Distribution Dynamics of Recombinant Lactobacillus in the Gastrointestinal Tract of Neonatal Rats

Sujin Bao1,2,*, Libin Zhu2, Qiang Zhuang3, Lucia Wang4, Pin-Xian Xu4, Keiji Itoh4, Ian R. Holzman4, Jing Lin2,4,*

1 Saint James School of Medicine, Bonaire, The Netherlands Antilles, 2 Yuying Children's Hospital, Wenzhou Medical College, Wenzhou, China, 3 The First Affiliated Hospital, Wenzhou Medical College, Wenzhou, China, 4 Icahn School of Medicine at Mount Sinai, New York, New York, United States of America

Abstract

One approach to deliver therapeutic agents, especially proteins, to the gastro-intestinal (GI) tract is to use commensal bacteria as a carrier. Genus Lactobacillus is an attractive candidate for use in this approach. However, a system for expressing exogenous proteins at a high level has been lacking in Lactobacillus. Moreover, it will be necessary to introduce the recombinant Lactobacillus into the GI tract, ideally by oral administration. Whether orally administered Lactobacillus can reach and reside in the GI tract has not been explored in neonates. In this study, we have examined these issues in neonatal rats. To achieve a high level of protein expression in Lactobacillus, we tested the impact of three promoters and two backbones on protein expression levels using mRFP1, a red fluorescent protein, as a reporter. We found that a combination of an L-lactate dehydrogenase (ldhL) promoter of Lactobacillus sakei with a backbone from pLEM415 yielded the highest level of reporter expression. When this construct was used to transform Lactobacillus casei, Lactobacillus delbrueckii and Lactobacillus acidophilus, high levels of mRFP1 were detected in all these species and colonies of transformed Lactobacillus appeared pink under visible light. To test whether orally administered Lactobacillus can be retained in the GI tract of neonates, we fed the recombinant Lactobacillus casei to neonatal rats. We found that about 3% of the bacteria were retained in the GI tract of the rats at 24 h after oral feeding with more recombinant Lactobacillus in the stomach and small intestine than in the cecum and colon. No mortality was observed throughout this study with Lactobacillus. In contrast, all neonatal rats died within 24 hours after fed with transformed E. coli. Taken together, our results indicate that Lactobacillus has the potential to be used as a vehicle for the delivery of therapeutic agents to neonates.

Introduction

Probiotics are living microorganisms that confer a health benefit on the host upon ingestion in adequate amounts [1,2]. Lactic acid bacteria are a large group of bacteria that produce lactic acid as an end product of fermentation. They are commonly used as probiotics because they have long been used in food production and they are safe for human consumption [3]. While the mechanisms remain unclear, clinical studies have demonstrated that supplementation of lactic acid bacteria as a probiotic can significantly reduce the incidence of neonatal necrotizing enterocolitis (NEC), a devastating disease affecting about 5–7% of infants with a birth weight less than 1500 grams and one of the significant causes of mortality and morbidity in premature infants [4,5,6,7].

Therapeutic designs aiming at promoting neonatal intestinal mucosa maturation and protection have become a strategy for prevention and treatment of NEC. A central question is how to deliver therapeutic agents to the GI tract, in part because expression of exogenous proteins in Lactococcus is well established [8,9], use of Lactobacillus in this aspect has lagged behind. In particular, expression of exogenous proteins at a high level in Lactobacillus has long been a challenge. However, Lactobacillus offers some advantages over Lactococcus. For example, Lactobacillus species are commensals of the human gut while Lactococcus species are not [10]. In addition, Lactobacillus species typically have a temperature range of 37–41°C for optimal growth, close to the normal human body temperature, compared with 25–30°C for Lactococcus [11]. Further, for certain popular dairy products such as yogurt, Lactobacillus but not Lactococcus has been commonly used as a starter culture. As a result, we aimed at developing a Lactobacillus-based system to deliver therapeutic agents to the gastrointestinal tract of neonates. In this work, we used mRFP1, a red fluorescent protein, as a reporter to examine the impact of three different promoters and two different backbones on the expression level of mRFP1. In addition, we used mRFP1 as a marker to label Lactobacillus and examined the retention rate of the recombinant Lactobacillus in the GI tract of neonatal rats.
Materials and Methods

Bacteria

The following Lactobacillus strains were used for this work: Lactobacillus casei subsp. casei (ATCC 27139™), Lactobacillus acidophilus (ATCC 11976™) and Lactobacillus delbrueckii subsp. bulgaricus (ATCC 11842™) (Table 1). All these strains were purchased from ATCC (Manassas, Virginia). The Escherichia coli DH5α strain was purchased from Life Technologies (Grand Island, New York). Lactobacillus was cultured at 37°C in de Man-Rogosa-Sharpe (MRS) medium (BD-Difco, New Jersey). E. coli was cultured in the Circlegrow medium (MP Biomedicals, California). Transformation of E. coli followed a standard procedure [12]. Transformed E. coli was selected in the presence of 100 µg/ml ampicillin.

Transformation of Lactobacillus

Preparation of Lactobacillus competent cells followed a standard protocol provided by Jean-Marc Chatel (MICALIS, INRA, Domaine de Vilvert, Jouy en Josas cedex, France) with some modifications. Specifically, 1 ml of an overnight culture of each Lactobacillus strain was diluted into 100 ml of fresh prewarmed MRS broth. The culture was incubated at 37°C till the optical density (A600) reached 0.6–0.8. The culture was harvested by centrifugation. The cells were washed twice in 20 ml of ice-cold SMEB (0.286 M sucrose, 1 mM MgCl₂) before being resuspended in 1 ml of 1× SMEB. For each transformation, 0.5 µg of DNA was mixed with 200 µl of 100× concentrated bacteria. The bacterial suspension was electroporated using a Gene Pulser electroporator (Bio-Rad, California) in a cuvette with a 2-mm gap at 2.5 kV, 25 μF and 200 Ω. Immediately after electroporation, 1 ml of MRS medium was added to the suspension. After incubation at 37°C overnight, serial dilutions were plated on MRS plates containing 25 µg/ml of erythromycin and incubated at 37°C for 2 to 3 days in anaerobic jars.

Construction of plasmids

In this work, combinations of three promoters and two backbones were tested. Three promoters used: L-lactate dehydrogenase (ldhL) promoter of Lactobacillus sakei derived from pRV85 [13], erythromycin resistance gene promoter (emr) derived from pUCYTIT365N (GenBank accession No. AB119527) and P59 promoter [14]. The two backbones used were derived from pLEM415 [15] and pUCYTIT365N, respectively. All plasmids used in this work are summarized in Table 1. pLEM415-ldhL-mRFP1 and the other two pLEM415-derived plasmids were generated following a procedure illustrated in Fig.1. Briefly, ldhL-FLAG-mRFP1 that contains the ldhL promoter and FLAG-mRFP1 was amplified by using a 3-round linking PCR technique (Fig. 1): a) In the first round of PCR, FLAG-mRFP1 containing FLAG tag and mRFP1 coding sequences was amplified from pRSETB-mRFP1 [16] using two primers 311-ldhl-mRFP and 352-mRFP-NBC (Table 2); b) in the second round of PCR, the ldhL promoter sequence from pRV85 (gift of Monique Zagorec) was joined with FLAG-mRFP1 by allowing pRV85 and the product from the first round of PCR to anneal and extend; c) in the third round of PCR, the ldhL-FLAG-mRFP1 fragment was amplified from the second round of PCR product using two primers 351-ldhl-AXN and 352-mRFP-NBC (Table 2). The product from the final round of linking PCR was cloned into pGEM-T (Promega, WI), giving rise to pGEM-T-ldhL-mRFP1. After verification by DNA sequencing, the ldhL-mRFP1 fragment was released from pGEM-T-ldhL-mRFP1 and inserted into pLEM415 (gift of Pascale Serror) between the same sites, leading to pLEM415-ldhL-mRFP1 (Fig. 1). The emr promoter was amplified from pUCYTIT-T7 [17], a derivative of pUCYTIT365N, and joined with FLAG-mRFP1 by linking PCR using three primers: 353-emr-AXN, 355-emr-mRFP1 and 356-mRFP-NBC (Table 2); b) in the second round of PCR, the emr promoter sequence from pRV85 (gift of Pascale Serror) was joined with FLAG-mRFP1 by allowing pRV85 and the product from the first round of PCR to anneal and extend; c) in the third round of PCR, the emr-FLAG-mRFP1 fragment was amplified from the second round of PCR product using two primers 311-emr-AXN and 356-mRFP-NBC (Table 2). The P59 promoter sequence was amplified using three overlapping primers: 275-P59-a, 277-P59-b and 276-P59-c (Table 2). The P59 promoter was joined with

Table 1. Summary of bacterial strains and plasmids used in this work.

| Strains/plasmids | Features | References or sources |
|------------------|----------|----------------------|
| **Bacterial strains** | | ATCC 27139™ |
| Lactobacillus casei subsp. Casei | Transformation host | |
| Lactobacillus acidophilus | Transformation host | ATCC 11976™ |
| Lactobacillus delbrueckii subsp. Bulgaricus | Transformation host | ATCC 11842™ |
| Escherichia coli DH5α | Transformation host | Life Technologies |
| **Plasmids** | | |
| pRVS5 | Emr, containing the dhL promoter | |
| pRSETB-mRFP1 | AmpR, containing the FLAG-mRFP1 sequence | |
| pLEM415 | Emr; E. coli-Lactobacillus shuttle vector | |
| pUCYTIT365N | Emr; E. coli-Lactobacillus shuttle vector; containing the emr promoter | GenBank accession No. AB119527 |
| pUCYTIT-T7 | Emr, derivative of pUCYTIT365N | |
| pLEM415-ldhL-mRFP1 | Emr, AmpR; pLEM415 derivative; containing ldhL-FLAG-mRFP1 | This study |
| pLEM415-emr-mRFP1 | Emr, AmpR; pLEM415 derivative; containing emr-FLAG-mRFP1 | This study |
| pUCYTIT-S1 | Emr; derivative of pUCYTIT-T7; containing the emr promoter | |
| pUCYTIT-ldhL-mRFP1 | Emr; pUCYTIT-S1 derivative; containing ldhL-FLAG-mRFP1 | This study |
| pUCYTIT-emr-mRFP1 | Emr; pUCYTIT-S1 derivative containing FLAG-mRFP1 downstream of the emr gene | This study |
| pUCYTIT-P59-mRFP1 | Emr; pUCYTIT-S1 derivative; containing P59-FLAG-mRFP1 | This study |

Emr, erythromycin resistant; AmpR, ampicillin resistant.

doi:10.1371/journal.pone.0060007.t001
mRFP1 by linking PCR using three primers: 357-P59-AXN, 278-P59-mRFP and 352-mRFP-NBC (Table 2). emr-FLAG-mRFP1 and P59-FLAG-mRFP1 were cloned into pLEM415 in a manner similar to ldhL-FLAG-mRFP1.

To generate pUCYT derived plasmids, the T7 polymerase sequence in pUCYT-T7 was replaced by a short sequence in between XhoI and NheI sites: CTGTGAGAA, giving rise to pUCYT-S1. To generate pUCYT-ldhL-mRFP1, ldhL-FLAG-mRFP1 was released from pGEM-T-ldhL-mRFP1 using NheI and cloned into pUCYT-S1 at the same site. To generate pUCYT-emr-mRFP1, FLAG-mRFP1 was released from pGEM-T-mRFP1 (Bao, S., unpublished) using XbaI and cloned into pUCYT-S1 at the NheI site. To generate pUCYT-P59-mRFP1, P59-FLAG-mRFP1 was released from pGEM-T-P59-mRFP1 using NheI and inserted into pUCYT-S1 at the same site.

Determination of Lactobacillus distribution in the GI tract

Pregnant Sprague-Dawley rats were purchased from Taconic Laboratory, New York. Rats were housed in conventional solid-bottom polycarbonate cages (nominal floor area, 930 cm²) with standard stainless steel lids and hardwood chip bedding. Cages were changed once a week. Pelleted rat chow was provided ad libitum, and tap water was provided in a water bottle with a sipper tube. The environmental conditions in the animal room were as follows: temperature, 22 to 26 °C; relative humidity, 30% to 60%; lighting, 200 lm/m² at cage level; lights on, 07:00 to 19:00. The female rats were allowed to deliver naturally. On postnatal day 1, a total of 38 pups were divided into three groups. In Group 1 (control group), 12 pups received orally a single dose of saline. In Group 2 (first experimental group), 20 pups received bacterial suspension containing about 6.5×10⁸ CFU of recombinant Lactobacillus transformed with pLEM-415-ldhL-mRFP1. In Group 3 (second experimental group), 6 pups received a single dose of bacterial suspension containing about 6.3×10⁸ CFU of E. coli DH5α transformed with pGEM-T-ldhL-mRFP1. At 24, 48, 72 and 96 h after oral administration, three pups from Group 1 and five from Group 2 were sacrificed. The stomach, small intestine, cecum and colon were dissected. Whole segments together with luminal contents were homogenized in phosphate-buffered saline.

Figure 1. Construction of Lactobacillus-based expression vectors. To generate pLEM415-ldhL-mRFP1, the ldhL promoter and FLAG-mRFP1 were joined by using a 3-round linking PCR technique. The resulting ldhL-FLAG-mRFP1 fragment was then cloned into pGEM-T. Finally, the fragment was released from pGEM-T and cloned into pLEM415. Other expression vectors described in this work were generated in a similar manner. Refer to Materials and Methods for details.

doi:10.1371/journal.pone.0060007.g001
Table 2. Primers used in this work.

| Primers | Sequences | Application |
|---------|-----------|-------------|
| To amplify IdhL-FLAG-mRFP1: | | |
| 351-IdhL-AXN | TTAGGGCCCTCTGAGCTAGCACTGAGAAGTTGCTCTCCCCA | forward, linking PCR |
| 311-IdhL-mRFP | TCCCGGTGACGCTAGGACACTGAGAAGTTGCTCTCCCCA | forward, linking PCR |
| 352-mRFP-NBC | TTAAAGCAGTATCCTGACTGGTGGAGTGGCGGCCCT | reverse, linking PCR |
| To amplify emr-FLAG-mRFP1: | | |
| 353-emr-AXN | TTAGGGCCCTCTGAGCTAGCACTGAGAAGTTGCTCTCCCCA | forward, linking PCR |
| 355-emr-mRFP | TOTAAGAGGAAGGCTAGCATGAGAAGTTGCTCTCCCCA | forward, linking PCR |
| 352-mRFP-NBC | TTAAAGCAGTATCCTGACTGGTGGAGTGGCGGCCCT | reverse, linking PCR |
| To amplify the P59 promoter: | | |
| 275-P59-a | GGTTCCTCCGAGCTATGCTCAAAATTTGCTTCTCCTTCTTCTAATCCTTCAC | forward, PCR |
| 277-P59-b | TCAATTTCCTACGTAATTGTAAGACTGTGTCCTCTCCTCTACCTATGATTTA | forward, PCR |
| 276-P59-c | AATAATAGATGTTGAAATATAGTATT | reverse, PCR |
| To amplify P59-FLAG-mRFP1: | | |
| 357-P59-AXN | TTAGGGCCCTCTGAGCTAGCACTGAGAAGTTGCTCTCCCCA | forward, linking PCR |
| 278-P59-mRFP | ACCCTTTGCTCCGACGTATCAGATCAGTGAATCTACCTCCTCATCTAATGATAGT | reverse, linking PCR |
| 352-mRFP-NBC | TTAAAGCAGTATCCTGACTGGTGGAGTGGCGGCCCT | reverse, linking PCR |

Restriction sites introduced in primers are underlined. doi:10.1371/journal.pone.0060007.t002

(PBS), serially diluted and plated on MRS plates containing 25 μg/ml of erythromycin. Colonies were counted manually. All pups from Group 3 died at 24 h after oral feeding and no further experiments were performed. All procedures were carried out in accordance with the institutional guideline for the care and use of laboratory animals at the Icahn School of Medicine at Mount Sinai, New York.

Detection of mRFP1

Images of Lactobacillus colonies on MRS plates were recorded using a Nikon SMZ 1500 dissecting microscope equipped with a Nikon LV-TV camera (Nikon, Inc., New York). For fluorescence detection, the pellet of mRFP1 expressing cells was washed once and resuspended in PBS. Cells were fixed in 4% paraformaldehyde. After washing once with PBS, fixed cells were mounted on a slide and examined using an Axiosplan 2 epifluorescence microscope (Carl Zeiss, Inc., New York). Fluorescence images were recorded using an Axiocam digital camera (Carl Zeiss, Inc., New York).

For detection by the western blot, cells were collected from 24 h cultures inoculated using a single colony. Cell pellets were washed once and resuspended in 250 μl of STE solution (6.7% sucrose, 50 mM Tris and 1 mM EDTA). Cells were lysed by adding 250 μl of a lysis solution (200 mM NaOH and 1% SDS) in the presence of protease inhibitors for 5 minutes. Lysates were cleared by centrifugation. The total protein concentration was determined by a BCA assay (Thermo Scientific, New York). An equal amount of protein in a total of 0.9 mg from each sample was loaded onto an 8% SDS-PAGE gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche, Indiana) using a Bio-Rad electrotransfer system (Bio-Rad, California). The FLAG-mRFP1 protein was detected using an M2 anti-FLAG antibody (Sigma-Aldrich, Missouri). An anti-mouse IgG-HRP conjugate (Cell Signaling, Massachusetts) was used as the secondary antibody. Chemical luminescence signals were captured using the Fujifilm LAS-3000 imaging system equipped with a digital camera (Fuji Medical Systems USA, Connecticut). Intensities of proteins bands were quantified using ImageJ [18].

Results

Expression of mRFP1 at a high level in Lactobacillus casei

A green fluorescent protein (GFP) and its variants have been broadly used as a reporter or marker. However, a high rate of fluorescence quenching has been observed in GFP at acidic pH [19]. Since Lactobacillus cultures produce lactic acid, detection of GFP in Lactobacillus in some cases has been problematic [20]. To circumvent this problem, in this study we used mRFP1, a red fluorescent protein, known to tolerate a low pH [16]. To achieve a high level of protein expression in Lactobacillus, we tested the impact of three promoters and two backbones on protein expression since choices of the promoter and the backbone often have a significant impact on the expression level of a target gene. Each construct was electroporated into Lactobacillus casei. A high level of expression was observed with the ldhL promoter. The highest level of mRFP1 expression was found with pLEM415-ldhL-mRFP1, a construct that combines the pLEM415 backbone and the ldhL promoter (Fig. 2A–B). The mRFP1 expression was so high that bacterial colonies appeared pink under visible light (Fig. 2C–D). In contrast, a lower level of mRFP1 expression was observed with the emr promoter. The amount of the mRFP1 protein expressed by pLEM-emr-mRFP1 and pUCY4T-emr-mRFP1 was about 73% and 47%, respectively, of that by pLEM415-ldhL-mRFP1 (Fig. 2A–B). A substantially lower level of mRFP1 expression was observed with the P59 promoter. The amount of mRFP1 protein expressed by pLEM-P59-mRFP1 and pUCY4T-P59-mRFP1 was only 18% and 11%, respectively, of that by pLEM415-ldhL-mRFP1. The combination of the pUCY4T365N backbone and the P59 promoter led to the lowest level of expression.

Due to its ability to express an exogenous protein at a high level in Lactobacillus casei, pLEM415-ldhL-mRFP1 was used to label Lactobacillus casei for further analyses. In fact, the high level of
mRFP1 expression was not limited to *Lactobacillus casei*. When pLEM415-ldhL-mRFP1 was transformed into *Lactobacillus delbruekii* and *Lactobacillus acidophilus*, an equivalent level of mRFP1 was also observed (Fig. 3A–D and data not shown) and the colonies appeared pink under visible light, indicating pLEM415-ldhL-mRFP1 can be broadly utilized to label *Lactobacillus*.

**Dynamic localization of *Lactobacillus casei* in the GI tract of neonatal rats**

To test whether *Lactobacillus casei* can be retained in the GI tract of neonates, we introduced $6.5 \times 10^8$ CFU of mRFP1-labeled *Lactobacillus casei* into neonatal rats on post-natal day 1 by gavage. At 24 h after oral administration of a single dose of Lactobacillus, about 3% of bacteria ($1.9 \times 10^7$ CFU) were retained in the GI tract without taking into account proliferation of the bacteria in the GI tract. These bacteria were found across all segments of the GI tract (Fig. 4A–C), but bacterial distributions were not even along the GI tract. In fact, a larger number of *Lactobacillus casei* predominated in the stomach and small intestine than in the cecum and colon: about 30% ($5.6 \times 10^6$ CFU) and 35% ($6.7 \times 10^6$ CFU) were found in the stomach and small intestine compared with 14% ($2.6 \times 10^6$ CFU) and 20% ($3.6 \times 10^6$ CFU) in the cecum and colon, respectively (Fig. 4D). This pattern is very reminiscent of natural distribution of *Lactobacillus* in the human GI tract where *Lactobacillus* predominates in the small intestine in some elderly individuals [21]. This distribution pattern in the neonatal rats largely remains unchanged till 72 h after oral feeding. At 48 h, the total bacteria retained in the GI tract dropped to 0.5% ($3.4 \times 10^5$ CFU) (Fig. 4D). At 72 h, only very few bacteria (about 0.1%) were found in the GI tract (Fig. 4D). At 96 h, recombinant *Lactobacillus casei* was almost undetectable using our assay. Although these colonies retained the antibiotic resistance, we observed that only about 31% of colonies preserved fluorescence ($n = 332$) when a total of 1144 colonies were examined. It is not clear why over 2/3 of colonies retained the antibiotic resistance but lost fluorescence; we are currently investigating this issue. Noticeably, during the course of this study, no mortality was observed in neonatal rats treated with either saline or recombinant Lactobacillus. In contrast, all neonatal rats treated with the *E. coli* DH5α strain transformed with pGEM-T-ldhL-mRFP1 died within 24 h after gavage ($n = 6$), precluding its use as a delivery vehicle. Taken together, these results indicate that a portion of *Lactobacillus casei* can be retained in the GI tract of neonatal rats for at least 24 hours.

**Discussion**

In this study, we achieved a high level of protein expression in Lactobacillus by combining an ldhL promoter from *Lactobacillus sakei* with a backbone from pLEM415. In addition, we examined the expression of mRFP1 in 3 strains of Lactobacillus: *Lactobacillus casei, Lactobacillus delbruekii* and *Lactobacillus acidophilus*. Expression levels of mRFP1 in all these strains were high. Using Lactobacillus *casei* that expressed mRFP1, we showed that a portion of the recombinant bacteria was retained in the GI tract of neonatal rats for at least 24 hours. Our results indicate that the recombinant Lactobacillus has the potential to be used as a vehicle for the delivery of therapeutic agents to the mucosa of GI tract.

Low levels of protein expression in Lactobacillus have hindered the wide application of Lactobacillus in therapeutic design. As a result, in the past decade many efforts have been made to improve the level of protein expression in this genus of Gram-positive bacteria. To date, constitutive expression of GFP in Lactobacillus has been reported [20,22]. However, GFP protein is not
detectable under visible light and therefore its detection requires a fluorescence microscope. In addition, GFP and its variants are often sensitive to acidic pH. Due to quenching of GFP at a low pH [19], detection even with a microscope has been problematic. Although in some instances the GFP protein expressed in Lactobacillus can be visualized after taking additional measures such as neutralizing cells with a neutral buffer [20], the degree of success with these measures has been variable in our hands. To overcome these problems, we have used mRFP1 as a reporter, which is known to be stable at a low pH [16]. To achieve a high level of protein expression in Lactobacillus, we have tested different combinations of two backbones and three promoters. We found for a given backbone the ldhL promoter overall yielded the highest level of reporter expression and the P59 promoter the lowest. The emr promoter yielded an intermediate level of reporter expression. For a given promoter, the pLEM415 backbone conferred a higher level of expression than pUCYIT365N backbone. Consistently, the combination of the ldhL promoter and pLEM415 backbone yielded the highest level and the combination of the P59 promoter and pUCYIT365N backbone led to the lowest level of protein expression. While different expression levels conferred by different promoters with a given backbone clearly indicate differential strengths of the promoters, the reason why the same promoter gave rise to different levels of protein expression when combined with different backbones may not be straightforward. At this stage, it is not clear whether the copy number of pLEM415 was higher than that of pUCYIT365N in a host bacterial strain or whether the secondary structure of plasmid DNA resulting from a particular combination makes the same promoter in pLEM415 more accessible to the transcriptional machinery than in pUCYIT365N. Of note also is the unusual migrating behavior of the FLAG-mRFP1 protein, which has a molecular weight of 28 kDa. In a Western blot, this protein migrated at 65 kDa (Fig. 2A). One possibility is that the stop codon used in these constructs was leaky, leading to a fusion protein that is larger than FLAG-mRFP1. Alternatively, FLAG-mRFP1 formed a dimer under the SDS-PAGE condition.

Figure 3. mRFP1 can be expressed broadly in Lactobacillus. The pLEM415-ldhL-mRFP1 vector was transformed into L. casei (B) and L. delbrueckii (D). mRFP1 was detected in both strains. Wild type strains were used as control (A,C). mRFP1 expression (red) is shown in the left panels and DIC images (purple) in the middle. Merged views are shown in the right panels. Scale bars, 20 μm.

doi:10.1371/journal.pone.0060007.g003
Currently we do not have any further evidence to distinguish these possibilities. Our results demonstrate that Lactobacillus can be retained in all segments of the gastro-intestinal tract in neonatal rats for at least 24 hours after oral administration. In particular, a larger number of the recombinant bacteria were found in the stomach and small intestine than in the cecum and colon. One possibility is that the stomach and small intestine are preferred segments for Lactobacillus to colonize since Lactobacillus predominates in the small intestine in some individuals [21]. Alternatively, these results may reflect larger luminal surface areas of the stomach and small intestine than those of the cecum and colon. Further studies are needed to clarify this issue.

Notwithstanding this, we have noticed that the retention rate of Lactobacillus is low in the GI tract of neonatal rats. Even without taking into account proliferation of Lactobacillus in vivo, only about 3.0% of Lactobacillus was retained in the GI tract at 24 h after oral feeding. From a clinical perspective, a low retention rate may actually be advantageous since complete removal of recombinant Lactobacilli from the GI tract after oral administration within a limited period of time will be essential for eliminating any potential unwanted long-lasting side effect of recombinant Lactobacillus on the host after therapeutic treatments. On the other hand, for

Figure 4. Dynamic distribution of Lactobacillus in the GI tract of neonatal rats. Neonatal rats were fed with L. casei expressing mRFP1. Animals were sacrificed on Days 1, 2, 3 and 4 after gavage. The gastrointestinal homogenates were diluted and plated on MRS plate in the presence of erythromycin. Control rats were fed with saline only. Homogenates from experimental groups were plated on the left half of each plate and those from controls on the right half (A–C). Colonies were found in animals fed with labeled bacteria on Day 1. A) Stomach; B) cecum; C) colon. Scale bars, 10 mm. D) Entire colony counts. Samples were collected on Days 1 (blue), 2 (red) and 3 (green). Note: standard deviation was used to measure variability.

doi:10.1371/journal.pone.0060007.g004
therapeutic purposes, a retention rate of 3% may not be large enough to elicit strong immune responses in certain applications. One way to address this issue could be to administer neonates with multiple doses of recombinant Lactobacillus. Since Lactobacillus inhabits the small intestine, cecum and colon in humans [21,23,24], an alternative approach is to use Lactobacillus species with a high capacity to colonize the human GI tract. Further studies are needed to isolate those species that are also capable of expressing a target protein at a desired level. Notably, during the course of this study, no mortality was observed in neonatal rats fed with Lactobacillus. In contrast, all neonates administered with the transformed E. coli DH5α strain died within 24 hours after gavage, precluding the use of E. coli as a vehicle in neonates. Taken together, our results indicate the Lactobacillus is safe and has the potential to be used as a vehicle to deliver therapeutic agents to the gastro-intestinal tract of neonates.

Acknowledgments
We are grateful to Jean-Marc Chatel and Philippe Langella for suggestions and protocols. We thank Jay Pendse for help in imaging and critical reading of our manuscript. We also thank Pascale Serror, Monique Zagorec, Roger Y. Tsien and Takashi Ohtsuki for plasmids, and Michela Lizier for discussion and suggestions.

Author Contributions
Conceived and designed the experiments: SB JL. Performed the experiments: SB QZ LZ LW KI. Analyzed the data: SB. Contributed reagents/materials/analysis tools: PX IH. Wrote the paper: SB JL.

References
1. Khani S, Hosseini HM, Taheri M, Nourani MR, Imani Fooladi AA (2012) Probiotics as an alternative strategy for prevention and treatment of human diseases: a review. Inflamm Allergy Drug Targets 11: 79-89.
2. Kelly D, Mulder HE (2011) Gut microbiota and GI health and disease. J Pediatr Gastroenterol Nutr 53 Suppl 2: S32-34.
3. Vanderhoof JA (2000) Probiotics and intestinal inflammatory disorders in infants and children. J Pediatr Gastroenterol Nutr 30 Suppl 2: S84-30.
4. Neu J, Walker WA (2011) Necrotizing enterocolitis. N Engl J Med 364: 255-264.
5. Fanaroff AA, Stoll BJ, Wright LL, Carlo WA, Ehrenkranz RA, et al. (2007) Trends in neonatal morbidity and mortality for very low birthweight infants. Am J Obstet Gynecol 196: 141.e141-148.
6. Lin HC, Hsu CH, Chen HL, Chung MY, Hsu JF, et al. (2008) Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. Pediatrics 122: 693-700.
7. Deshpande G, Rao S, Patole S, Balara M (2010) Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics 125: 921-930.
8. Steidler L, Rottiers P (2006) Therapeutic drug delivery by genetically modified Lactococcus lactis. Ann N Y Acad Sci 1072: 176-186.
9. Wells JM, Mercenier A (2000) Macosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. Nat Rev Microbiol 6: 349-362.
10. Martinez P, Rambaud JC (1993) Potential of using lactic acid bacteria for therapy and immunomodulation in man. FEMS Microbiol Rev 12: 207-220.
11. Douwat P, Cochu A, Ehrlich SD, Gruss A (1997) Characterization of Lactococcus lactis UV-sensitive mutants obtained by ISS1 transposition. J Bacteriol 179: 4473-4479.
12. Sambeirok J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.
13. Gory L, Montel MC, Zagorec M (2001) Use of green fluorescent protein to monitor Lactobacillus sakei in fermented meat products. FEMS Microbiol Lett 194: 127-133.
14. van der Vossen JM, van der Leij D, Vanema G (1987) Isolation and characterization of Streptococcus cremoris Wg2-specific promoters. Appl Environ Microbiol 53: 2452-2457.
15. Forn M, Heye T, Ludir M, Rainaud P, Ducluzeau R, et al. (1997) Isolation and characterization of a plasmid from Lactobacillus fermentum conferring erythromycin resistance. Plasmid 37: 199-203.
16. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, et al. (2002) A monomeric red fluorescent protein. Proc Natl Acad Sci U S A 99: 7877-7882.
17. Kowahara A, Arita M, Kusuhara Y, Suido M, et al. (2010) Lactobacillus-mediated RNA interference in nematode. J Biosci Bioeng 109: 189-192.
18. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9: 671-675.
19. Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem 67: 509-544.
20. Lizier M, Sarra PG, Cauda R, Larchini F (2010) Comparison of expression vectors in Lactobacillus reuteri strains. FEMS Microbiol Lett 308: 8-13.
21. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benyo Y (2005) Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. J Med Microbiol 54: 1093-1101.
22. Yu QH, Dong SM, Zhu WY, Yang Q (2007) Use of green fluorescent protein to monitor Lactobacillus in the gastro-intestinal tract of chicken. FEMS Microbiol Lett 275: 207-213.
23. Marteau P, Pochart P, Dore J, Beaufays F, Bernalier A, et al. (2001) Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol 67: 4939-4942.
24. Thadeepalli H, Lous MA, Bach VT, Matsui TK, Mändal AK (1979) Microflora of the human small intestine. Am J Surg 138: 845-850.