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Research Paper

Use of green fluorescent protein to monitor *Lactobacillus plantarum* in the gastrointestinal tract of goats

Xufeng Han[1], Lei Wang[1], Wei Li[1], Bibo Li[1], Yuxin Yang[1], Hailong Yan[2], Lei Qu[2], Yulin Chen[1]

[1]College of Animal Science and Technology, Northwest A&F University, People's Republic of China, China.
[2]College of Life Science, Yulin University, People's Republic of China, China.

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Abstract

The experiment aimed to specifically monitor the passage of lactobacilli *in vivo* after oral administration. The green fluorescent protein (GFP) gene was cloned downstream from the constitutive p32 promoter from *L. lactis subsp. cremoris* Wg2. The recombinant expression vector, pLEM415-gfp-p32, was electroporated into *Lactobacillus plantarum* (*L. plantarum*) isolated from goat. Green fluorescent protein (GFP) was successfully expressed in *L. plantarum*. After 2 h post-administration, transformed *Lactobacillus* could be detectable in all luminal contents. In the rumen, bacteria concentration initially decreased, reached the minimum at 42 h post-oral administration and then increased. However, this concentration decreased constantly in the duodenum. This result indicated that *L. plantarum* could colonize in the rumen but not in the duodenum.

Key words: green fluorescent protein, gastro-intestinal tract, *Lactobacillus plantarum*, goat.

Introduction

Lactobacilli are Gram-positive anaerobic or facultative aerobic rods, which can be isolated from human and animal tissues, plants and materials of plant origin, and sewage and fermented products (Coeuret *et al.*, 2003; Lee *et al.*, 2009; Azadnia *et al.*, 2011). Particular lactobacilli strains are considered to present beneficial effects on human and animal health. Therefore, several species of lactobacilli are used as probiotics (Forestier *et al.*, 2001). Lactobacilli are generally regarded as safe (GRAS) by the FDA and are thus regarded as potential vehicles for oral vaccination (Mercenier *et al.*, 1996; Scheppler *et al.*, 2002). Moreover, they can stimulate local cell immunity of intestinal and humoral immunity (Biller *et al.*, 1995; Christensen *et al.*, 2002; Servin, 2004). To provide a beneficial effect on the health of the hosts, the probiotic strain must be able to survive in the gastro-intestinal tract (Fuller, 1992). Therefore, knowing the distribution and movement of lactobacilli in the gastro-intestinal tract, as well as in which tract the strain functions, is necessary. Marking the lactobacilli and tracing them by using green fluorescence protein (GFP) is a good method because GFP presents the advantage of being an auto-fluorescent protein that does not require a substrate. In addition, GFP allows for real-time detection in living cells (Kitts *et al.*, 1995). This method has been applied in monogastric animals but has yet to be reported in ruminants (Yu *et al.*, 2007; Wang *et al.*, 2011).

In the present study, we constructed a constitutive expression vector for lactobacilli by using GFP as the reporter protein. We then electroporated the recombinant into *L. plantarum*, which was isolated from Shanbei White cashmere goats. Finally, the goat was fed with the transformants by using GFP as a visible marker for tracking this strain introduced into the gastrointestinal tract and observing its colonization capability.

Materials and Methods

Bacteria, plasmid and growth conditions

*Escherichia coli DH5*α was used for the construction and propagation of plasmid and grown in Luria-Bertani

Send correspondence to Y. Chen. College of Animal Science and Technology, Northwest A&F University, No. 22 Xinong Road, Yangling, 712100 Shaanxi, P.R. China. E-mail: chenyulindk@163.com.

(LB) medium at 37 °C. *Lactobacillus* strains were originally isolated from the rumen of goats and were used as recipient strains for genetic construction and grown without shaking in an MRS medium at 37 °C at static conditions. The bacteria were identified as *L. plantarum* via both phenotypic and genotypic methods (Table 1). When appropriate, antibiotics were added to the culture medium. For *E. coli* and *Lactobacillus*, ampicillin (Amp) was used at final concentrations of 100 and 50 μg /mL, respectively.

In this study, the replicative plasmids, namely, pLEM415, pMG36e, and pEGFP-N1, were used (Table 1). pLEM415 is an *E. coli-L. reuteri* shuttle vector (Serror *et al.*, 2002) that contained a multi-cloning site and genes for Amp resistance. pMG36e contained the p32 promoter from *L. lactis subsp. cremoris* Wg2 (Van de Guchte *et al.*, 1989).

Restriction endonucleases, T4 DNA ligase, and Taq polymerase were purchased from TaKaRa Biotechnology (Dalian, China) and used according to the recommendations of the manufacturers.

PCR amplifications

The *gfp* fragment was obtained by PCR amplification from pEGFP-N1, and the primers used were gfpF (5'-ATACCGCGGATGGTGAGCAAGGGCGAG-3') and gfpR (5'-GCCGGAGCTCTTACTTGTACAGCTCG TCCATGC-3').The *p32* promoter was obtained via PCR amplification from pMG36e, and the primers used were p32F (5'-TGCTCTAGAAATTCGGTCCTCGGGATAT GATAAG-3') and p32R (5'-TCCCCGCGGGAATTTTT CTGCTGAAACGATTGCCAT-3'). Restriction sites added at the 5'-end of each primer are underlined. Agarose gel electrophoresis of plasmid DNA and PCR fragments was performed using the procedure described by Sambrooket *et al.* (1989). These fragments were recovered via gel extraction by using a DNA Gel-extraction kit (OMEGA, USA).

Construction of expression plasmid carrying the *gfp* gene

The PCR products of *gfp* and *p32* were cloned into the pMD19 T vector and then sequenced. Subsequently, the recombinant pMD19 T-gfp was digested by *Sac*II and *Sac*I and then ligated with *Sac*II-*Sac*I-restricted pLEM415 to construct the recombinant pLEM415-gfp. The recombinant pMD19 T-p32 was then digested by *Xba*I and *Sac*II and then ligated with *Xba*I-*Sac*II-restricted pLEM415-gfp to construct the recombinant pLEM415-gfp-p32. Thus, the resulting recombinant, pLEM415-gfp-p32, carried the *gfp* gene under the control of the *p32* promoter. The structure of pLEM415-gfp-p32 was verified via restriction analysis, and the *p32* and *gfp* fragment were verified via DNA sequencing.

Electrotransformation procedures

The pLEM415-gfp-p32 was propagated by transformation into *E. coli* DH5α competent cells according to manufacturer's instructions. *E. coli* transformants were selected on LB agar plates containing Amp. DH5α harbouring pLEM415-gfp-p32 was proliferated at 37 °C for 12 h with shaking in LB broth containing Amp. pLEM415-gfp-p32 was purified from *E. coli* cultures via the alkaline lysis method (Sambrook *et al.*, 1989). *L. plantarum* electrocompetent cells were prepared based on a previously described method (Mason *et al.*, 2005). A 50 μL concentrated cell suspension was electroporated using a Gene Pulser electroporator (Bio-Rad, Hemel Hempstead, UK) in cuvettes with a 0.2 cm electrode gap (Flowgen, Ashby de la Zouch, UK) with up to 1 μg of plasmid DNA (at a concentration of 100 μg mL^{-1}). Except where stated, electroporation parameters were 2.0 kV, 200 Ω parallel resistance, and 25 μF capacitance. For phenotypic expression, the cells were diluted immediately into 1 mL of MRS broth in a 2 mL vial that was pre-warmed to 37 °C. After 3 h of incubation, serial dilutions were plated onto MRS agar containing Amp. Plasmids from lactobacilli transformants were

Table 1 - Plasmids and bacterial strains.

Plasmids or strain	Relevant characteristics	Antibiotic resistance	Reference or source
Plasmid			
pGFP-N1	plasmid containing gfp gene	Amp^r	Jia *et al.* (2006)
pMG36e	*E. coli-LAB* shuttle vector, containing P32 promoter	Em^r	Van de Guchte *et al.* (1989)
pLEM415	*E. coli -L. reuteri* shuttle vector	Amp^r Erm^r	Serror *et al.* (2002)
pLEM415-gfp	pLEM415 containing gfp gene	Amp^r Erm^r	This study
pLEM415-gfp-p32	pLEM415-gfp containing p32 gene	Amp^r Erm^r	This study
Bacterial strains			
Escherichia coli DH5α	Transformation host		Invitrogen
Lactobacillus plantarum	Transformation host		Goat rumen

Em^r: erythromycin resistance; Amp^r: ampicillin resistance.

isolated following the method described by Anderson and McKay (1983).

Observation of fluorescence

For the observation of fluorescent bacteria, lactobacilli cultured overnight under investigation were washed three times in phosphate-buffered saline (PBS, pH = 7.5) and smeared on microscope slides. Prior to observation, the slides were treated by overlaying with 20% glycerine. An epifluorescent microscope (AMG) equipped with a GFP filter set (excitation 470 nm; emission 505 nm to 530 nm) was used to visualize fluorescent bacteria. Lactobacilli without pLEM415-gfp-p32 were used as a negative control. To confirm the stability of the replicative plasmid pLEM415-gfp-p32 in lactobacilli, the transformants were tested after culturing continuously for 100 generations at non-selective conditions.

Intestinal distribution of *L. plantarum*-GFP in goats

To monitor the distribution of *L. plantarum*-GFP *in vivo*, eight Shanbei white cashmere goats with fistulas were used in our experiment. Each goat was installed with three fistulas, namely, rumen, duodenum, and ileum fistula. Four goats were orally inoculated with 1 mL of PBS containing 10^9 cfu of *L. plantarum* harbouring pLEM415-gfp-p32. Other goats were orally inoculated with 1 mL of PBS as a blank control. After 2, 6, 24, 48, and 72 h, samples from luminal contents in the rumen, duodenum, and ileum were serially diluted in PBS and cultured on MRS plates containing Amp overnight at 37 °C. Bacteria concentrations (cfu mL^{-1}) in rumen, duodenum, and ileum were determined according to the number of colonies in MRS plates. The data were analysed by the general linear model procedures of SPSS software (SPSS Inc., Chicago, IL, USA). All experimental procedures with goats used in the present study had been given prior approval by the Experimental Animal Manage Committee of Northwest A&F University.

Results

Verification of Recombinant plasmid

Recombinant plasmid pLEM415-gfp-p32 extracted from DH5a was digested by *Sac*II-*Sac*I and *Xba*I-*Sac*II, respectively. After enzyme digestion, we performed agarose gel eletrophoresis to identity the DNA fragments. The result of agarose gel eletrophoresis showed that two DNA fragments were about 750 and 200 bp, which were consistent with the sizes of *gfp* and *p32* fragment (Data not show). In addition, we also performed agarose gel eletrophoresis to check the PCR products amplified from pLEM415-gfp-P32 by primer *gfp* and *p32*, which were about 750 and 200 bp as well. Taken together, these results showed that the expression plasmid of lactobacilli was successfully constructed.

Observation of fluorescence

For the observation of fluorescent bacteria, transformed bacteria were washed in PBS (pH = 7.5) and observed via direct fluorescence microscopy, and bacteria without pLEM415-gfp-p32 were used as a negative control. The result showed that the transformed bacteria expressed fluorescence, whereas no fluorescence was observed in the negative control (Figure 1). Furthermore, we demonstrated that GFP was detectable in clones of transformed lactobacilli after subculturing for 100 generations without antibiotic. The results suggested that GFP could be stably expressed in the recombinant lactobacilli *in vitro*.

Monitoring *L. plantarum*-GFP in the gastrointestinal tract of goat

After 2, 6, 24, 48, and 72 h of inoculating goats with the transformant, the geometric means of $\log_{10}$ concentrations of bacteria in rumen, duodenum, and ileum were analysed (Figure 2). At 2 h post-administration, transformed lactobacilli can be detectable in all luminal contents. In the rumen, the concentrations of bacteria initially decreased, reached minimum at 42 h, and then increased. In the duodenum, however, a constant decrease was observed. Compared with those in other gastrointestinal sections, the transformant colonized in the ileum were highest at any time but without regular changes.

Discussion

A breakthrough in the transformation of *Lactobacillus* strains was made when Chassy and Flickinger used electroporation to introduce plasmid and phage DNA into *L. casei* (Bringel *et al.*, 1989). Since then, methods for the transformation of *Lactobacillus* have been rapidly developed and widely applied (Lin and Savage, 1986; Bringel and Hubert, 1990; Posno *et al.*, 1991; Bhowmick and Steele, 1993; Mason *et al.*, 2005; Palomino *et al.*, 2010), although few studies have optimized this procedure for lactobacilli. Numerous studies have shown that various parameters, including the competent cell preparation, electrical parameters, and host specificity, can influence the transformation efficiency of *Lactobacillus* (Hashiba *et al.*, 1990; Wei *et al.*, 1995; Serror *et al.*, 2002).

At present, no standard protocol of competent cell preparation has been established. Numerous studies have shown that culture, suspension, and resuscitation medium of competent cells should be adjusted according to different species of lactic acid bacteria. When competent cells were established according to the procedure described by Mason *et al.* (2005), the initial transformation frequencies were low (Table 2). Thus, we modified the suspension by adding 0.4 M sucrose and 20% glycerine, which resulted in improved transformation efficiencies (Table 2).

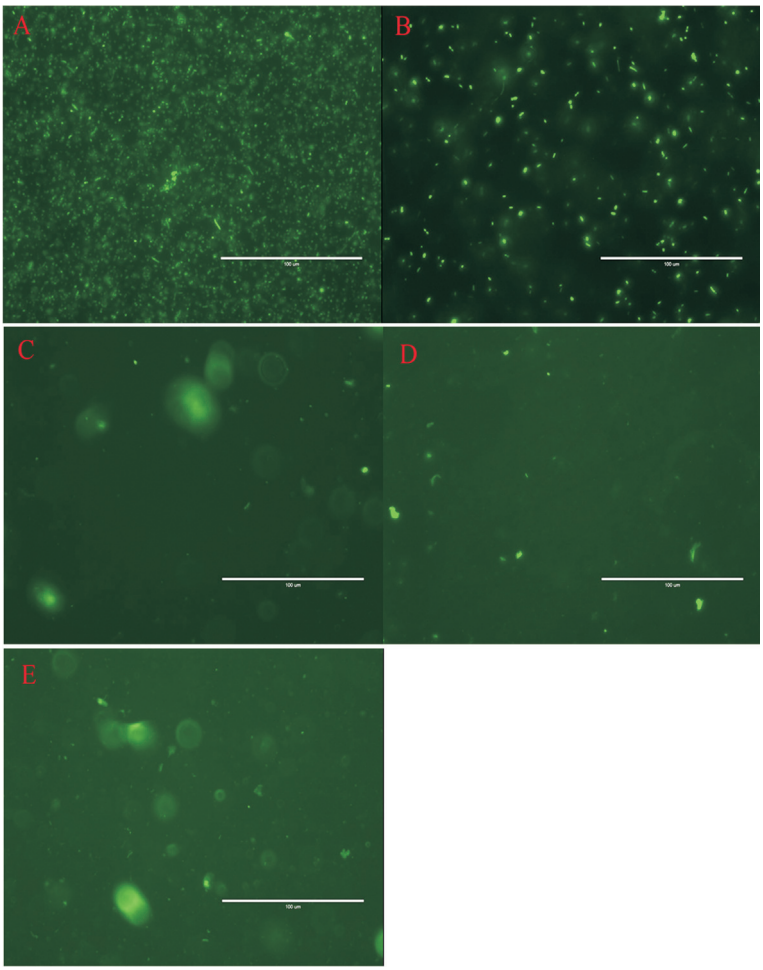

Figure 1 - Fluorescent detection of *L. plantarum*-GFP under fluorescence microscopy. A: fluorescent *E. coli* DH5α cultured in LB B: *L. plantarum*-GFP cultured in MRS; C to E: contents of rumen, duodenum, and ileum from goat inoculated with *L. plantarum*-GFP.

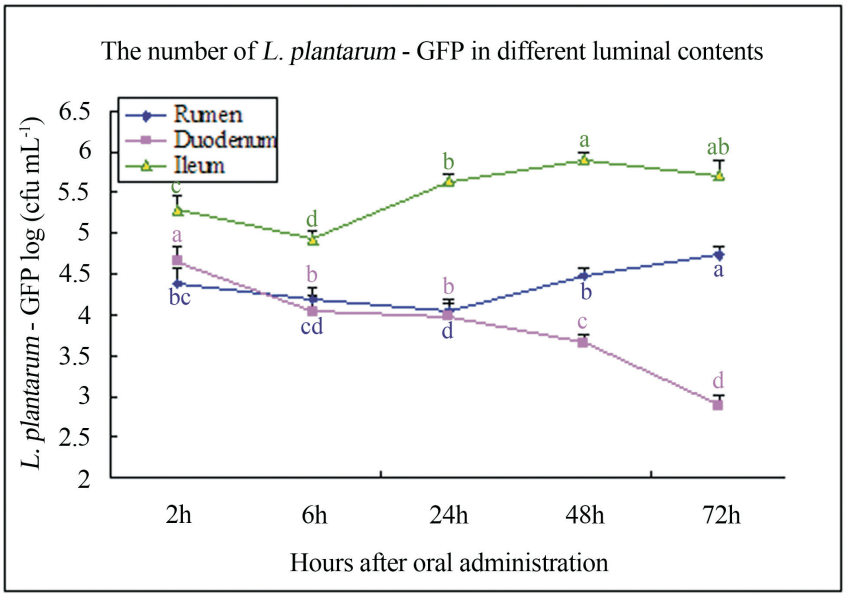

Figure 2 - Trend of the amount of the recombinant strain in the gastrointestinal tract (log (cfu mL^{-1})). Data presented are the means of four goats.

Table 2 - Transformation frequency of different lactobacilli.

Voltage (kV)	Transformation frequency[a] (cfu/μg plasmid DNA)	
	Mason procedure	Modified procedure
1.5	1.1×10^2	4.3×10^3
2.0	2.7×10^2	8.6×10^3
2.5	Not detected[b]	3.4×10^3

[a]Data are obtained from three experiments.
[b]Less than 10 transformants per μg plasmid DNA.

Electrical parameters are another important factor that affects electrotransformation efficiency. The optimization of electrical conditions was inconsistent in a previous study (Teresa *et al.*, 2004). Considering the different suspension used, electrical parameters need be optimized to obtain the highest transformation efficiency and a minimum of cell destruction at the same time. In this study, an optimum transformation efficiency was obtained at a pulse strength of 2.0 kV, 200 Ω parallel resistance, and 25 μF capacitance (Table 2).

Gory *et al.* (2001) demonstrated that the expression of GFP did not alter *L. sakei* growth and the GFP-*L. sakei* strains can be monitored both at laboratory growth conditions and in dry sausage samples. Yu *et al.* (2007) orally inoculated chicken with D17-GFP and found that D17-GFP could propagate and persist at a high level in the gastro-intestinal tract after 2 h. Wang *et al.* (2011) orally administered GFP-*L. lactis* WH-C1 to mice and found that this *Lactococcus* strain could exist in all gastro-intestinal tracts for extended periods. The above tests both showed that the recombinant *Lactobacillus* mainly colonized in the downstream of gastro-intestinal tract. By contrast, trends in the number of *Lactobacillus* in this study indicated that *Lactobacillus* could colonize in the rumen but not in the duodenum. Whether lactobacilli could colonize in the ileum requires further studies in the future. Whether exogenous *Lactobacillus* can adhere in the intestinal mucosa has been considered as one of the selection criteria for probiotic strains (Klaenhamme, 1982; Collins *et al.*, 1998). Thus, our findings indicate that *Lactobacillus* from rumen can perform an important function in goats as probiotic or expression and delivery vehicles for recombinant proteins.

In addition, the GFP expression of *Lactobacillus* was successfully applied to animal testing because it offers a simple and rapid method to detect the colonization ability of LAB. This result will aid in better understanding a series of problems, such as the study of relationship between microorganisms and intestinal epithelial cells at the cellular level and the effects on the resident microbiota. In addition, our study will enable us to better understand the distribution and movement of microorganisms in the gastro-intestinal tract.

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