Use of Luciferase Genes as Biosensors To Study Bacterial Physiology in the Digestive Tract

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A method based on the use of the bacterial luciferase genes was developed in order to investigate *Lactococcus lactis* gene expression in the mouse digestive tract. Germfree mice were monoassociated with different strains containing transcriptional fusions of promoters with the luciferase genes. Our results demonstrate that this method is readily applicable to the study of promoter strength and physiology of bacteria in the digestive tract.

Lactic acid bacteria are widely used in food technology and may have a so-called "probiotic effect" (4, 10). However, the metabolic activities of these bacteria in the digestive tract (DT) remain uncertain, and this fact impairs our understanding of the beneficial effect of these organisms on health. The lactic acid bacteria adapt their metabolism to the environment by synthesizing proteins whose activities are required for survival and, eventually, proliferation. The level of transcription of regulated promoters reflects the need of the cells for particular functions according to the physiological state and environment stimulation. The activity of regulated promoters, therefore, reflects the physiological state of the cells.

Moreover, the use of lactic acid bacteria as vectors for new molecules with targeted activity in hosts is being investigated (5, 11, 18). Being able to measure promoter strength in the DT is a prerequisite for expression of heterologous proteins at suitable levels. This paper presents an experimental model for studying promoter activities in the DT by using the bacterial luciferase gene as a reporter gene.

Lactococcus lactis was grown in M17 (17) or chemically defined medium (CDM) (13) supplemented with erythromycin (5 μg/ml). Thermoresistant *Bacillus subtilis* spores were used as transit markers (2). The *L. lactis* strains used are derivatives of NCFB2118 (National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom) containing integrative plasmids or transposons. These elements carry the *lux* genes from *Vibrio harveyi* fused with *L. lactis* promoters as described previously (15). A fragment from pJIM2366 (15) containing the fusion of *lux* genes and P$_{ald}$, the promoter for the acetolactate decarboxylase gene from *L. lactis* (fragment from position 24152 to 24716 from GenBank sequence accession no. V92974), with an erythromycin marker was subcloned in the *Hind*III site present in *tetM* from Tn*916* carried by pBluescript (Stratagene, CF). This construction was used to replace the *tetM* gene from Tn*916* by transformation of a derivative of *B. subtilis* 168 (1). JIM5192 was obtained by introducing the transposon into NCFB2118 by conjugation. JIM5206, JIM5213, and JIM5460 are transformants of NCFB2118 containing the *luxAB* gene fused with the *his* promoter (P$_{his}$; segment fragment from position 1 to 1296 from GenBank sequence accession no. M90760), a truncated derivative of the *his* promoter (P$_{hisD3}$; segment fragment from position 1 to 948), and the *mleS* promoter (P$_{mle}$; segment fragment from po-

sition 1 to 468 from GenBank sequence accession no. X75982), respectively. The plasmids used to integrate these fusions into the chromosome are derivatives of pORI28 (9), and the method used to select single crossover events has been described previously (6).

Germfree C3He/J mice (10 mice per group) were reared in a Trexler isolator fitted with a rapid transfer system (La Calhène, Vélizy, France). All materials introduced into the isolator were sterilized by irradiation or heating. Each mouse received 0.5 ml of a 24-h culture of *L. lactis* (about 4×10^8 CFU/ml) by the orogastric route. Malate inoculation was performed as follows: each mouse received orogastrically 0.5 ml of malate (10% in 0.15 M NaCl) at 10 and 12 a.m. Fecal samples were collected at 2 p.m., and the samples were diluted 1/3 with sterile distilled water. Luciferase activities (Lux) and *L. lactis* counts were estimated from these dilutions as previously described (15). Promoter strength was expressed as the logarithm of the number of relative light units per 10^8 CFU.

Different groups of germfree mice inoculated with a single *L. lactis* strain (gnotoxenic mice) were studied. One day after inoculation, the bacteria were established in the DT, and their populations remained stable (about 10^8 CFU/g of fecal sample) for several months. It has been shown previously that this animal model allows *L. lactis* to reach a stable population level in fecal samples (7). However, the metabolic activities of the bacteria were unknown.

In the present study, luciferase genes were used as biosensors to study the physiology of *L. lactis* established in DT. The bacterial luciferase gene has been shown to be a potential reporter gene in *L. lactis* (3, 15). Light emission with intact cells requires nonaldehyde as the substrate and reduced flavin mononucleotide (FMN) produced by the bacteria (12). The availability of reduced FMN depends on the physiological state of the cells, a condition that restricts the use of *luxAB* reporter genes to fully viable cells (8).

We first studied *L. lactis* JIM5213. In this strain the *lux* genes are under control of P$_{hisD3}$, a truncated derivative of the histidine operon promoter which is deregulated in CDM with or without histidine (Fig. 1A). The amount of Lux activity per *L. lactis* cell was on the same order of magnitude in laboratory media and in feces of gnotoxenic mice colonized with *L. lactis* (Fig. 1A). This showed that *L. lactis* is metabolically active and contains nonlimiting amounts of reduced FMN when it is established in the DT of gnotoxenic mice.

The *lux* construction JIM5206 contains the wild-type histidine promoter (P$_{his}$), which is turned off and on in CDM with and without histidine, respectively (Fig. 1B). In gnotoxenic mice, the strength of this promoter was even less than its strength in CDM

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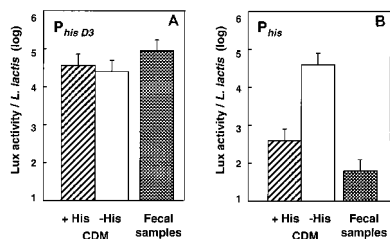
FIG. 1. Comparison of the expression of the P_{his} promoters in culture broth (CDM with and without histidine) and in gnotoxenic mice colonized with *L. lactis*. The following two forms of the P_{his} promoter were used: P_{hisD3}, which is the truncated form of P_{his} (A), and P_{his}, which is the wild-type promoter (B). Lux activities and bacterial counts were determined for each mouse fecal sample. The vertical bars represent the standard errors of the means.

with histidine (Fig. 1B). The repression of P_{his} was presumably mostly due to the presence of histidine in fecal samples at a concentration slightly higher than the concentration in CDM (600 and 360 nmol/g in DT and CDM, respectively).

In *L. lactis* JIM5192, *lux* genes are under the control of P_{ald}, which is induced in the presence of the three branched-chain amino acids (isoleucine, leucine, and valine [ILV]) (Fig. 2A). The Lux activity in fecal samples was similar to the Lux activity observed in CDM lacking ILV (Fig. 2B). However, the concentrations of free ILV were similar in the fecal samples and CDM (400 to 500 nmol/g in DT and CDM, respectively), suggesting that the absence of the branched-chain amino acids was not responsible for the low level of promoter expression. However, P_{ald} has reduced activity under certain stress conditions. This response is known to be induced by amino acid starvation and low pH (14). Fusions with other promoters that reveal specifically different stress responses should be tested to determine the factor(s) involved in P_{ald} closure in DT.

We tested our ability to stimulate *L. lactis* in the DT. To do this, we used JIM5460 containing a fusion with P_{mle}, the promoter for malolactic fermentation genes, which is induced by malate (16). Gnotoxenic mice harboring JIM5460 received malate orally, and the Lux activity of *L. lactis* was measured in fecal samples. The uninduced P_{mle} activities were low and similar in fecal samples and CDM (Fig. 3). A 40-fold increase was observed after malate was ingested. This increase was significant, although it was 10-fold lower than the increase observed in CDM with malate (Fig. 3). Activation was transient, as the Lux activity reached the uninduced level 1 day after supplementation and was again inducible another day later (data not shown). It appears that *L. lactis* is able to adapt its metabolism to this diet additive. The induction in the DT was not as effective as the induction in the laboratory medium, possibly because the malate given orally may have been absorbed in the small intestine, leading to reduced malate availability for *L. lactis* in the cecum.

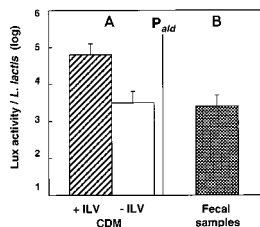To our knowledge, our work considering the total *L. lactis*

FIG. 2. Comparison of the expression of the P_{ald} promoter in culture broth (CDM with and without the branched-chain amino acids ILV) (A) and in gnotoxenic mice colonized by *L. lactis* (B). The vertical bars represent the standard errors of the means.
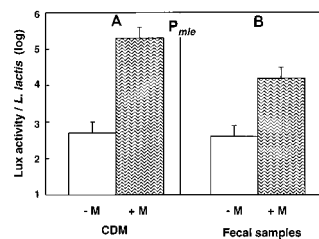

FIG. 3. Induction by malate (M) of the expression of the P_{mleS} promoter in CDM (A) and in gnotoxenic mice colonized by *L. lactis* (B). The vertical bars represent the standard errors of the means.

population in the DT is the first work which reports an assessment of promoter strength in the DT. More information on the metabolic activity of bacterial cells in the DT could be obtained by using promoters specific for other pathways and responding to different signals. Knowledge concerning the expressed genes and metabolic activities of microorganisms ingested with food is important, as it has been proposed that some microorganisms are beneficial to human health (4, 10). This new approach for studying bacterial metabolism in vivo could help determine the role of microorganisms as probiotic organisms.

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