp_0237 and lp_0775 can be translated to the in vivo situation in the mouse duodenum and strongly suggest that contact with bile is the inducing environmental factor in vivo.

## DISCUSSION

In this paper we describe the growth, morphological, and genetic responses of *L. plantarum* WCFS1 to bile salts. A stepwise increase in the porcine bile concentration resulted in a stepwise decrease in the maximal growth rate and the final $OD_{600}$ of *L. plantarum*. The observed gradual decrease in the growth rate coincided with the gradually increasing severity of changes in morphology, including bulky structures on the cell surface, the formation of membrane vesicles, and clumping of the cells. Moreover, the observed formation of ghost cells suggests that cell wall integrity was lost after addition of bile, possibly leading to leakage of intracellular material from the cells and a disturbed energy balance. The bile-induced morphological changes in *L. plantarum* are very similar to those observed in *P. freudenreichii* (20). Furthermore, leakage of proteins from cells after bile treatment was previously observed in other LAB, including *P. freudenreichii* and *Lactobacillus acidophilus* (20, 22). Since a porcine bile concentration of 0.1% had only a marginal effect on the growth rate but nevertheless resulted in severe morphological changes, this physiologically relevant concentration was used in the rest of the experiments.

The previously constructed *alr* complementation library (5) was exploited for identification of clones containing *L. plantarum* chromosomal fragments that harbor promoter elements conditionally activated by bile. This approach resulted in the identification of 30 unique loci harboring 31 putative genes whose expression is potentially induced by bile. The putative genes identified as bile inducible were organized in six functional categories (Table 2). Strikingly, 11 of these ORFs encode proteins involved in membrane- and cell wall-associated functions. The induction of this relatively high number of genes involved in cell envelope functions is in agreement with the observed morphological changes in *L. plantarum* in the presence of bile. Notably, genes encoding putative functions involved in fatty acid and cell wall biosynthesis have previously been identified as important for the bile resistance of *E. faecalis* (3). The group of genes encoding membrane-associated functions includes the genes encoding three possible exporter proteins, namely, lp_0085, lp_2564, and lp_3160. The latter gene is annotated as a multidrug transporter gene, suggesting a possible role in the export of bile or bile-derived compounds. Similarly, lp_2564, encoding a protein with significant homology to a permease in *Bacillus cereus*, could play a role in the export of bile. Finally, lp_0085, encoding a putative efflux protein, might be involved in maintenance of the electrochemical membrane potential under bile-induced stress conditions. Remarkably, the importance of efflux pumps in bile resistance was previously demonstrated in several bacteria, including *Escherichia coli* and *L. monocytogenes* (2, 15). The genes encoding three cell wall-associated functions identified here include a putative lysozyme gene and two genes annotated to encode fragments of a possible muramidase. Moreover, BlastP analysis of lp_3154 demonstrated that there was 35% identity with the gene encoding a choline binding protein from *Streptococcus pneumoniae*. The chemical structure of choline is similar to that of bile. Therefore, the protein encoded by lp_3154 could be important in the defense of *L. plantarum* against bile salts.

Five genes identified here as bile-inducible genes are annotated as having functions involved in redox reactions (namely, four oxidoreductases and a redox protein acting as a regulator of disulfide bond formation), suggesting that bile-induced redox balance disturbance and/or oxidative stress occurs. Notably, a gene encoding a function involved in a redox reaction was previously recognized as important during bile stress in *E. faecalis* (3). Another group of five genes encoding regulatory functions was identified as bile inducible in *L. plantarum*. Remarkably, two of these genes (lp_2484 and lp_3344) belong to the *marR* family of regulators. In several bacteria, including *E. coli* and *Salmonella enterica* serovar Typhimurium, the MarA and MarR proteins mediate the expression of a diverse set of genes involved in multidrug resistance, including genes encoding multidrug efflux proteins (14, 32). The lp_3344 gene product exhibits 28% identity with MarR from *S. enterica* serovar Typhimurium. Moreover, the conserved hypothetical protein encoded by lp_3415 exhibits 30% identity with MarA from *S. enterica* serovar Typhimurium. Therefore, lp_3344 and lp_3415 might be involved in the regulation of multidrug transporters in *L. plantarum*, possibly including the two transporters encoded by lp_2564 and lp_3160 mentioned above (Table 2). Next to lp_3415 four other conserved genes and one unique hypothetical protein gene were identified in the screening for bile-inducible *L. plantarum* ORFs. The role of these genes in bile resistance remains to be determined.

Bile induction could reflect the in situ conditions encountered by *L. plantarum* during passage through the gastrointestinal tract. Two striking findings in this context are lp_0237 and lp_0775, which we identified as bile-inducible genes in the in vitro *alr* complementation screening analysis described here; these genes have been identified previously in *L. plantarum* as genes that are induced in vivo during passage through the

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mouse GI tract (4). By using quantitative reverse transcription-PCR, the in vitro induction by bile and the in vivo induction in the duodenum of a mouse model system compared to laboratory conditions could be established for these genes. In conclusion, in this paper we provide valuable data on the in vitro genetic response of *L. plantarum* to bile. The experiments described here demonstrated that simplified in vitro mimicking of complex environmental niches can result in the identification of genes that are relevant in situ in these niches. Moreover, this approach potentially provides clues to the environmental trigger involved in the in situ regulation of specific genes, which should enable future unraveling of the genetic behavior of *L. plantarum* during passage through (specific parts of) the GI tract.

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## REFERENCES

1. **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.
2. **Begley, M., C. G. Gahan, and C. Hill.** 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Appl. Environ. Microbiol. **68:**6005–6012.
3. **Breton, Y. L., A. Maze, A. Hartke, S. Lemarinier, Y. Auffray, and A. Rince.** 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis.* Curr. Microbiol. **45:**434–439.
4. **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.
5. **Bron, P. A., S. M. Hoffer, S. Van II, W. M. De Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310–317.
6. **Chou, L. S., and B. Weimer.** 1999. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus.* J. Dairy Sci. **82:**23–31.
7. **Christiaens, H., R. J. Leer, P. H. Pouwels, and W. Verstraete.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. Appl. Environ. Microbiol. **58:**3792–3798.
8. **De Boever, P., and W. Verstraete.** 1999. Bile salt deconjugation by *Lactobacillus plantarum* 80 and its implication for bacterial toxicity. J. Appl. Microbiol. **87:**345–352.
9. **Duran Quintana, M. C., P. Garcia Garcia, and A. Garrido Fernandez.** 1999. Establishment of conditions for green table olive fermentation at low temperature. Int. J. Food Microbiol. **51:**133–143.
10. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol. Microbiol. **45:**1095–1106.
11. **Enan, G., A. A. el-Essawy, M. Uyttendaele, and J. Debevere.** 1996. Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. Int. J. Food Microbiol. **30:**189–215.
12. **Flahaut, S., J. Frere, P. Boutibonnes, and Y. Auffray.** 1996. Comparison of the bile salts and sodium dodecyl sulfate stress responses in *Enterococcus faecalis.* Appl. Environ. Microbiol. **62:**2416–2420.
13. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20:**3304–3309.
14. **Grkovic, S., M. H. Brown, and R. A. Skurray.** 2002. Regulation of bacterial drug export systems. Microbiol. Mol. Biol. Rev. **66:**671–701.
15. **Gunn, J. S.** 2000. Mechanisms of bacterial resistance and response to bile. Microbes Infect. **2:**907–913.
16. **Hofmann, A. F., G. Molino, M. Milanese, and G. Belforte.** 1983. Description and simulation of a physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids in man. J. Clin. Investig. **71:**1003–1022.
17. **Hofmann, A. F., and K. J. Mysels.** 1992. Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and $Ca^{2+}$ ions. J. Lipid Res. **33:**617–626.
18. **Hyronimus, B., C. Le Marrec, A. H. Sassi, and A. Deschamps.** 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. Int. J. Food Microbiol. **61:**193–197.
19. **Kleerebezem, M., J. Boekhorst, R. Van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. De Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. USA **100:**1990–1995.
20. **Leverrier, P., D. Dimova, V. Pichereau, Y. Auffray, P. Boyaval, and G. Jan.** 2003. Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. Appl. Environ. Microbiol. **69:**3809–3818.
21. **Mallonee, D. H., W. B. White, and P. B. Hylemon.** 1990. Cloning and sequencing of a bile acid-inducible operon from *Eubacterium* sp. strain VPI 12708. J. Bacteriol. **172:**7011–7019.
22. **Noh, D. O., and S. E. Gilliland.** 1993. Influence of bile on cellular integrity and beta-galactosidase activity of *Lactobacillus acidophilus.* J. Dairy Sci. **76:**1253–1259.
23. **Palumbo, E., C. F. Favier, M. Deghorain, P. S. Cocconcelli, C. Grangette, A. Mercenier, E. E. Vaughan, and P. Hols.** 2004. Knockout of the alanine racemase gene in *Lactobacillus plantarum* results in septation defects and cell wall perforation. FEMS Microbiol. Lett. **233:**131–138.
24. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.
25. **Pfaffl, M. W., G. W. Horgan, and L. Dempfle.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. **30:**e36.
26. **Phan-Thanh, L., and T. Gormon.** 1997. Stress proteins in *Listeria monocytogenes.* Electrophoresis **18:**1464–1471.
27. **Rince, A., Y. Le Breton, N. Verneuil, J. C. Giard, A. Hartke, and Y. Auffray.** 2003. Physiological and molecular aspects of bile salt response in *Enterococcus faecalis.* Int. J. Food Microbiol. **88:**207–213.
28. **Ruiz-Barba, J. L., J. C. Piard, and R. Jimenez-Diaz.** 1991. Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations. J. Appl. Bacteriol. **71:**417–421.
29. **Schmidt, G., and R. Zink.** 2000. Basic features of the stress response in three species of bifidobacteria: *B. longum*, *B. adolescentis*, and *B. breve.* Int. J. Food Microbiol. **55:**41–45.
30. **Shah, N. P.** 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci. **83:**894–907.
31. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin-10. Science **289:**1352–1355.
32. **Sulavik, M. C., M. Dazer, and P. F. Miller.** 1997. The *Salmonella typhimurium mar* locus: molecular and genetic analyses and assessment of its role in virulence. J. Bacteriol. **179:**1857–1866.
33. **Tanaka, H., K. Doesburg, T. Iwasaki, and I. Mierau.** 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. J. Dairy Sci. **82:**2530–2535.
34. **Tannock, G. W., A. Tangerman, A. Van Schaik, and M. A. McConnell.** 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. Appl. Environ. Microbiol. **60:**3419–3420.
35. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823–828.
36. **Wells, J. E., and P. B. Hylemon.** 2000. Identification and characterization of a bile acid 7α-dehydroxylation operon in *Clostridium* sp. strain TO-931, a highly active 7α-dehydroxylating strain isolated from human feces. Appl. Environ. Microbiol. **66:**1107–1113.

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