Differential Activities of Four *Lactobacillus casei* Promoters during Bacterial Transit through the Gastrointestinal Tracts of Human-Microbiota-Associated Mice

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In a previous study using fusion of the deregulated lactose promoter *lacTp and reporter genes, we suggested that *Lactobacillus casei* could initiate de novo protein synthesis during intestinal transit. In order to confirm this finding and extend it to other promoters, we adopted a reverse transcriptase quantitative PCR (RT-QPCR) approach combined with a transcriptional fusion system consisting of luciferase genes under the control of four promoters (*ccpA*, *dlt*, *ldh*, and *lacT**) from *L. casei* DN-114 001. Promoter expression was monitored during cell growth, and variable luciferase activities were detected. In 3-day cultures, all the genetically modified strains survived but without exhibiting luciferase activity. Luciferase mRNA levels determined by RT-QPCR analysis (RNA/CFU) were not significant. The cultures were administered to human-microbiota-associated mice, and the feces were collected 6 h later. *L. casei* promoters *lacTp** and *ldhp* initiated mRNA synthesis during gastrointestinal transit. The promoters, *ccpAp* and *dltP*, exhibited no luciferase activity, nor was de novo-synthesized luciferase mRNA detected in the feces. *L. casei* seems to adapt its physiology to the gastrointestinal tract environment by modulating promoter activities. The approach (fecal transcriptional analysis) described herein may, moreover, be of value in studying gene expression of transiting bacteria in human fecal specimens.**

Lactobacillus casei has attracted considerable interest as a probiotic over the last few years (15, 19). The strain *L. casei* DN-114 001 has been shown to alleviate acute diarrhea in healthy children (21, 22), to modulate the production of proinflammatory cytokines in Crohn's disease ex vivo (3), and to enhance the immune system during bacterial gastrointestinal (GI) transit (20). However, little is known about the survival of *L. casei* species and their physiological status in the environment of the GI tract.

Probiotics are usually ingested with fermented milk during the late stationary phase of the bacterial cycle. At the onset of the stationary phase, the overall rate of protein synthesis has been shown to drop precipitously to a rate equivalent to approximately 0.2% of the exponential growth rate (13). These changes are concomitant with a decrease in overall mRNA and DNA synthesis (13). The behavior of such metabolically inactive cells in the GI environment was thus investigated with a view to identifying the mechanisms underlying their action on health.

To carry out this work, we developed a genetic approach using transcriptional fusion of luciferase genes (*luxA* and *luxB*) and selected promoters to study the response of lactic acid bacteria to the GI tract environment (5). The same approach was then extended to a probiotic microorganism, *L. casei* DN-114 001, to study its response to the GI tract in the human-microbiota-associated (HMA) mouse model using transcriptional fusion of the modified *L. casei lacTEGF* operon promoter and *luxA* and *luxB* genes from *Photobacterium luminescens*. Resulting data suggest that *L. casei* can initiate de novo

protein synthesis during transit with the diet (18). However, determining luciferase activity necessitates both enzyme synthesis and the environmental conditions required for luciferase activity (FMNH₂ availability, pH). The aims of the present study were to validate results previously obtained with transcriptional fusion (18) by quantifying luciferase mRNA in the GI tract while investigating the activity of other *L. casei* promoters in the GI tract.

A sensitive and easy-to-perform method of quantifying *L. casei* mRNA in culture and fecal samples was developed. Four *L. casei* promoters involved in different metabolic pathways were selected among promoter sequences available in GenBank: the deregulated lactose operon promoter (*lacTp**) (1, 18), the promoter of L-(+)-lactate dehydrogenase (*ldhp*) (25), the *ccpA* promoter (*ccpAp*) (16), and the *dlt* operon promoter (*dltP*) (17).

Protein CcpA is a central catabolite regulator expressed constitutively in *L. casei* cultures (16). Some researchers (16), however, accept the possibility of autoregulation of its synthesis. The *dlt* operon is necessary for cell growth in *L. casei*, as its inactivation generates defective cell separation and aberrant morphology (17) and its promoter has been reported to be weak in a gram-positive bacterium (26). In *Bacillus subtilis*, peak expression of the *dlt* operon occurs during logarithmic growth prior to down-regulation by the SpoOA and AbrB global regulators (23). In *L. casei*, the promoter *ldhp* is known to be a highly efficient and constitutive promoter (25). In *Lactobacillus helveticus*, the *ldh* gene is strongly expressed in the logarithmic phase, but in the stationary phase, the level of *ldh* transcripts decreases rapidly (30).

Direct quantitative transcript analysis of fecal extracts from HMA mice confirmed that *L. casei* can initiate mRNA synthesis during transit through their GI tracts (18). Moreover, dif-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
<i>E. coli</i>		
LLB1	<i>supE Δthi (lac-proAB) hsdD5 (F' traD36 proAB lacI^qZΔM15) pcnB</i>	14
TG1 RepA	TG1 with <i>repA</i> from <i>E. coli</i> EC101 integrated in the chromosome	9
<i>L. casei</i>		
DN-114 001	<i>L. casei</i> Danone strain	CNCM ^a no. I-1518
DN-240 041	DN-114 001, Em ^r , <i>lacTp*::luxAB</i> on pDN26 integrated in the chromosome (* <i>lacT</i> promoter without the ribonucleic antiterminator sequences) (1)	18
DN-240 047	DN-114 001, Em ^r , <i>ccpAp::luxAB</i> on pDN75 integrated in the chromosome	This study
DN-240 048	DN-114 001, Em ^r , <i>dltP::luxAB</i> on pDN76 integrated in the chromosome	This study
DN-240 049	DN-114 001, Em ^r , <i>ldhP::luxAB</i> on pDN77 integrated in the chromosome	This study
Plasmids		
pBluescript II	Amp ^r , M13ori pBR322ori	Stratagene
pDN69	Amp ^r , 241-bp fragment of <i>L. casei</i> containing <i>ccpA</i> cloned in pBluescript II	This study
pDN70	Amp ^r , 267-bp fragment of <i>L. casei</i> containing <i>dlt</i> cloned in pBluescript II	This study
pDN71	Amp ^r , 525-bp fragment of <i>L. casei</i> containing <i>ldh</i> cloned in pBluescript II	This study
pIL4232	Amp ^r , <i>luxAB</i> genes from <i>P. luminescens</i> cloned in pBluescript II	This study
pIL4240	Amp ^r Em ^r , fusion of pIL4232 with pWV01(<i>ΔrepA</i>)	This study
pIL4242	Em ^r , ori (pWV01 <i>ΔrepA</i>), integrative vector derived from pIL4240	This study
pDN72	Amp ^r Em ^r , fusion of pDN69 and pIL4242	This study
pDN73	Amp ^r Em ^r , fusion of pDN70 and pIL4242	This study
pDN74	Amp ^r Em ^r , fusion of pDN71 and pIL4242	This study
pDN75	Em ^r , derivative of pDN72 carrying <i>ccpAp::luxAB</i>	This study
pDN76	Em ^r , derivative of pDN73 carrying <i>dltP::luxAB</i>	This study
pDN77	Em ^r , derivative of pDN74 carrying <i>ldhP::luxAB</i>	This study

^a CNCM, Collection Nationale de Culture de Microorganismes.

ferential activities of the four promoters were detected in the GI environment.

MATERIALS AND METHODS

Bacterial strains, media, and animals. Table 1 shows the bacterial strains and plasmids used in this study. *Escherichia coli* strains LLB1 and TG1 RepA were cultured aerobically in Luria-Bertani medium at 37°C. *L. casei* strains, derived from strain DN-114 001 (Collection Nationale de Culture de Microorganismes no. I-1518), were cultured in MRS medium at 37°C (6). When required, the concentrations of antibiotics added to the media were 100 µg of ampicillin (ICN)/ml or 100 µg of erythromycin (Merck, Darmstadt, Germany)/ml to select *E. coli* transformants and 5 µg of erythromycin/ml or 5 µg of chloramphenicol (ICN)/ml for *L. casei*. For transcription inhibition studies, a concentration of 100 µg of rifampin (Merck)/ml was used. The HMA mice used in this study were obtained as previously described (18).

DNA. The method for plasmid and genomic DNA extraction was based on that of Sambrook et al. (29). Restriction endonucleases and T4 DNA ligase were obtained from Fermentas (Vilnius, Lithuania). *Taq* DNA polymerase was obtained from Takara Bio, Inc. (Otsu Shiga, Japan). All the enzymes were used per the manufacturers' instructions. *L. casei* was transformed by electroporation using a Gene-Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) as previously described (24).

Plasmid and strain constructs. Transcriptional fusions of three promoters from *L. casei* DN-114 001, *ccpAp*, *dltP*, and *ldhP*, fused to *P. luminescens luxA* and *luxB* genes were constructed and incorporated in the chromosome of *L. casei* DN-114 001 (Table 1 and Fig. 1). For the integrative transcriptional fusion with luciferase genes, pIL4242 was first constructed. This plasmid consists of *luxA* and *luxB* genes inserted into pOri. In this integrative vector, the *luxA* and *luxB* genes are followed by the *E. coli* *rrnB* terminator sequence and RepA protein, produced in *trans*, is required for it to replicate in *E. coli* or *L. casei*. The three promoters were PCR amplified (Table 2) and fused upstream of the luciferase genes in pIL4242. The final integrative plasmids, pDN75, pDN76, and pDN77, contained, respectively, the promoters *ccpAp*, *dltP*, and *ldhP* (Table 1 and Fig. 1). These plasmids were integrated as previously described (18) in the DN-114 001 chromosome by homologous recombination. The corresponding strains are listed in Table 1. The integrants were analyzed by PCR using primers located in the

related genes and in *luxB*. The correct insertions of *ccpAp::luxAB*, *dltP::luxAB*, and *ldhP::luxAB* were checked using primer pairs OLC33-OLC52, OLC33-OLC71, and OLC33-OLC72, respectively. The degenerated primers, OLC71 and OLC72 (Table 2), were designed by homologous sequence analysis of *dlt* and *ldh* genes from several lactic acid bacteria. The DN-240 041 (*lacTp*::luxAB*) strain had previously been constructed (18).

Luminescence determination. (i) **Measurement in culture.** To demonstrate that pH interferes with luciferase activity, overnight *L. casei* cultures in MRS medium did not exhibit luminescence but a 10-fold dilution of the same culture in fresh medium immediately generated luciferase activity (7). The initial method of measuring luminescence was therefore modified (5): the cultures in MRS medium were systematically diluted 1:10 in fresh medium, and luciferase activity was determined within less than 1 min, immediately after the addition of 5 µl of decanaldehyde (Sigma, Detroit, Mich.) to 1 ml of diluted broth culture (5). Light emission was measured in a Luminometer (LB9501; Berthold, Bad Wilbad, Germany) in arbitrary units, relative light units (RLU).

(ii) **Measurement in fecal samples.** Feces were rapidly 10-fold diluted in saline buffer after sampling. Luciferase measurements were then immediately performed on 1 ml of diluted fecal samples after addition of 5 µl of decanaldehyde.

(iii) **Luciferase activity expression.** For growth kinetics, luciferase activities in RLU were divided by values of absorbance determination at 600 nm (*A*₆₀₀) so that luciferase activities were expressed as RLU per *A*₆₀₀. For the comparison of luciferase activity in the inoculum and feces, the luciferase activities in RLU were divided by the number of CFU determined at the same time in each sample. The values were multiplied by 10⁶ so that results were expressed as log µRLU per CFU.

Growth kinetics. MRS medium was inoculated with 1% 2-day-old *L. casei* culture. The cultures were incubated at 37°C. Samples were collected at regular intervals and 10-fold diluted for *A*₆₀₀ (model 6400 spectrophotometer; Jenway, Dunmow, Essex, United Kingdom), luminescence determination (LB9501; Berthold), and agar plate count of the bacteria.

Study of promoter activities. *L. casei* DN-240 041, DN-240 047, DN-240 048, and DN-240 049 cultures in MRS medium were incubated at 37°C for 3 days. The inoculum size was selected so that the cultures obtained were in exponential phase after 1 day. For each strain, bacterial viability and promoter activity were determined daily in culture and posttransit through the GI tracts of HMA mice. Promoter activities were studied by luciferase activity determination and lucif-

FIG. 1. Genetic constructs. Step 1: Promoter insertion in pBlueScript II SK(+). XbaI-SmaI-cut PCR *ccpA* and *dlt* promoter or EcoRI-BamHI for *ldh* was used. This fragment was inserted into the XbaI-SmaI or EcoRI-BamHI sites of pBlueScript II SK(+), forming the plasmid pBS-Promoter. Step 2: Fusion with a luciferase vector. EcoRI-PstI-cut pBS-Promoter for *ccpA* and *dlt* or SacI for *ldh* was used. The resulting plasmid was fused with pIL4242 digested with the same enzymes, forming pLux-Promoter. Step 3: pBlueScript removal. BssHII-cut pLux-Promoter and the 5.3-kb fragment were ligated, giving the final plasmid.

erase mRNA quantification. Culture samples were diluted 1:10 in prewarmed medium containing erythromycin and incubated at 37°C. The experiment was carried out with or without the addition of rifampin to the media. Counts and luminescence determinations were performed over a period of 4 h postdilution. HMA mice were orally administered about 0.5 ml of *L. casei* culture at time zero. The feces were individually collected 6 h later. Counts, luminescence determination, and RNA extraction were performed on feces diluted 1:10 in saline buffer.

RNA extraction. The protocol developed was based on the isolation of the bacterial fraction from feces or broth culture followed by cell lysis and chloroform-phenol extraction.

(i) **Fecal samples.** A total of 0.1 g of the freshly collected fecal sample was resuspended in 1 ml of Tris-EDTA (TE). The suspension was centrifuged at $500 \times g$ for 1 min (Sigma 3K15) to remove the fiber. The supernatants were transferred to new tubes and then filtered (5- μ m pore size; Millipore) to remove small particles. The effectiveness of bacterial fraction isolation was estimated by total plate counts conducted on the suspension of freshly collected fecal samples and on the supernatants containing the microbial fraction by using MRS-erythromycin medium. The filtrate, containing about 80% of the microbial fraction, was centrifuged at $7,000 \times g$ and 4°C for 2 min. The supernatant was eliminated, and the pellet was washed twice and resuspended in 100 μ l of TE. The RNA was then extracted as described below.

(ii) **Culture samples.** Cultures were centrifuged at $7,000 \times g$ and at 4°C for 2 min. The pellets were washed twice and suspended in 100 μ l of TE. The RNA was isolated using the Macherey Nagel kit (Nucleospin RNAII) per the manufacturer's instructions but with modifications and partially adapted steps: 350 μ l of buffer RA1 (supplied with the kit), 5 μ l of β -mercaptoethanol, and 300 mg of glass beads (each $\leq 106 \mu$ m in diameter; Sigma) were added to the resuspended bacteria. The tubes containing the glass beads were shaken twice at 4,500 rpm for 40 s in a Fast Prep instrument (FP 120, Bio101; Savant, Holbrook, N.Y.) with rapid cooling on ice between steps. After shaking, the tubes were centrifuged at $7,000 \times g$ for 5 min. The supernatants were transferred to new tubes, and extraction was conducted per the manufacturer's protocol. The RNA was rapidly subjected to chloroform-phenol extraction and ethanol precipitation. The pellets

were washed with 70% ethanol and resuspended in 60 μ l of RNase-free water. Genomic DNA contamination was removed by DNase treatment (DNA free; Ambion Europe).

In vitro synthesis of luciferase messenger. The *P. luminescens* gene encoding *luxA* was PCR amplified from plasmid pIL4242 with primers OLC15 and OLC16. The PCR product was ligated to a T7 phage promoter adapter (Lig'nScribe; Ambion Europe). The ligation product was used in subsequent PCR amplification with the T7-promoter-specific primer P1 (supplied with the kit) and OLC16 to generate a sense strand transcription template. The T7-driven in vitro transcription reaction was performed using a standard transcription assay (MEGA script; Ambion Europe). The synthetic luciferase RNA was quantified using a spectrophotometer (Biophotometer; Eppendorf) and stored at -80°C. The concentrated RNA was diluted for the reverse transcriptase quantitative PCR (RT-QPCR) assays.

Reverse transcription. First-strand cDNA synthesis was conducted with random hexamers (New England Biolabs) by using 0.25 to 0.50 μ g of DNase I-treated total RNA. For each experiment, 10-fold dilutions (10^5 to 10^9 copies/ μ l) of in vitro-transcribed luciferase RNA were reverse transcribed concomitantly with the samples to generate QPCR standard curves. For the RT assays, Moloney murine leukemia virus RT (Invitrogen) was used per the manufacturer's instructions. Negative controls without RT were conducted for all samples in order to exclude any genomic DNA contamination.

SYBR green QPCR. In order to assess promoter activity during the GI transit of *L. casei*, real-time QPCR was conducted on samples. Luciferase messenger quantification was performed on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Primer Express version 1.0 from Applied Biosystems was used to design luciferase primers. A 198-bp PCR fragment generated by using primers OLC15 and OLC32 was selected for all the assays (see Results section). QPCR was conducted on 10 μ l of 10-fold-diluted reverse-transcribed cDNA and 15 μ l of $1 \times$ PCR mix (SYBR green I PCR core kit; Applied Biosystems) with optimum concentrations of primers. The thermal cycling conditions were as follows: 2 min at 50°C and then 10 min at 95°C followed by 40 cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 60°C. For each run, a standard dilution of a luciferase reverse-transcribed RNA fragment con-

TABLE 2. Primers used in this study

Primer	Accession no.	Sequence	Description ^a
Promoter amplification			
OLC1	U28137	5'-GCTCTAGAGCTCGCGATCTGGCTTAAAG-3'	<i>ccpAp</i> Fwd
OLC2	U28137	5'-TCCCCCGGGGCAATTGATTTTCTCCTTAGTCG-3'	<i>ccpAp</i> Rev
OLC3	M83993	5'-GCTCTAGACGTGGGGTGACAAGATGTTTC-3'	<i>dltP</i> Fwd
OLC8	M83993	5'-TCCCCCGGGGATCAATCGCTGTAATAACATTATC-3'	<i>dltP</i> Rev
OLC39	AF351136	5'-CCGGAATTCTGTGCGATTGCTTTGAGATT-3'	<i>ldhP</i> Fwd
OLC41	AF351136	5'-CGCGGATCCGAATAACTTTTGGTGATCC-3'	<i>ldhP</i> Rev
Integrand analysis			
OLC33	M90093	5'-GATATGAATATGCTGAAAGATA-3'	<i>luxB</i> Fwd
OLC52	U28137	5'-GCTAAACCAGCTTGGTAGCT-3'	<i>ccpA</i> Rev
OLC71	M83993, AAZ01000003, AP004824	5'-CCATCACGNATTTCCATAAT-3'	<i>dlt</i> Rev
OLC72	AF351136, AAK01000273, U78634, AF054624, AF436734, M72545	5'-TCMARTGAAGTACCTGARCC-3'	<i>ldh</i> Rev
Luciferase and other primers			
OLC15	M90093	5'-CGGGATCCAAATTTGGAACTTTTGTCTTACA-3'	<i>luxA</i> Fwd
OLC16	M90093	5'-AATCTGCAGCTAATATAATAGCGAACGTTGTT-3'	<i>luxA</i> Rev
OLC17	M90093	5'-CGGGATCCAAATTTGGATTGTTCTTCCTTAA-3'	<i>luxB</i> Fwd
OLC18	M90093	5'-AATCTG CAGTTAGGTATATTCCATGTGGTAC-3'	<i>luxB</i> Rev
P1	See kit	5'-GCTTCCGGCTCGTATGTTGTGTGG-3'	T7p

^a Fwd, forward; Rev, reverse.

taining a known quantity was included for cDNA quantification. A linear relationship was obtained by plotting the threshold cycle against the logarithm of a known amount of initial luciferase RNA. The equation of the best-fit line was determined by linear regression analysis. The plotting made it possible to determine QPCR effectiveness. The quantities of target copies contained in an unknown sample were determined by extrapolation from the linear regression of the standard curve. A negative control (no template control) and RNA samples which had not undergone the RT step (RT⁻) were included in each run. For each cDNA quantification, the number of copies was determined in triplicate and the mean was used for analysis. The specificity of the PCR was checked by analyzing the reaction mixtures by using 3% agarose gel electrophoresis.

Repeatability and relative gene expression. For all the promoters, the experiments were conducted in duplicate or triplicate using three mice per experiment. Six to nine mice were thus used to determine the activity of each promoter following HMA GI transit. Only samples with a constant total RNA concentration between 0.25 and 0.50 µg/µl were used for RT-QPCR (approximately 80% of the samples). Concomitantly with RNA isolation, *L. casei* cells were counted on MRS-erythromycin cultures. For each sample, the number of cDNA copies (an indicator of mRNA quantity) was divided by the number of bacterial CFU (mRNA/CFU).

RESULTS

In vitro luciferase expression in *L. casei* under the control of various promoters. (i) **Luciferase activities during *L. casei* multiplication.** The growth of each constructed strain was monitored in triplicate over a 24-h period by A_{600} determination (Fig. 2A). The exponential growth phase continued to an A_{600} of about 5.0. The calculated generation times were 2.25 h for DN-240 041 (*lacT**), 2.5 h for DN-240 049 (*ldh*) and DN-240 048 (*dlt*), and 2.75 h for DN-240 047 (*ccpA*). In the cultures, luciferase activities of the four promoters were very different (Fig. 2B). The highest activity was observed with the *ldh* promoter (*ldhP*) (3×10^5 RLU/ A_{600}), while the activity of the modified *lacT** promoter (*lacTp**) was threefold lower. The activities detected with the *ccpA* and *dlt* promoters (*ccpAp* and *dltP*) were, respectively, 8 and 25 times lower than that of *ldhP* but markedly above the limit of detection. The relative

luciferase expressions per CFU were 2.7, 2.2, 1.8, and 1.3 log µRLU/CFU for DN-240 049 (*ldh*), DN-240 041 (*lacT**), DN-240 047 (*ccpA*), and DN-240 048 (*dlt*), respectively. For all of the promoters, the highest luciferase activity was observed during early exponential growth. A gradual decline subsequently occurred. Luminescence declined during the switch from the mid- to late-exponential-growth phase ($A_{600} = 1.5$ to 2.0). No luciferase activity was detected during the stationary phase (A_{600} of about 5).

(ii) **Luciferase activities from 3-day-old *L. casei* cultures.** We sought to determine, in culture, the minimal time for restarting luciferase activity from 3-day-old cultures. The four constructed strains were cultured for 3 days and then diluted in fresh medium, and the luciferase activity was monitored over a 3.5-h period (Table 3). During that short interval, no significant multiplication was detected (data not shown), as had already been observed (18). However, luciferase activity was resumed more rapidly by DN-240 049 (*ldh*) and DN-240 041 (*lacT**) (after 2 h) than by DN-240 047 (*ccpA*) and DN-240 048 (*dlt*) (3.5 h). For all the genetic constructs, the addition of rifampin blocked luciferase activity without affecting *L. casei* viability.

(iii) **Luciferase activity under the control of different promoters in HMA mice.** The strains expressing luciferase under the control of *ldhP* and *ccpAp* were incubated for 3 days in MRS medium. On days 1, 2, and 3, HMA mice were orally administered the culture and luciferase activity/CFU was estimated in the dosing preparation and mouse feces 6 h postdosing (Fig. 3). On days 2 and 3, no luciferase activity was detected in the dosing preparation but resumption of activity was observed in the feces. Posttransit, the luciferase expression induced by the *ldh* promoter was about fivefold greater than that induced by *ccpAp*.

The same experiment with the four transcriptional fusion

FIG. 2. *L. casei* growth and promoter activities in MRS medium. Strains DN-240 041 (*lacT**, triangles and solid lines), DN-240 049 (*ldh*, squares and dotted lines), DN-240 048 (*dlt*, squares and solid lines), and DN-240 047 (*ccpA*, triangles and dotted lines) were cultured at 37°C, and samples were removed at timed intervals for A_{600} (A) and luminescence (B) determinations. Luciferase activity was determined immediately after the addition of 5 μ l of decanaldehyde to 1 ml of culture. The values obtained were expressed as RLU per A_{600} . The error bars represent the standard errors of the means. OD₆₀₀, optical density at 600 nm.

constructs was repeated using a 3-day-old bacterial culture and testing in HMA mice (Table 4). Posttransit luciferase activity resumption was observed for DN-240 049 (*ldh*) and DN-240 041 (*lacT**) but not detected with the other promoters.

Use of RT-QPCR to determine luciferase mRNA in *L. casei*.
(i) Reproducibility of RNA isolation and use of a synthetic luciferase RNA standard. RNA isolation was determined on 0.1 g of feces containing 10^7 to 10^8 *L. casei*. The effectiveness of extraction from a larger (1 g) fecal sample was inferior (data not shown). Repeated RNA extraction from 0.1 g of HMA mouse fecal samples consistently generated similar results, demonstrating the satisfactory between-assay reproducibility of the method (325 ± 40 ng/ μ l). The chloroform-phenol-treated total RNA remained stable when stored at -80°C . With respect to pure cultures, reproducible quantities of RNA were obtained from 10^8 to 10^9 bacteria. After further DNase treatments, samples that had not been reverse transcribed (RT⁻) showed no amplification, indicating the absence of contaminating genomic DNA.

In order to plot standard RNA curves, specific luciferase

RNA was synthesized in vitro. The in vitro transcription yield ranged from 10^{11} to 10^{12} copies of synthetic RNA per microliter. This was verified to assure that PCR effectiveness did not fluctuate with the initial amount of RNA. In order to ensure that the experimental conditions were the same for both the standards and the samples during RT-QPCR and in order to consider the rate of the RT step, synthetic luciferase RNA was used for the standard curves (8).

(ii) Adaptation of real-time QPCR to the digestive tract contents for *L. casei* mRNA quantification. In order to confirm the results obtained in previous work based on luciferase activities (18), *L. casei* gene expression was quantified by adapting a sensitive and accurate method for use on the contents of the GI tract. RT-QPCR was selected on the basis of the small quantity of total RNA it required compared to other methods, such as Northern hybridization. To detect the expression of the four genes against a high endogenous microbiota mRNA background, a fragment of the luciferase genes which had previously been fused with the promoters of interest was targeted in RT-QPCR. Since luciferase genes were absent from the HMA

TABLE 3. Luciferase activities of 3-day-old *L. casei* cultures diluted in fresh medium^a

	Culture diluted (1:10) in MRS				Culture diluted (1:10) in MRS+Rif	
	log CFU/ml (T = 0 to 3 h 30 min)	log μ RLU/CFU (T = 0 h)	log μ RLU/CFU (T = 2 h)	log μ RLU/CFU (T = 3 h 30 min)	log CFU/ml (T = 0 to 3 h 30 min)	log μ RLU/CFU (T = 0 to 3 h 30 min)
<i>ldhp</i>	8.32 ± 0.1	nd	1.32 ± 0.1	ND	8.20 ± 0.3	nd
<i>lacTp*</i>	8.33 ± 0.2	nd	1.67 ± 0.2	1.8 ± 0.2	8.30 ± 0.1	nd
<i>ccpAp</i>	8.14 ± 0.2	nd	nd	0.6 ± 0.1	8.00 ± 0.3	nd
<i>dltp</i>	8.40 ± 0.2	nd	nd	0.7 ± 0.1	8.30 ± 0.1	nd

^a Three-day-old culture samples were incubated at 37°C after dilution (1:10) in prewarmed MRS medium supplemented or not with rifampin. Bacterial counts and luminescence determinations were conducted over 4 h postdilution. The results are presented as the means $\pm$ standard errors of the means. nd, not detected; ND, not done.

FIG. 3. Analysis of luciferase expression in *L. casei* post-HMA mouse GI transit. The mice were orally administered 0.5 ml of a preparation containing *L. casei* (10^8 to 10^9 CFU/ml) culture and spores (same quantity) of *B. subtilis* (transit marker) daily at 10 a.m. The feces were collected 6 h later. The luminescence and luciferase mRNA molecules per CFU were determined on the dosing preparation, and feces were diluted 1:10. The results for strains DN-240 049 (*ldh*) (A) and DN-240 047 (*ccpA*) (B) are shown. ¹, molecules of luciferase mRNA per CFU; Nd, not detected; ³, below the limit of detection. The error bars represent the standard errors of the means.

mice GI tract, the transcriptional fusion approach made the specific quantification of promoter activity possible. Real-time PCR assays were conducted using the DNA-binding dye SYBR green I. For the five pairs of primers tested, satisfactory results were obtained with the OLC15-OLC32 pair. PCR effectiveness was around 81.2%, and no primer dimer artifacts for the negative control were observed. All of the RT-QPCR products yielded a single band of the expected magnitude on agarose gel. The specificities of the signals detected from GI tract contents in RT-QPCR were confirmed (data not shown) with HMA mice not exposed to luciferase-containing *L. casei*. The limit of detection for mRNA molecules per number of CFU was about 10 copies of luciferase mRNA per CFU.

(iii) Direct analysis of luciferase transcripts post-GI transit. The between-assay variability of messenger quantification for the DN-240 049 strain is shown in Table 5. Two different experiments performed on six mice (three per experiment) generated reproducible results with 15.8% variability (Table 5). The expression levels of the four genes of *L. casei* post-GI transit, determined by RT-QPCR, are summarized in Fig. 3 and Table 4. For each gene, the expression level was determined in six to nine mice. The number of luciferase mRNA

copies quantified by RT-QPCR posttransit was compared to that present in the initial dosing preparation (Fig. 3 and Table 4). After 2 days of incubation at 37°C, *luxA* and *luxB* transcripts were detected by RT-QPCR in 2-day-old *L. casei* cultures in MRS medium while no luciferase activity was detected (Fig. 3). For strains DN-240 049 (*ldh*) and DN-240 047 (*ccpA*), the number of mRNA copies was almost the same in the 2-day-old cultures (96 and 26 copies/CFU, respectively) and posttransit in the feces (84 and 30 copies/CFU, respectively) (Fig. 3). In consequence, 3-day-old cultures were used to investigate de novo protein synthesis in the GI tract. In these cultures, the viable bacteria count was around 10^9 CFU/ml (Table 4). For all strains, the number of luciferase mRNA copies per CFU was below the level of detection in 3-day-old cultures (Table 4). In the 3-day-old posttransit cultures, a marked increase in luciferase transcripts was detected for DN-240 049 (*ldh*) (27 copies/CFU) and DN-240 041 (*lacT**) (118 copies/CFU), reflecting de novo synthesis (Table 4). For DN-240 047 (*ccpA*) and DN-240 048 (*dlt*), no signal was detected by RT-QPCR on the post-transit fecal samples (Table 4). These results confirm and reinforce those obtained by luminescence determination.

TABLE 4. Analysis of the expression of the four promoters in 3-day-old cultures and post-HMA mouse GI transit^a

	3-day-old cultures			HMA mouse feces		
	log CFU/ml	log μRLU/CFU	RNA/CFU ^b	log CFU/mL	log μRLU/CFU	RNA/CFU ^b
<i>ldhp</i>	9.23 ± 0.3	nd	nd	8.60 ± 0.2	1.36 ± 0.1	27 ± 4
<i>lacTp</i> *	9.03 ± 0.2	nd	nd	8.60 ± 0.1	1.60 ± 0.2	118 ± 54
<i>ccpAp</i>	9.25 ± 0.2	nd	nd	8.73 ± 0.2	nd	nd
<i>dltp</i>	9.30 ± 0.3	nd	nd	8.52 ± 0.2	nd	nd

^a Forced feeding and determinations were conducted as described in the legend to Fig. 3. For each gene, the result shown is the mean of six to nine determinations. nd, not detected.

^b Molecules of luciferase mRNA per CFU. The results are presented as the means ± standard errors of the means.

TABLE 5. Variability of luciferase mRNA quantities in HMA mouse feces^a

Expt	Mouse 1	Mouse 2	Mouse 3	Mean
1	38	31	10	26
2	19	35	27	27
Both				27 ± 4

^a The forced feeding and determinations were conducted as described in the legend to Fig. 3. The numbers of molecules of luciferase mRNA per CFU for strains DN-240 049 (*ldh*) are shown.

DISCUSSION

This study was designed to investigate the physiological status of a probiotic bacterium, *L. casei* DN 114 001, during GI transit. In a previous study (18), we used transcriptional fusion of *luxA* and *luxB* genes and the *lacT** promoter to demonstrate that *L. casei* does not multiply in the HMA mouse GI tract model but that it does survive. The comparison of the luciferase activity of 3-day-old cultures, preingestion versus post-transit, together with protein synthesis inhibition in cultures suggests that *L. casei* can initiate de novo protein synthesis during GI transit with the diet. In the previous study, one promoter was investigated and the evidence of de novo synthesis in the GI tract was indirect. The present study went into greater depth by using four *L. casei* promoters suspected of exhibiting different degrees of effectiveness in vitro. The activity ranking determined in vitro (*ldhp* > *lacTp** > *ccpAp* > *dltp*) was consistent with that reported in previous studies (16, 25, 26). Moreover, a molecular approach consisting of RT-QPCR was applied to the transcriptional fusion constructs to clearly show de novo mRNA synthesis by bacteria during GI transit.

Use of RT-QPCR has rendered investigation of in vivo gene expression during infectious processes possible (28, 31). The application of QPCR methods to GI contents has mainly focused on the detection or quantification of exogenous bacterial genomes (2, 11), while transcriptional analysis has not previously been addressed. The molecular approach developed for this study made quantification of specific mRNA in a fecal extract possible for the first time. In order to detect the expression of the four genes against a high background level of endogenous microbiota mRNA (32), a fragment of the luciferase genes previously fused with the promoters of interest was targeted in RT-QPCR. The *luxA* and *luxB* genes were absent from the HMA mouse GI tract. It was thus possible to quantify the luciferase messengers specifically and estimate promoter activities in a different manner. Since analysis at transcriptional level does not necessarily indicate whether luciferase proteins are synthesized in the GI tract, this approach was combined with luminescence determination.

When mice were administered 2-day-old culture preparations, the luciferase activity determinations showed potential de novo synthesis in the GI tract for three promoters. These results were not, however, confirmed by mRNA analysis. Although luciferase mRNA remained present in these cultures, luciferase activity was not detected. The presence of detectable mRNA about 10 to 20 h after the onset of the stationary phase, in line with previous studies (33), suggests that the luciferase activity detected posttransit may originate from enzymes al-

ready synthesized before ingestion. In this context, the RT-QPCR approach generated further information to combine with the luciferase determinations. Hence, the absence of luciferase activity in 3-day-old cultures was shown to be concordant with the nonsignificant level of luciferase messengers detected. The facts that no luciferase mRNA was present in the initial 3-day-old culture and that luciferase activity regeneration was prevented by RNA polymerase inhibition suggest that the detection of luminescence is clearly concomitant with de novo mRNA synthesis. The *L. casei* cells were metabolically inactive when administered to the mice. During intestinal transit, they produced luminescence and therefore proceeded to de novo synthesis under the control of the *ldh* and *lacT** promoters. These results were confirmed by direct in vivo transcription analysis. The positive activities of the deregulated *lacT** and *ldh* promoters in the GI tract strongly suggest that at least the primary basal metabolism of the bacteria is activated during transit. However, exposure to the GI environment involves physiological adaptation, since functions unnecessary for basal metabolism (*ccpA* and *dlt* promoters) were not activated. *L. casei* does not multiply in the GI tract (18). It is therefore consistent that the bacterium does not activate *dlt* operon transcription since the resulting proteins are involved in cell shape (10) and, indirectly, in cell division. Despite the presence of autochthonous microbiota (32) and the stress generated by exposure to gastric acid and bile (12), the GI tract environment does not appear to impair the physiology of *L. casei* or its viability. However, the bacterium seems likely to carefully control promoter activity while transiting through the GI tract.

Several studies have recently investigated probiotic adaptation in the highly competitive ecosystem of the GI tract, and different approaches have been used to identify specific gene expressions. In vivo expression technology has been adapted to screen for specific in vivo-induced genes of *Lactobacillus plantarum* (4) and *Lactobacillus reuteri* (34) in the murine GI tract. Two-dimensional electrophoresis and DNA microarray studies, with methods adapted to GI contents (35), are promising approaches to identify the thousands of genes expressed simultaneously in vivo. The methods enable rapid identification of the functions activated in the gut, but they need to be combined with an accurate method for targeted studies. In such a context, the RT-QPCR approach developed in this study may be of value. The sensitivity of RT-QPCR has been shown to be superior to that of DNA microarrays (27) and may thus ensure detection of low-level gene expression.

The present study is the first to investigate a probiotic in a physiological state in the GI tract by direct transcription analysis of fecal samples. In line with previous studies, we have confirmed that *L. casei* can initiate protein synthesis during GI transit and that the bacterium modulates gene expression in a setting of physiological adaptation to environmental change. With a view to linking beneficial probiotic functions to new protein synthesis and obtaining specific information on probiotic adaptation, the RT-QPCR approach described herein, combined with other methods, is promising.

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