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

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Received 27 June 2006/Accepted 20 October 2006

*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 mannosereceptor 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, 169).
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 _ivi_ 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 _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 5 ml sterile phosphate-buffered saline (PBS) solution 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 were compared to 16S rRNA sequences from the Ribosomal Database Project (11). The numbers of _L. plantarum_ cells contained in the mouse digestive tract. (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 µM of each deoxynucleotide 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-primed first-strand cDNA agarose gel run (no-template control) and remaining chromosomal DNA (RT reactions in which Superscript III was omitted). Spiking experiments in which _L. plantarum_ WCFS1 cDNA 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_ gene transcripts during mouse intestinal transit. Housekeeping genes (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). 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 [CT] value). CT 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 CT values was used for further analysis. Real-time PCR amplification efficiencies were determined for each gene by standard curves generated by plotting the starting RNA concentration against the observed CT 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/CTslope) (Table 1) (26). To estimate the number of _L. plantarum_ cells contained in the mouse digestive tract, the dynamic activities of _L. plantarum_ in the intestinal tract.
<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene</th>
<th>Function</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>E value</th>
<th>Source or reference</th>
</tr>
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<td></td>
<td></td>
<td></td>
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<td>16S rRNA</td>
<td>SSU ribosome (5 copies)</td>
<td>TGATCCTTGGCTCAGGACGAA</td>
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<td>7</td>
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<td>gyrB</td>
<td>DNA gyrase B</td>
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<td>GAATCCCAGACCGTTACAA</td>
<td>1.90</td>
<td>This study</td>
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<td>lp_0537</td>
<td>ldhL</td>
<td>l-Lactate dehydrogenase</td>
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<td>CCGATGTGGTCAGTTAGAAG</td>
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<td>This study</td>
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<td>lp_0727</td>
<td>groES</td>
<td>Chaperone</td>
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<td>CTTCACGGCTGGGGTCAACTT</td>
<td>1.97</td>
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<tr>
<td>lp_1027</td>
<td>ftsA2</td>
<td>Elongation factor G</td>
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<td>pcrA</td>
<td>ATP-dependent DNA helicase</td>
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<td>AAGGTCGCGTGCTGCTAGT</td>
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<td>This study</td>
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<td>lp_1898</td>
<td>pfr</td>
<td>6-Phosphofructokinase</td>
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<td>ileS</td>
<td>Isoleucine-tRNA ligase</td>
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<td>This study</td>
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<td>lp_2301</td>
<td>recA</td>
<td>Recombinase A</td>
<td>GCCGAAACAGCATCAGGAGG</td>
<td>TATCCACCTCGGCACGTAA</td>
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<tr>
<td><strong>ivi gene</strong></td>
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<td></td>
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<td>lp_0017</td>
<td>proA</td>
<td>Gulantate-5-semialdehyde dehydrogenase</td>
<td>CGTGAGTTTTCGCCAGATC</td>
<td>CATGCGCAGAATCACCATGAGG</td>
<td>2.00</td>
<td>This study</td>
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<td>lp_0237</td>
<td>argG</td>
<td>Integral membrane protein (hypothetical)</td>
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<td>ACGGGTGCAGAAGAAG</td>
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<td>7</td>
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<td>lp_0419</td>
<td>argH</td>
<td>Membrane-bound protease, immunity protein</td>
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<td>1.91</td>
<td>This study</td>
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<tr>
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<td>argG</td>
<td>Argininesuccinate synthase</td>
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<td>Cell surface protein precursor</td>
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<td>clpC (mecB gene)</td>
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<td>2.00</td>
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<td>celB</td>
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<td>TCGATCTTTGCGTGGATGT</td>
<td>1.91</td>
<td>This study</td>
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<td>celB</td>
<td>Cell surface protein</td>
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<td>This study</td>
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<tr>
<td>lp_1603</td>
<td>hem</td>
<td>Hemolysin homologue</td>
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<td>CGTGAGGCAATCAGCATGA</td>
<td>1.99</td>
<td>This study</td>
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<td>lp_3055</td>
<td>copA</td>
<td>Copper-transporting ATPase</td>
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<td>1.95</td>
<td>This study</td>
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<tr>
<td>lp_3176</td>
<td>pkn2</td>
<td>Serine-threonine protein kinase</td>
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<td>ram2</td>
<td>Rhamnosidase</td>
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<td>1.94</td>
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<td>pkn2</td>
<td>Serine-threonine protein kinase</td>
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<td>1.94</td>
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<td>lp_3662</td>
<td>alcohol dehydrogenase</td>
<td>Alcohol and acetaldehyde dehydrogenase</td>
<td>GCCGCACCTGGCAATGCAAT</td>
<td>TCGTGGCAGTAATGTTTCTT</td>
<td>1.99</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Designated gene number for the annotated *L. plantarum* WCFS1 chromosome.
of the digestive tract. Fecal samples collected before inoculation

were very similar (two of seven). Hence, colony enumeration on MRS agar en-

sures that three fecal isolates were

formed colonies on MRS agar (Fig. 2). 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 $2 \times 10^9$ *L. plantarum* cells in the stomach and over $5 \times 10^6$ 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 $10^6$ *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)
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* WCFS1 gene transcript abundance. 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>
<thead>
<tr>
<th>Gene</th>
<th>Stomach</th>
<th>SI-1</th>
<th>SI-2</th>
<th>SI-3</th>
<th>Cecum</th>
<th>Colon</th>
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<td><em>adhE</em></td>
<td>6.03</td>
<td>6.10</td>
<td>6.85</td>
<td>5.68</td>
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<td>−0.61</td>
<td>−0.44</td>
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<td>0.23</td>
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<td><em>proA</em></td>
<td>1.06</td>
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<td><em>ram2</em></td>
<td>0.79</td>
<td>BD</td>
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<td>3.53</td>
<td>BD</td>
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<td>3.15</td>
<td>1.89</td>
<td>2.28</td>
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</table>

Average change (n-fold) in expression levels (log2) 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. 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.](http://aem.asm.org/)
This study revealed that L. plantarum WCFS1 undergoes dynamic changes in *ini* 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). 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 mouse.

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 *Lactobacillus* 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 *ini* 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 *ini* gene transcripts. 16S rRNA is among the most commonly used cellular products used for transcript normal-
However, 16S rRNA was not a suitable normalizer for the mouse intestinal samples because it is not specific for \textit{L. plantarum} WCFS1 and is much more abundant than mRNA. In comparison, the detection limit for \textit{L. plantarum} WCFS1 housekeeping gene transcripts was similar to that found for the \textit{ivi} gene transcripts. We previously compared the expression levels of \textit{L. plantarum} WCFS1 housekeeping genes in actively dividing, stationary-phase, and nutrient-starved \textit{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 \textit{L. plantarum} RNA. Therefore, we used this approach to analyze \textit{L. plantarum} transcript levels in mice.

\textit{copA} and \textit{adhE} were the most highly induced \textit{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 \textit{ivi} genes are consistently up-regulated in the mouse intestinal tract over time. While \textit{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 \textit{copA} gene for \textit{L. plantarum} during its residency in the intestine was recently confirmed in studies whereby \textit{L. plantarum} \textit{copA} deletion mutants showed reduced levels of persistence and survival in mice (8). \textit{AdhE} is predicted to function as a bifunctional alcohol and acetaldehyde dehydrogenase involved in metabolism during fermentation. Although the relevance of this gene for \textit{L. plantarum} intestinal activities remains to be established, \textit{adhE} was also found to be up-regulated in pathogenic \textit{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 \textit{copA} and \textit{adhE} were up-regulated throughout the entire digestive tract, most other \textit{ivi} genes were preferentially induced in the small intestine. This result is in agreement with the commonly held assumption that \textit{L. plantarum} is among the most predominant and active microorganisms in the small intestine. Several \textit{ivi} genes encoding membrane-bound proteins were among those that were most highly up-regulated at this location. Both \textit{lp}_0800 and \textit{lp}_2940 encode proteins containing LPXTG-like motifs (LPQTNE) involved in anchoring them extracellularly to the \textit{L. plantarum} cell wall (4). The protein product of \textit{lp}_1403 is also likely exported outside the cell, but it lacks any known binding domains. Finally, \textit{lp}_0237 is anno-

\begin{figure}
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\caption{Relative expression levels of \textit{lp}_0237, \textit{lp}_0800, \textit{lp}_1403, and \textit{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 \textit{L. plantarum} WCFS1. The normalized gene transcript amounts in the intestinal sections were compared to those found for \textit{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.}
\end{figure}
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 a result of a relatively small sample size 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 ivi 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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