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Spatial and Temporal Expression of *Lactobacillus plantarum* Genes in the Gastrointestinal Tracts of Mice▽†

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Lactobacillus plantarum **is a common inhabitant of mammalian gastrointestinal tracts, and *L. plantarum* strain WCFS1 is a human isolate with a known genome sequence. *L. plantarum* WCFS1 survives intestinal passage in an active form, and its transit time and transcriptional activities were monitored in 15 BALB/c mice at 2, 4, 6, 8, and 24 h after being fed a single intragastric dose of this organism. Enumeration of viable cells isolated from fecal material revealed that the majority of the *L. plantarum* inoculum transited the mouse intestine within 4 h after ingestion. Three mice were sacrificed at each time point, and total RNA was isolated from the mouse intestinal compartments (stomach through colon). Quantification of *L. plantarum* 16S rRNA by quantitative real-time reverse-transcription-PCR revealed that *L. plantarum* was present at elevated levels in the stomach and small intestine for at least 4 h following ingestion and for over 8 h in the cecum and colon. We also examined the expression of 9 *L. plantarum* housekeeping genes and 15 *L. plantarum* in vivo-inducible (*ivi*) genes previously identified by recombination-based in vivo expression technology to be induced in the mouse gastrointestinal tract. The relative expression levels of the *ivi* genes increased up to 350-fold in the mouse intestine compared to levels observed for *L. plantarum* WCFS1 cells grown in a rich laboratory medium. Moreover, several genes displayed intestinal compartment-specific (small intestine versus colon) activities. These results confirm that *L. plantarum* displays specific and differential responses at various sites along the mammalian intestine.**

The human gastrointestinal tract contains over 10^{13} bacterial cells, comprising more than 500 different species (13; for a review, see reference 40). These microorganisms perform many critical functions, including digestion and assimilation of nutrients, protection against pathogen colonization, activation of immunological surveillance signals, regulation of fat storage, and stimulation of intestinal angiogenesis (3, 39).

Members of the genus *Lactobacillus* are commonly found in human and animal gastrointestinal tracts and are considered to be among the most dominant organisms colonizing the small intestine (SI). Lactobacilli belong to the lactic acid bacteria (LAB), which comprise a variety of microorganisms applied to a variety of industrial and artisanal dairy, meat, and plant fermentations. Some selected strains of *Lactobacillus* are believed to be beneficial to human and animal health and are marketed as probiotics (28). While consumer interest in probiotics is growing (1), the activities of probiotics in the gastrointestinal tract and the mechanisms by which they exert their health-modulating effects remain largely unknown (16).

The genome of *Lactobacillus plantarum* WCFS1 was the first *Lactobacillus* genome to be sequenced (15). This strain originates from human saliva and displays good survival and persistence properties in the human gastrointestinal tract compared to other LAB (36). In-depth genome annotation has provided molecular maps and detailed catalogues of metabolic pathways for this organism (14, 31, 34). Additionally, whole-genome genotyping of different *L. plantarum* strains by using *L. plantarum* WCFS1-based DNA microarrays revealed variable genomic regions that are probably involved in strain-specific adaptation to different habitats (20). Such studies also enabled genotype-phenotype matching, leading to the identification of the *L. plantarum* gene encoding a mannose-specific adhesion (25). This *L. plantarum* adhesion might protect the host from infection by competing with pathogenic bacteria for attachment at mannose-receptor binding sites on the intestinal epithelial surface.

The specific activities of *Lactobacillus* in the gastrointestinal tract are being investigated through the application of in vitro- and in vivo-based approaches. In vitro genetic screening strategies, including promoter trapping (6, 7) and transcriptome analysis (9), were used to identify bile- and salt-inducible genes of *L. plantarum* WCFS1 and acid tolerance genes of *Lactobacillus acidophilus* (2). Because low pH, bile acids, and increasing osmolarity are encountered by bacteria during gastrointestinal tract transit, the genes identified in these studies are likely to encode functions that enable *Lactobacillus* to compete and survive better in this harsh environment. Several strains of *Lactobacillus* also appear to be metabolically active in vivo in the intestine. *Lactobacillus casei* was shown to actively transcribe genes involved in central metabolism and initiate de novo protein synthesis at various sites along the mouse intestine (21, 23). Moreover, application of in vivo expression technology (IVET) resulted in the identification of gut-inducible genes of *Lactobacillus reuteri* and *L. plantarum* WCFS1 in mouse model systems (5, 38). Three *L. reuteri* genes with in vivo-inducible (*ivi*) gene activities were identified, one of which (encoding methionine sulfoxide reductase) was found to be required for competitive intestinal colonization of mice (37,

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38). In total, 72 *L. plantarum* WCFS1 *ivi* genes were identified using a recombination-based IVET (R-IVET) approach (5). These genes encode various classes of proteins, including those involved in nutrient acquisition and synthesis, transcription regulation, and adaptation to environmental stresses (5).

The R-IVET approach identified *L. plantarum* WCFS1 genes that are expressed at a low level in culture medium but are induced in the mouse intestine. However, *ivi* gene expression levels and the intestinal locations at which these genes were most highly expressed remained to be determined. Here, we address this issue by using real-time reverse-transcription (RT)-PCR to measure the transcript abundances of 15 *L. plantarum* WCFS1 *ivi* genes during mouse intestinal transit. This analysis confirmed that most *ivi* genes, and not *L. plantarum* housekeeping genes, are up-regulated in vivo. Moreover, the expression levels of several *ivi* genes differed considerably between the mouse intestinal compartments, thereby illustrating the dynamic activities of *L. plantarum* in the intestinal tract.

MATERIALS AND METHODS

Preparation of bacterial strain and administration to mice. Wild-type *L. plantarum* WCFS1 (15) was grown at 37°C in 200 ml Mann Rogosa Sharpe (MRS) broth (Difco, Surrey, United Kingdom) (12) without aeration (14.5 h). The cells were collected by centrifugation for 10 min at 600 × *g* and then resuspended in MRS broth to a final volume of 2.5 ml. This cell suspension was divided into separate aliquots for RNA isolation, viable-cell-number determination, and inoculation into mice. Fifteen 7-week-old female BALB/c mice (Wageningen University, Harlan, Horst, The Netherlands) were fed directly into the stomach by gavage with 100 μl of the *L. plantarum* cell suspension and placed in groups of three into separate cages (time zero). The mice were housed in standard cages with free access to tap water and mouse chow throughout the duration of the experiment. The mice were sacrificed by cervical dislocation in groups of three at 2, 4, 6, 8, and 24 h after inoculation. An additional group of three mice were sacrificed 4 h after being fed sterile MRS broth (100 μl). The mouse digestive tracts were immediately excised, sectioned, frozen in liquid nitrogen, and stored at −80°C until RNA isolation. The animal welfare committee of Wageningen University (Wageningen, The Netherlands) approved the experimental protocol used in this study.

Enumeration of viable lactobacilli in mouse feces. Mouse fecal material was collected before and after intragastric gavage. To ensure an absence of fecal contamination from earlier time points, the mice were transferred into cages containing fresh bedding at the time point prior to sacrifice. Approximately 0.1 g of mouse fecal material was placed into tubes containing 3 ml sterile phosphate-buffered salt solution (30) and 1.0 g glass beads (2 mm) for homogenization by vortexing. Numbers of viable cells in fecal material were determined by plating serial dilutions of the suspension on MRS agar, followed by aerobic incubation for 3 days at 30°C. For species identification, the V1-to-V3 regions of the 16S rRNA genes of individual colonies were amplified by PCR according to standard procedures (33). Purified PCR products were then sequenced, and these sequences were compared to 16S rRNA sequences from the Ribosomal Database Project (11).

RNA isolation. Total RNA was isolated from *L. plantarum* and mouse tissues (weighing between 0.1 and 0.7 g) by using a bead-beating procedure as described previously, with the exception that the frozen samples were placed directly in ice-cold tubes containing 0.5 g of 0.1 mm zirconia/silica beads (Biospec, Bartlesville, OK), 0.5 ml phenol, and 0.5 ml TE (Tris-EDTA) buffer containing 1% sodium dodecyl sulfate and 0.01% macaloid clay (Kronos Titan GmbH, Germany) (7). RNA was further purified and treated with DNase I on QIAGEN RNeasy Midi columns (QIAGEN, Venlo, The Netherlands) according to the manufacturer's protocol. The concentration and quality of isolated RNA were assessed by spectrophotometry at absorbance levels of 260 and 280 nm (Ultrospec 2000; GE Healthcare, Diegem, Belgium) and by analysis with a 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). All bioanalyzer profiles showed distinct peaks for mouse 28S and 18S rRNA, whereas measurable levels of 23S and 16S rRNA (typical 23S-versus-16S rRNA ratio of 1.7) were observed only for the cecum and colon samples.

Primer design. All primers used in this study are listed in Table 1. Primers were designed using Primer 3 (29) and the software package Primer Express (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The presence of secondary structures, including possible primer-dimers, was evaluated using NetPrimer (Premier Biosoft International, Palo Alto, CA). All primers were designed to have melting temperatures of 58 to 60°C according to Primer Express and amplicon sizes between 70 and 130 bp. The specificities of the primers to *L. plantarum* WCFS1 were evaluated by nucleotide similarity searches with the BLAST algorithm for short, nearly exact matches at the NCBI website (http://www.ncbi.nlm.nih.gov) (19). In silico comparisons and PCR amplification products confirmed that the 16S rRNA gene primer set was specific for both *L. plantarum* and the closely related species *Lactobacillus pentosus* but not other *Lactobacillus* species (data not shown).

cDNA construction and real-time PCR assays. To eliminate remaining genomic DNA contamination, a second DNase treatment (DNase I; Invitrogen, Breda, The Netherlands) was included for all RNA samples prior to first-strand cDNA synthesis. For every 5 μg of total RNA, 20-μl reaction mixtures containing 200 U Superscript III reverse transcriptase (Invitrogen), 40 U RNaseOUT RNase inhibitor (Invitrogen), 1 mM of each deoxynucleoside triphosphate, and 2 pmol of each gene-specific primer complementary to the mRNA of specified genes (Table 1) were prepared. RT reactions were performed in duplicate and contained 3 ng or 60 ng total RNA collected from *L. plantarum* WCFS1 cells grown in laboratory culture and either 80 ng or 12 μg mouse digestive tract total RNA depending on whether subsequent PCRs would be performed for detection of 16S rRNA or protein-encoding gene transcript analysis, respectively. RT was carried out according to the manufacturer's protocol. The resulting cDNA samples were stored at −20°C until use.

Real-time PCR amplification was performed in 96-well plates on an ABI Prism 7700 sequence detection system (Applied Biosystems), using the double-stranded DNA intercalating fluorescent agent SYBR green for product detection. Each well contained SYBR green Master Mix (Applied Biosystems), 200 nM of each primer, and a template. Templates consisted of cDNA products equivalent to either 0.01 or 1 ng RNA from the *L. plantarum* WCFS1 laboratory culture and either 2 or 200 ng mouse digestive tract total RNA. PCR amplification was initiated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 55°C for 60 s. Control PCRs were included to detect background contamination (no-template control) and remaining chromosomal DNA (RT reactions in which Superscript III was omitted). Spiking experiments in which *L. plantarum* WCFS1 RNA was added to the mouse-derived RNA samples confirmed that real-time RT-PCR amplification was not inhibited, even when these reactions were initiated with large amounts of mouse-derived RNA.

PCR specificity and product detection were checked postamplification by examining the dissociation curves of the PCR products. These melting curve profiles were generated by first heating the samples to 95°C and then cooling them to 55°C and slowly heating them at 2°C/min to 95°C for detection of SYBR green fluorescence. Melting curve profiles were analyzed and compared using Dissociation Curve software 1.0 (Applied Biosystems).

Schema for analysis of *L. plantarum* gene transcripts in the mouse intestine. Real-time RT-PCR was performed for the quantification of 15 *L. plantarum ivi* genes and 9 housekeeping gene transcripts in the stomach and small intestine (regions SI-1 to SI-3) at 2 h and in the cecum and colon at 8 h after the ingestion of *L. plantarum* WCFS1 (Fig. 1A). To determine whether *L. plantarum* gene transcripts were measurable in the small intestine at other time points, equivalent amounts of compartment-specific, total RNA from individual mouse samples were combined for real-time RT-PCR transcript analysis (Fig. 1A). Transcripts of some genes were detected in the small intestine samples at 4 h but not at 6 and 8 h, and therefore, the analysis was expanded to quantify these gene transcripts among individual mice at this time point (Fig. 1B). Finally, a smaller set of highly abundant housekeeping and *ivi* gene transcripts were also examined in the ceca and colons of individual mice at all possible time points to obtain a comprehensive view of their expression through time and location in the digestive tract (Fig. 1C).

Data analysis. Real-time PCR quantification is based on the number of cycles required for amplification-associated fluorescence to reach the detection threshold (as expressed by the cycle threshold [C_T] value). C_T values were obtained for each reaction by manually setting the baseline to a level at which fluorescence was noticeably above the background level and exponential amplification was under way. Two independent RT reactions and real-time PCR amplifications were performed for each gene in every sample, and the average of the observed C_T values was used for further analysis. Real-time PCR amplification efficiencies were determined for each primer pair by standard curves generated by plotting the starting RNA concentration against the observed C_T values over a 1×10^4-fold dilution range of cDNA input. The slope of the calibration curve was used to determine the reaction efficiency (E) as $E = 10^{-1/\text{slope}}$ (Table 1) (26).

To estimate the number of *L. plantarum* cells contained in the mouse digestive

TABLE 1. Gene targets and primers

Locus tag[a]	Gene	Function	Forward primer	Reverse primer	*E* value	Source or reference
General						
	16S rRNA	SSU ribosome (5 copies)	TGATCCTGGCTCAGGACGAA	TGCAAGCACCAATCAATACCA	1.90	7
lp_0006	*gyrB*	DNA gyrase B	GGAATTGATGAAGCCCTAGCAG	GAATCCCACGACCGTTATCA	1.90	This study
lp_0537	*ldhL*	L-Lactate dehydrogenase	TGATCCTCGTTCCGTTGATG	CCGATGGTTGCAGTTGAGTAAG	1.92	This study
lp_0727	*groES*	Chaperone	CCCAAAGCGGTAAGGTTGTT	CTTCACGCTGGGGTCAACTT	1.97	This study
lp_1021	*rpoB*	DNA-directed RNA polymerase	CACCGTACCCGTAGAAGTTATGC	GGAGACCTTGATCCAAGAACCA	1.91	This study
lp_1027	*fusA2*	Elongation factor G	CCCATGATGGTGCTTCACAA	TCGTGGCAGCAGAGGTAATG	1.89	7
lp_1144	*pcrA*	ATP-dependent DNA helicase	AGGAGGTCTGGGTCTCAACG	AAGGTCCGTTGCTCGCTAGT	1.95	This study
lp_1898	*pfk*	6-Phosphofructokinase	GTGGCGACGGTTCTTACCAT	CCCTGGAAGACCAATCGTGT	1.93	7
lp_2187	*ileS*	Isoleucine-tRNA ligase	GGCACCTTACGTTCCTGGTT	CGTCATCTTCTTGCGGTCAT	1.90	This study
lp_2301	*recA*	Recombinase A	GGCAGAACAGATCAAGGAAGG	TATCCACTTCGGCACGCTTA	1.91	This study
ivi gene						
lp_0017	*proA*	Gulamate-5-semialdehyde dehydrogenase	CGTGAGTTTGCCAGATCCAA	CATGCCGATAACACCTAATGG	2.00	This study
lp_0237		Integral membrane protein (hypothetical)	GTACTGATATGGTTGTCGGGAATTA	ACGGGTGCGTAGAAGAAGC	1.92	7
lp_0419	*plnI*	Membrane-bound protease, immunity protein	CGCTTCGATGATGACTGCTT	CTGACCATACGGGCTGTGAT	1.91	This study
lp_0775	*argG*	Argininosuccinate synthase	GCTCTTGCACCGGATATCAA	TTTCTTCTTCCCGTGACCAGT	2.00	7
lp_0800		Cell surface protein precursor	CGATTAATGCGGCAACAACA	CCGGTTGTTCAGCCTTTGAG	1.90	This study
lp_1019	*clpC*	*clpC* (*mecB* gene)	TTCGCAAGCTAGGTGTCAGTG	AGGTTGGCGTTCCTTCAGTC	2.00	This study
lp_1164	*celB*	Cellobiose PTS, EIIC	GGGCATCTTCCTCGCACTAT	TCGATCTCCTGGTGGATGTGT	1.91	This study
lp_1403		Cell surface protein	AGTCCCAGTCGATGCTAACG	CGTCAGGCGAATACAACCAT	1.99	This study
lp_1603	*hem*	Hemolysin homologue	TGGTTCAGTCGTTGCCCTAA	AACAGCAGGATCACGGACAA	1.82	This study
lp_2940		Cell surface protein precursor	ATGGCACGGTCAGTTTAGCA	TTGCACCGCTTGTGTTACCT	1.88	This study
lp_3055	*copA*	Copper-transporting ATPase	CGCACTTGTGACCACTTTCG	TTCCGCTTCCTTGGCTTGTA	1.95	This study
lp_3176	*pkn2*	Serine-threonine protein kinase	CAACGGAGCGATCTATATTCGT	GCCCATGTTGAATCCTGTGT	1.94	This study
lp_3473	*ram2*	Rhamnosidase	CAACCACGCTGACGTTACCA	CCGTGACCACTGGATTGCTA	1.91	This study
lp_3660	*rbsK3*	Ribokinase	TTATTGGTGCGGTTGGTGAC	TCTTTGTGATCCCTGCCAAG	1.93	This study
lp_3662	*adhE*	Alcohol and acetaldehyde dehydrogenase	GCCGCACTGGACAATCATA	TCGCTGGCGTAGATGTTCTT	1.99	This study

[a] Designated gene number for the annotated *L. plantarum* WCFS1 chromosome.

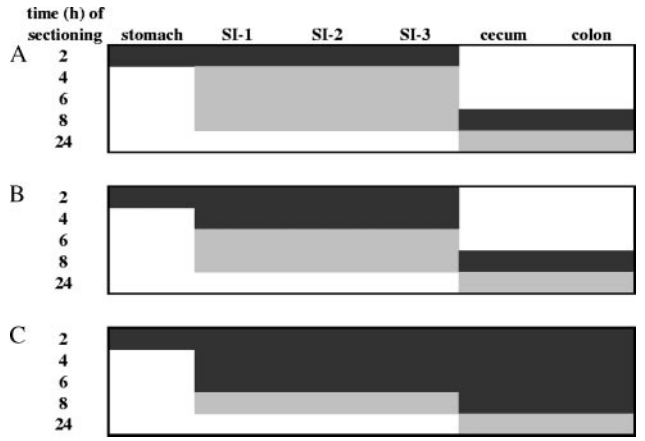

FIG. 1. Mouse intestinal tract samples selected for gene transcript analysis. Real-time RT-PCR was performed on RNA from individual mice ($n = 3$) (black panels) or pooled samples (gray panels). (A) The minimal number of samples in which a housekeeping or *ivi* gene was examined. (B) *argG*, *clpC*, *pkn2*, *plnI*, *proA*, *pts14C*(*celB*), *rbsK3*, *ram2*, lp_0237, lp_0800, lp_1403, and lp_2940 were also monitored in the small intestines of individual mice at 4 h. (C) *groES*, *fusA*, *ldhL*, *pfk*, *rpoB*, *copA*, and *adhE* were monitored as described for panels A and B, with the addition of the small intestine at 6 h and the cecum and colon at 2, 4, and 6 h.

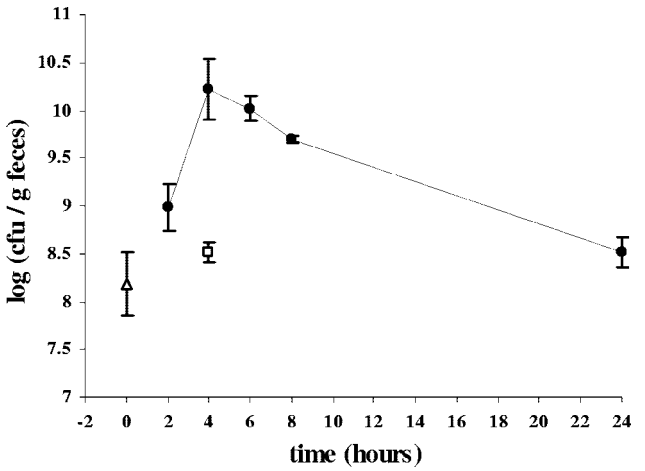

FIG. 2. Enumeration of viable *Lactobacillus* cells and related LAB isolated from feces of mice immediately prior to the start of the experiment (open triangle) and inoculated with either *L. plantarum* WCFS1 (closed circles) or sterile MRS broth (open square). Shown are the averages ± standard deviations for feces excreted by three mice during the time period prior to sacrifice.

tract compartments, the C_T values obtained for *L. plantarum* 16S rRNA in these samples were first compared to those for samples containing known amounts of *L. plantarum* WCFS1 total RNA. The estimated *L. plantarum* total RNA in the mouse samples was then correlated to the corresponding number of *L. plantarum* cells based on the observation that we typically isolated approximately 0.02 pg total RNA per laboratory culture-grown *L. plantarum* cell.

To identify endogenous reference genes, a panel of nine *L. plantarum* housekeeping genes (Table 1) was evaluated for gene expression stability according to pair-wise variation procedures outlined for the geNorm approach (35). Following this method, we calculated a normalization factor based on the geometric mean of the linear-transformed expression values for the three most stable housekeeping genes. These values were then used to express the relative amounts of the *ivi* and housekeeping gene transcripts in the mouse-derived RNA samples compared to those in *L. plantarum* WCFS1 grown in MRS broth.

RESULTS

Enumeration of viable *Lactobacillus* in mouse feces. A single dose of wild-type *L. plantarum* WCFS1 (2×10^{10} cells grown in MRS broth) (12) was fed by gavage directly into the stomachs of 15 BALB/c mice (time zero). During the subsequent 24 h, mouse fecal material was collected periodically to determine the time required for *L. plantarum* WCFS1 to transit through the digestive tract. Fecal samples collected before inoculation of *L. plantarum* and after control inoculation with sterile culture medium contained on average 10^8 cells/g feces able to form colonies on MRS agar (Fig. 2). 16S rRNA gene sequence analysis of seven colonies with distinct colony morphologies indicated that three fecal isolates were *L. plantarum*, while the others were very similar (>97% nucleotide identity) to either *Lactobacillus murinus* (two of seven) or *Enterococcus hirae* (two of seven). Hence, colony enumeration on MRS agar enabled the monitoring of the endogenous *Lactobacillus* and related LAB populations contained in the feces as well as the *L. plantarum* WCFS1 fed to the mice.

The number of fecal bacteria able to grow on MRS agar increased within 2 h after ingestion of *L. plantarum* WCFS1 (Fig. 2). Over the next 2 hours, this population reached its highest level at 10^{10} cells/g feces. Subsequently, after 6 h, the population started to decline and continued to decline in time until colony counts reached preinoculation levels. 16S rRNA gene sequence analysis confirmed that the observed population dynamics were the result of the excretion of *L. plantarum* WCFS1 into the mouse feces (data not shown).

Transit of *L. plantarum* through intestinal compartments. At 2, 4, 6, 8, and 24 h after ingestion of *L. plantarum* WCFS1, three mice were sacrificed and the stomach, small intestine (in three parts approximately representing the duodenum [SI-1], jejunum [SI-2], and ileum [SI-3]), cecum, and colon of each mouse were collected for RNA isolation. In order to monitor the dynamics of *L. plantarum* transit through the different intestinal compartments, we applied real-time RT-PCR to measure the levels *L. plantarum* 16S rRNA present in the samples, using primers described previously (Table 1) (7). Estimates of the number of *L. plantarum* cells were obtained by correlating the real-time RT-PCR signals for 16S rRNA in the mouse samples to those obtained for reactions in which the number of *L. plantarum* WCFS1 cells and the RNA template were known. This approach revealed that the control mice harbored approximately 2×10^5 *L. plantarum* cells in the stomach and over 5×10^6 cells in the cecum and colon (per gram tissue) (Fig. 3).

Upon inoculation of *L. plantarum* WCFS1, the number of *L. plantarum* cells in the different intestinal compartments increased by 2 to 3 orders of magnitude compared to what was found for the control mice (Fig. 3). The amounts of *L. plantarum* in the stomach and small intestine remained large for at least 4 h but then declined to background levels, indicating that the majority of *L. plantarum* WCFS1 cells were no longer present in these compartments. In contrast, the cecum and colon contained approximately 10^9 *L. plantarum* cells per gram tissue for at least 8 h (Fig. 3). Remarkably, in one mouse sacrificed at 24 h, the stomach and the upper duodenum (SI-1)

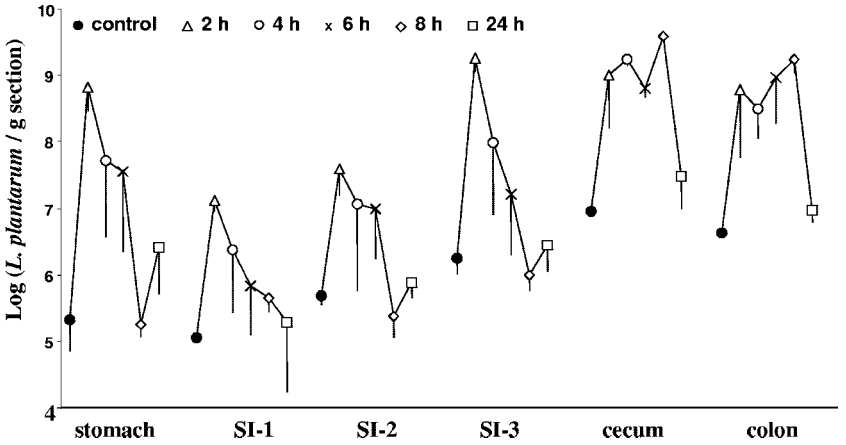

FIG. 3. Population dynamics of *L. plantarum* cells contained in mouse digestive tract sections as determined by 16S rRNA-targeted real-time RT-PCR. Each point represents the average ± standard error of the mean values for three mice at each time point.

contained elevated levels of *L. plantarum*. This result could be explained by the fact that mice are coprophilic (consume their own feces), and hence, their fecal material likely served as a secondary source of *L. plantarum* inoculum.

***L. plantarum* housekeeping gene expression levels in the mouse intestine.** The transcript abundances of the *ivi* genes were compared between the intestinal samples and the *L. plantarum* WCFS1 cells that were used to gavage the mice (time zero). To control for errors introduced by differences in the concentrations of *L. plantarum* total RNA isolated from the mouse intestine and laboratory-grown cultures, the observed gene expression values were first normalized to those for endogenous *L. plantarum* reference housekeeping gene transcripts. Housekeeping genes were evaluated on the basis that the gene transcript was detected in mouse samples collected from mice fed *L. plantarum* WCFS1 and that it was stably expressed relative to all other housekeeping genes examined. Real-time RT-PCR of RNA isolated from the intestinal compartments of the control mice did not result in a detectable product for *L. plantarum* housekeeping or *ivi* genes. In contrast, transcripts for five of the nine housekeeping genes were present in measurable levels in the intestinal compartments of mice fed *L. plantarum* WCFS1 (Fig. 1A). Transcripts for *ileS*, *pcrA*, *gyrB*, and *recA* were not consistently detected (see Fig. S2 in the supplemental material) and were therefore not included in subsequent analyses. Pair-wise comparisons (35) of the expression values obtained for the five remaining housekeeping genes indicated that *fusA*, *rpoB*, and *pfk* were the most stably expressed housekeeping genes among *L. plantarum* cells contained in the mouse intestine and grown in MRS medium. Sample-specific normalization factors comprising the geometric mean of their expression values were used to quantify *L. plantarum* WCFS1 gene transcript abundance.

Analysis of the real-time RT-PCR data revealed that most *L. plantarum* housekeeping genes were not differentially regulated (see Fig. S1 and S2 in the supplemental material). Only the transcription of *gyrB* and *ldhL* was induced more than twofold, on average, in the stomach and SI-1 (*gyrB*) or cecum and colon (*ldhL*). Conversely, *groES* was down-regulated at the distal end (SI-3) of the small intestine, cecum, and colon of most mice.

In vivo-inducible (*ivi*) gene expression levels in the mouse intestine. A total of 15 *ivi* genes, selected to represent the diversity of cellular activities identified in the R-IVET screen, were analyzed for their in vivo expression levels by real-time RT-PCR (Table 2). Strikingly, *copA*, encoding a putative copper-transporting ATPase, and *adhE*, encoding bifunctional alcohol dehydrogenase, were highly induced in all intestinal compartments and at all time points examined (Table 2 and Fig. 4). These loci were up-regulated in the mouse stomach, and their expression remained induced at similar levels throughout the digestive tract. The gene encoding a lytic enzyme, annotated as a putative hemolysin, *hem*, was the only *ivi* gene that was consistently down-regulated in all intestinal samples examined (Table 2). All other *ivi* genes examined exhibited at least modest levels of induction in the mouse intestine, and in some cases, this activity was dependent upon the intestinal tract compartment. For example, *L. plantarum* genes en-

TABLE 2. *ivi* gene expression in mice[a]

Gene	*ivi* expression level at indicated site					
	Stomach	SI-1	SI-2	SI-3	Cecum	Colon
adhE	**6.03**	**6.10**	**6.85**	**5.68**	**6.43**	**7.12**
argG	0.52	1.76	1.88	1.24	0.58	0.25
celB	−0.09	BD	1.73	0.76	−0.08	0.29
clpC	0.16	0.99	0.94	0.37	−0.36	−0.06
copA	**4.80**	**4.51**	**4.81**	**3.67**	**4.22**	**4.38**
hem	−0.61	−0.44	−0.56	−0.71	−1.00	−1.38
pkn2	0.23	**1.93**	**1.76**	**1.81**	−0.18	−0.02
plnI	1.06	1.82	4.31	4.28	**2.23**	**2.60**
proA	0.52	0.39	0.69	0.34	−0.25	−0.50
rbsK3	**2.70**	BD	4.86	**3.51**	**2.27**	**2.04**
ram2	0.79	BD	4.15	3.53	BD	0.11
lp_0237	0.18	1.88	**2.84**	**2.26**	0.88	0.70
lp_0800	0.27	0.86	0.49	1.22	−0.27	−0.20
lp_1403	−0.79	1.86	**3.08**	1.14	−0.34	0.11
lp_2940	0.77	BD	3.15	**1.89**	**2.28**	1.17

[a] Average change (*n*-fold) in expression levels ($\log_2$) for *L. plantarum ivi* genes in mouse intestinal compartments compared to those in MRS culture medium (time zero). The average includes all mice and time points examined. The values in bold indicate gene transcripts that could be detected in three or more individual mice and were, on average, more than twofold induced according to the one-tailed *t* test ($P < 0.05$). BD, below detection.

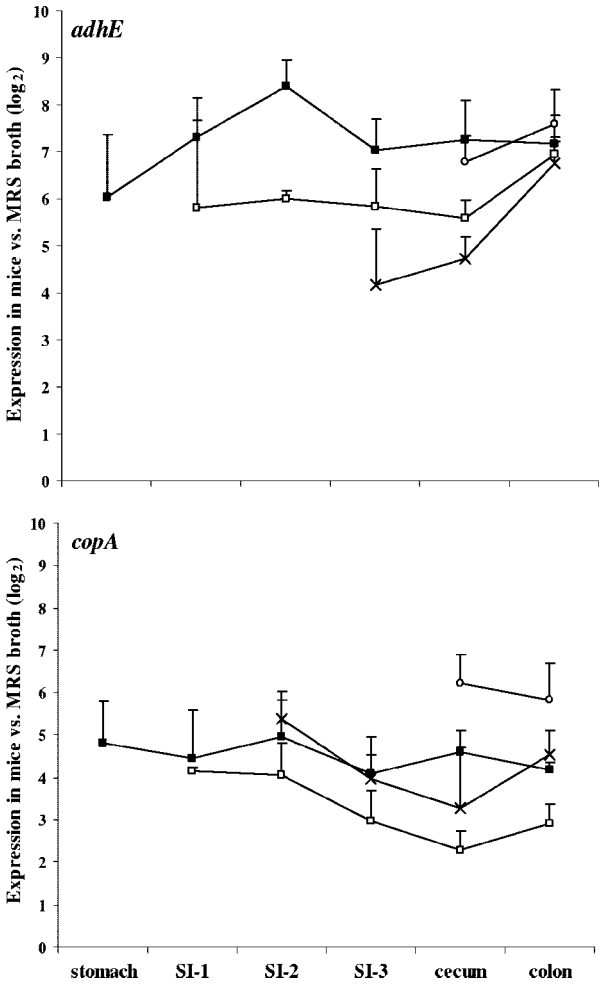

FIG. 4. Relative expression levels of the *copA* and *adhE* genes in the mouse intestine at 2 h (■), 4 h (□), 6 h (×), and 8 h (○) after inoculation of *L. plantarum* WCFS1. The normalized gene transcript amounts in the intestinal sections were compared to those found for *L. plantarum* in the mouse inoculum (time zero). Expression values are reported as the averages ± standard errors of the means for three mice at each time point.

coding cell surface and membrane-bound proteins, the lp_0237, lp_0800, lp_1403, and lp_2940 proteins, were induced primarily in the small intestine (Table 2 and Fig. 5). Although there was considerable mouse-to-mouse variation and transcripts were not detected in all samples, three of these genes (lp_0237, lp_0800, and lp_1403) were induced along the length of the small intestine, whereas lp_2940 was most active at the distal end of this compartment and in the cecum. Similar to the cell surface proteins, genes encoding protein kinase Pkn2 and argininosuccinate synthetase ArgG (Table 2) (see Fig. S3 in the supplemental material) were typically induced in the small intestine. Genes that appeared to be up-regulated but for which transcripts were detected in only a few intestinal samples included *plnI* and *rbsK*, encoding a plantaricin immunity protein and ribokinase, respectively (Table 2) (see Fig. S3 in the supplemental material). Finally, transcripts for *clpC* and *proA* were detected in the intestine but were not present in significantly larger amounts than those found in *L. plantarum* cells contained in MRS broth (Table 2) (see Fig. S3 in the supplemental material).

DISCUSSION

This study revealed that *L. plantarum* WCFS1 undergoes dynamic changes in *ivi* gene expression in the digestive tracts of mice. To aid the quantitative detection of *L. plantarum* gene transcripts, the transit of *L. plantarum* through the mouse intestinal compartments was also measured. The number of viable *Lactobacillus* cells in the mouse feces increased within 2 h after inoculation of *L. plantarum* WCFS1, reached its highest level in the subsequent 2-h period, and returned to preinoculation levels within 24 h (Fig. 2). These transit dynamics are similar to those reported for *L. casei* and *Bacillus subtilis* spores fed to human flora-associated mice (22). Because *L. plantarum* WCFS1 was not selectively monitored on the MRS medium used here, it is not possible to conclude whether this organism was still present once the number of *Lactobacillus* cells in the mouse feces returned to the initial level. A previous study found that an antibiotic-resistant variant of *L. plantarum* WCFS1 could still be detected in the feces of mice for up to 7 days after inoculation (24). Therefore, it remains likely that while the majority of the *L. plantarum* WCFS1 cells transited the digestive tract rather rapidly in the present study, a small but persistent population of this organism was retained in the mice.

Consistent with the number of *Lactobacillus* cells present in the mouse feces, real-time RT-PCR of *L. plantarum* 16S rRNA isolated from the intestinal compartments confirmed substantial increases in the number of *L. plantarum* cells 2 h after inoculation of *L. plantarum* WCFS1 (Fig. 3). Transit of this organism was most rapid through the mouse stomach and small intestine (regions SI-1 through SI-3), and within 6 h, the numbers of *L. plantarum* returned to preinoculation levels at these sites. In contrast, the cecum and colon retained large amounts of *L. plantarum* for at least 8 h. These transit dynamics are in agreement with analyses of *L. plantarum* WCFS1 housekeeping and *ivi* gene transcripts such that they could be detected only in mouse compartments containing elevated numbers of *L. plantarum* cells.

Based on the *L. plantarum* intestinal tract population sizes estimated according 16S rRNA-based real-time RT-PCR analysis and the signals obtained for individual gene transcripts in the different samples, the detection limit for *L. plantarum* mRNA was in the range of 10^7 cells per intestinal compartment. This range is similar to what was found for other DNA-based real-time PCR detection applied to fecal samples (18). Because there was considerable mouse-to-mouse variation in the intestinal transit of *L. plantarum* WCFS1, particularly through the small intestine, the approach taken here of collecting mouse intestinal compartments at multiple time points proved to be essential for determining which samples were likely to contain detectable levels of *L. plantarum* gene transcripts.

Because different quantities of *L. plantarum* total RNA were isolated from the mice, it was necessary to normalize the real-time RT-PCR data collected for the *L. plantarum* housekeeping and *ivi* gene transcripts. 16S rRNA is among the most commonly used cellular products used for transcript normal-

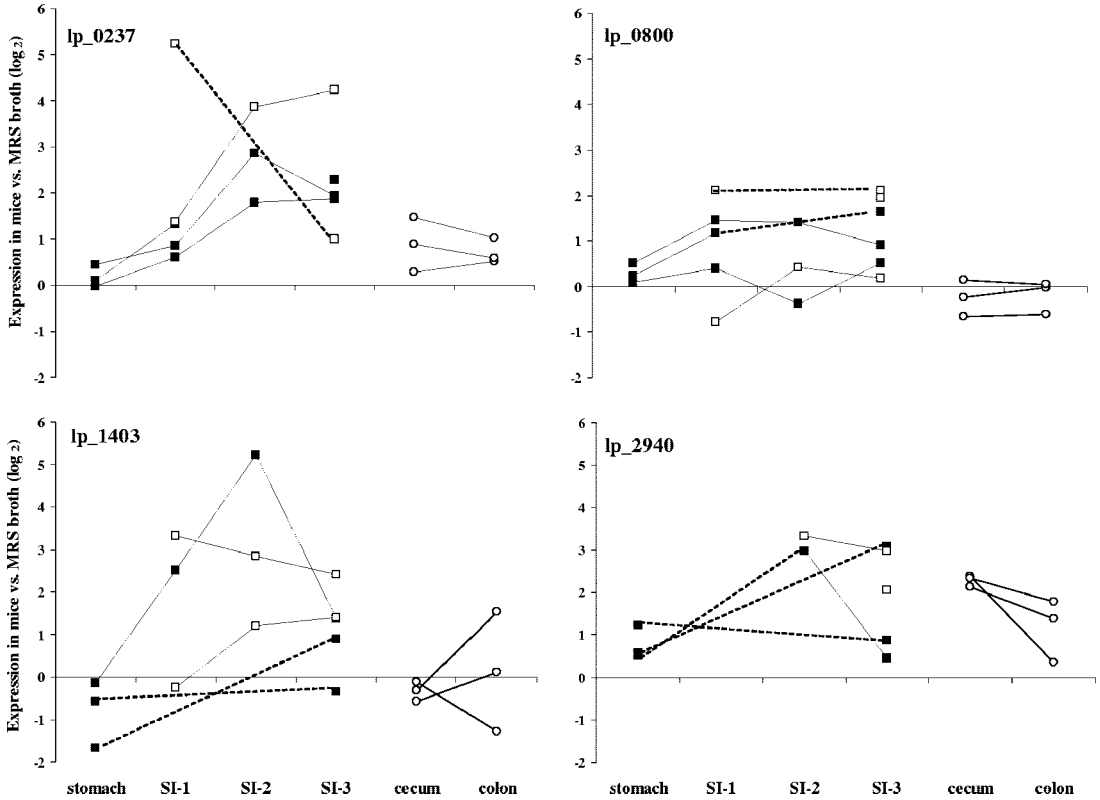

FIG. 5. Relative expression levels of lp_0237, lp_0800, lp_1403, and lp_2940 in the stomachs and small intestines of individual mice at 2 h (■) and 4 h (□) and in the ceca and colons at 8 h (○) after inoculation of *L. plantarum* WCFS1. The normalized gene transcript amounts in the intestinal sections were compared to those found for *L. plantarum* in the mouse inoculum (time zero). Expression values for individual mice are connected by solid or dotted lines, depending on whether transcripts were detected in consecutive or nonconsecutive intestinal sections, respectively.

ization (10). However, 16S rRNA was not a suitable normalizer for the mouse intestinal samples because it is not specific for *L. plantarum* WCFS1 and is much more abundant than mRNA. In comparison, the detection limit for *L. plantarum* WCFS1 housekeeping gene transcripts was similar to that found for the *ivi* gene transcripts. We previously compared the expression levels of *L. plantarum* WCFS1 housekeeping genes in actively dividing, stationary-phase, and nutrient-starved *L. plantarum* cells in vitro (M. L. Marco and M. Kleerebezem, submitted for publication). Real-time RT-PCR of the housekeeping gene transcripts revealed considerable variation in their abundance levels according to several data normalization methods. Only internal pair-wise comparisons made according to the geNorm approach accounted for variations in cell viability, fluctuating rRNA levels, and total extracted *L. plantarum* RNA. Therefore, we used this approach to analyze *L. plantarum* transcript levels in mice.

copA and *adhE* were the most highly induced *ivi* genes examined in this study, exhibiting between 5- and 350-fold-higher levels of expression throughout the mouse digestive tract. Interestingly, the expression levels of these genes were similar within the intestinal compartments at the different time points examined, suggesting that these *ivi* genes are consistently up-regulated in the mouse intestinal tract over time. While *copA* is annotated as a putative copper-transporting ATPase, its cation specificity and direction of transport are not known. Remarkably, genes involved in cation transport were frequently identified in other IVET screens in other bacteria (27). In addition, the importance of the *copA* gene for *L. plantarum* during its residency in the intestine was recently confirmed in studies whereby *L. plantarum copA* deletion mutants showed reduced levels of persistence and survival in mice (8). AdhE is predicted to function as a bifunctional alcohol and acetaldehyde dehydrogenase involved in metabolism during fermentation. Although the relevance of this gene for *L. plantarum* intestinal activities remains to be established, *adhE* was also found to be up-regulated in pathogenic *Escherichia coli* K1 during intestinal colonization (17) and therefore is likely involved in bacterial adaptation to the changing environment and nutritional resources available in the gastrointestinal tract.

While *copA* and *adhE* were up-regulated throughout the entire digestive tract, most other *ivi* genes were preferentially induced in the small intestine. This result is in agreement with the commonly held assumption that *L. plantarum* is among the most predominant and active microorganisms in the small intestine. Several *ivi* genes encoding membrane-bound proteins were among those that were most highly up-regulated at this location. Both lp_0800 and lp_2940 encode proteins containing LPXTG-like motifs (LPQTNE) involved in anchoring them extracellularly to the *L. plantarum* cell wall (4). The protein product of lp_1403 is also likely exported outside the cell, but it lacks any known binding domains. Finally, lp_0237 is anno-

tated as an integral membrane protein of an unknown function. Both this gene and *argG* were previously confirmed to be up-regulated in the mouse small intestine in independent real-time RT-PCR experiments (7). These genes were also found to be induced in the presence of bile salts and hence may be responding to these compounds in the small intestine (7).

Most *L. plantarum ivi* genes appeared to be only moderately induced in the intestinal tract. Although this observation might be due to uniform and relatively small changes in *L. plantarum* WCFS1 *ivi* gene expression in the intestine, it is also possible that only a fraction of the total *L. plantarum* WCFS1 cells was responding to the stimulus required for individual *ivi* gene expression. As real-time RT-PCR provides an average of gene activities for all cells from which RNA was isolated, it is likely that some *L. plantarum* cells contained large amounts of the *ivi* gene transcripts, while many others were either not sensing the stimulus necessary for *ivi* gene expression or were metabolically inactive and unable to initiate a response. Such genes would be identified by R-IVET because this screening technique requires only single or small groups of *L. plantarum* cells to be activated at these loci in order to be identified as being in vivo induced. Therefore, although the hemolysin homolog appeared to be down-regulated in the mouse digestive tract according to the real-time RT-PCR studies performed here, it remains plausible that this gene was induced in *L. plantarum* cells located in specific intestinal microsites. A second factor that might have influenced the observed *ivi* gene expression levels is that the relative gene transcript abundance was dependent on the in vitro condition to which they were compared. Comparisons of transcript levels found in stationary- and exponential-phase *L. plantarum* cells revealed that many *ivi* genes were more highly expressed in the stationary-phase cells fed to the mice (see Table S1 in the supplemental material). However, independent of the growth phase to which the in vivo data were compared, the conclusion remains that the *L. plantarum ivi* genes examined here were more highly expressed in the mouse gastrointestinal tract than in culture medium.

Real-time RT-PCR has yet to be commonly applied to confirm and quantify the expression levels of genes identified in an IVET-based approach (27). However, this technique proved to be valuable for the study of *L. plantarum* in the mouse intestine, whereas other, less-sensitive methods may not have detected specific *L. plantarum* gene transcripts. Genome-wide transcript analyses using DNA microarrays also provide opportunities for even more comprehensive and integrative views of bacterial activities occurring within the intestinal tract. The potential of this approach was exemplified by studies reporting full-genome transcriptome profiles of *Bacteroides thetaiotaomicron* residing in the ceca of germfree mice (32). However, only real-time RT-PCR permits direct measurements of bacterial gene expression in densely populated, species-rich digestive tracts. Application of such complementary techniques will improve our understanding of bacterial gene expression in situ in the gastrointestinal tract and aid in the development of molecular models for describing bacterial activities in vivo and the corresponding host responses. Such models will be required for our understanding of the mechanisms by which probiotic strains exert their functional benefits in the consumer and will ultimately enable the selection or construction of improved strains with predestined health benefits.

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