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Published in:
Microorganisms

Link to article, DOI:
10.3390/microorganisms8030410

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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Article

Application of Recombinase-Based In Vivo Expression Technology to Bifidobacterium longum subsp. longum for Identification of Genes Induced in the Gastrointestinal Tract of Mice

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Received: 11 January 2020; Accepted: 10 March 2020; Published: 13 March 2020

Abstract: Bifidobacteria are one of the major components in human gut microbiota and well-known as beneficial microbes. However, clarification of commensal mechanisms of bifidobacteria in the intestines is still ongoing, especially in the presence of the gut microbiota. Here, we applied recombinase-based in vivo expression technology (R-IVET) using the bacteriophage P1 Cre/loxP system to Bifidobacterium longum subsp. longum 105-A (B. longum 105-A) to identify genes that are specifically expressed in the gastrointestinal tract of conventionally raised mice. Oral administration of the genomic DNA library of B. longum 105-A to conventionally raised mice resulted in the identification of 73 in vivo-induced genes. Four out of seven tested genes were verified in vivo-specific induction at least in the cecum by quantitative reverse transcription PCR. Although there is still room for improvement of the system, our findings can contribute to expanding our understanding of the commensal behavior of B. longum in the gut ecosystem.

Keywords: bifidobacteria; Bifidobacterium longum subsp. longum; R-IVET; in vivo gene expression; Cre recombinase; gut microbiota; qRT-PCR

1. Introduction

Bifidobacteria are one of the major components in the gut microbiota of humans, especially infants [1–3]. Currently, more than 10 species of Bifidobacterium are known to colonize the human gut [4]. However, the occurrence of each in the gut differs depending on the species [5]. Bifidobacterium longum subsp. longum (B. longum) is one of the most representative human gut-associated bifidobacteria. A recent study has reported that B. longum is the most ubiquitously and highly distributed among bifidobacteria across the human lifespan [6]. B. longum is also prevalent across various mammalian species [7]. Certain strains of B. longum are reported to provide hosts with health benefits [8,9]. Considering such ecological roles of B. longum, clarification of why and how they colonize the gut and interact with the host is important. The importance of several proteins (enzymes) in terms of bifidobacterial gut colonization and/or host physiology has been reported in B. longum so far.
Several glycosidases and transporters are involved in the proliferation of *B. longum* in the gut through assimilation of host glycans and dietary fibers [10,11]. The cell surface fimbrial protein binds to host colonic mucin to possibly enhance the colonization ability in the gut [12]. A serine protease inhibitor produced by *B. longum* possesses immune-modulating properties in the host [13]. Nevertheless, in vivo commensal mechanisms of *B. longum*, especially in the presence of the gut microbiota, are still unexplored, probably because of the limitation of comprehensive information on in vivo gene expression [14,15]. Even for other *Bifidobacterium* species, in vivo transcriptome analyses, such as DNA microarray and RNA-sequencing, are limited [16–18].

An effective approach to resolve this issue is to use recombinase-based in vivo expression technology (R-IVET) that enables identification of bacterial genes expressed specifically in vivo or in specific environmental conditions [19–24]. Basic R-IVET applies the Cre/*loxP* site-specific recombination system from bacteriophage P1 (Figure 1) [19]. In R-IVET, an antibiotic resistance gene that is sandwiched by two *loxP* sites is inserted into the chromosome of the host strain. A promoterless Cre gene located downstream of a random DNA fragment from the host genome is provided by a plasmid. Promoter activity of the DNA fragment induces the Cre expression and the site-specific recombination between two *loxP* sites results in exclusion of the antibiotic resistance gene from the chromosome. Consequently, based on evaluation of the antibiotic susceptibility of strains, in vivo-induced genes can be identified. One of the characteristics for R-IVET is that in vivo expression can be evaluated in each single cell by the irreversible recombination reaction. Therefore, this technology is advantageous to detect in vivo-induced genes, including transiently and locally expressed genes, even in low persistent bacterial strains in certain environments. The data obtained by R-IVET can provide valuable information to understand in vivo bacterial behavior, especially when integrated with other types of transcriptomic data such as DNA microarray and RNA-sequencing.

**Figure 1.** Overview of the recombinase-based in vivo expression technology (R-IVET) system constructed in this study. The *loxP*-Sp strain harbored a *loxP*-Sp<sup>R</sup>-*loxP* cassette that was inserted between BL105A<sub>1451</sub> and BL105A<sub>1452</sub> on the chromosome of *B. longum* 105-A. Random DNA fragments of *B. longum* 105-A were independently inserted upstream of the promoterless Cre gene in pBFK86. The resulting plasmids were introduced into the *loxP*-Sp strain, generating the genomic DNA library consisting of ~120,000 clones. The library was cultured in Sp-containing medium to exclude Sp<sup>S</sup> strains in which the Cre gene was expressed by the DNA fragment with in vitro promoter activity. The library was then administered orally to mice and collected from feces. Finally, the Sp<sup>S</sup> strains in which the Cre gene had been expressed during passage through the gastrointestinal tract were identified to determine in vivo-induced gene promoters. Sp<sup>R</sup>, spectinomycin resistance; Sp<sup>S</sup>, spectinomycin sensitive; Cm<sup>R</sup>, chloramphenicol resistance.
Here, we applied the R-IVET system to *B. longum* 105-A to identify genes that are specifically expressed in vivo. Oral administration of the genomic DNA library of *B. longum* 105-A to conventionally raised mice resulted in identification of 73 genes induced in the gastrointestinal tract. Quantitative reverse-transcription PCR (qRT-PCR) analysis verified the in vivo-induced expression of four out of seven tested genes in the cecum of the mice. These findings can contribute to advance our understanding of commensal mechanisms of *B. longum* in the gut ecosystem.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The representative bacterial strains used in this study are listed in Table 1. The *Escherichia coli* DH5α strain was used as a DNA cloning host and grown aerobically in Luria-Bertani (LB) medium. *B. longum* 105-A (JCM 31944; RIKEN BioResource Research Center [25]) was anaerobically grown at 37 °C in a half concentration of de Man, Rogosa, and Sharpe (MRS) medium [26] supplemented with 0.34% (w/v) sodium ascorbate and 0.02% (w/v) cysteine-HCl (1/2MRSCS medium). Anaerobic cultivation was carried out in an anaerobic chamber (80% N₂, 10% CO₂, and 10% H₂; Coy Laboratory Products, Inc., Grass Lake, MI, USA) or a closed pouch with an AnaeroPack (Mitsubishi Gas Chemical, Tokyo, Japan). When necessary, antibiotics were added to the media as follows: spectinomycin (Sp; 75 µg/mL) and chloramphenicol (Cm; 10 µg/mL for *E. coli* and 2.5 µg/mL for *B. longum*), if not indicated.

**Table 1.** Representative bacterial strains used in this study.

<table>
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<tr>
<th>Strain/Description</th>
<th>Source or Reference</th>
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<td>E. coli DH5α</td>
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<td>E. coli DH5α</td>
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<td>deoR, recA1,</td>
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<td></td>
<td>endA1, hsdR17(ρK−mK+), phoA,</td>
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<td>supE44, X−, thi-1, gyrA96, relA</td>
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<td><em>Bifidobacterium</em></td>
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<td>B. longum subsp. longum 105-A (JCM 31944)</td>
<td>Human fecal isolate</td>
</tr>
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<td>loxP-Sp strain</td>
<td>B. longum 105-A derivative strain harboring loxP-Sp&lt;sup&gt;R&lt;/sup&gt;-loxP cassette on the chromosome, Sp&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Sp<sup>R</sup>: spectinomycin resistance.

2.2. Animal Experiments

Animal experiments were approved by the Animal Use Committee of Hokkaido University (no. 17-0050, approved on 15 March 2018). Animals were maintained following the Hokkaido University guidelines for the care and use of laboratory animals. Five-week-old female BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in standard plastic cages in a temperature-controlled environment (23 ± 2 °C) with a 12-h light/dark cycle and allowed free access to water and food. For the first and second R-IVET experiments, mice (*n* = 4 per each experiment) were acclimatized for 1 week by feeding on a standard chow diet (MR stock; Nosan Corporation, Yokohama, Japan). After administration of the R-IVET library, the mice continuously fed on the same diet.

For the third and fourth R-IVET experiments, an AIN-93G control diet (Supplementary Table S1) was fed to the mice (*n* = 2 per each experiment) for 2 weeks before the administration. On the day of R-IVET library administration, the mice were fed the AIN-93G-based diet containing 6% (w/w) 1-kestose (kindly provided by B Food Science Co., Ltd., Tokyo, Japan) at the expense of maltodextrin to increase the persistence of *B. longum* 105-A in the mouse intestines. After the administration, the mice
continuously fed on the same diet. BALB/c mice (n = 6) were also acclimatized and reared in the same manner for qRT-PCR analysis of the identified genes in the screening described in Section 2.7.

2.3. Generation of the B. longum Strain for the R-IVET System

The Sp resistance (Sp$^{R}$) gene flanked by two loxP sites (loxP-Sp$^{R}$-loxP) was inserted between BL105A_1451 (putative aminotransferase) and BL105A_1452 (galactoside transport protein) on the chromosome of B. longum 105-A by double-crossover recombination as described previously [27]. Sanger sequencing was carried out to confirm that mutation other than those expected had not occurred. The resulting mutant was designated as the loxP-Sp strain.

The plasmid for integration of loxP-Sp$^{R}$-loxP was constructed as described below. The Sp$^{R}$ gene was amplified by PCR from pBS423 [27] and inserted into the SwaI site (between two loxP sequences) of pULwL [28], which yielded pBFH23. Then, two homologous DNA regions to the BL105A_1451 locus (designated HR1) or BL105A_1452 locus (HR2) were amplified by PCR from B. longum 105-A genomic DNA and inserted into the EcoRI and BamHI sites of pBFH23, respectively. The resulting plasmid, pBFH35, was used as a DNA template for PCR amplification of a DNA fragment containing HR1, loxP-Sp$^{R}$-loxP, and HR2 in this order. The fragment was inserted into pBFS423A_repA [27] lacking the Sp$^{R}$ gene, which was amplified by inverse PCR. The primers and DNA cloning strategies are listed in Table 2 nos. 1–5.

2.4. Construction of a Plasmid Harboring the Cre Gene for the R-IVET System

2.4.1. Cloning of a Promoterless Cre Gene with an RBS

A Cm resistance gene was amplified by PCR from pBFS38 [29] and cloned into the Scal- and NsiI-digested pKKT427 [30] fragment containing the pTB6 replicon and pUC ori, resulting in the E. coli-Bifidobacterium shuttle vector pBFS63. Then, promoterless Cre genes with different ribosome-binding sites (RBSs) (RBS$^{h4}$, 5$'$-GAAGGATGCT-3$'$; RBS$^{h3}$, 5$'$-GAAGGATGC-3$'$) [31] were amplified by PCR from bacteriophage P1 genomic DNA [32] and inserted into the BglII site of pBFS63, generating pBFH65-5 and pBFK71, respectively. pBFH65-4 with a spontaneous RBS mutation (5$'$-GAGGATGCT-3$'$, hereafter designated RBS$^{h4'}$) was also obtained incidentally. The primers and DNA cloning strategies are indicated in Table 2 nos. 6–8.

2.4.2. Insertion of a Transcriptional Terminator

The following four transcriptional terminators were used for the analysis: T$^{lass}$ (a terminator for the lactic acid synthesis operon of Lactococcus lactis [33]); T$^{rps9}$ (putative terminator for the 30S ribosomal protein S9 gene of B. longum 105-A); T$^{leaB}$ (terminator for the 3-isopropylmalate dehydrogenase gene of Corynebacterium glutamicum ATCC 13032 [34]); T$^{clpP}$ (modified terminator for the clpP operon of Bifidobacterium breve UCC2003 [35]). T$^{lass}$, T$^{rps9}$, and T$^{leaB}$ were amplified by PCR and inserted into the BglIII site of pBFK71 (upstream of the promoterless Cre gene), yielding pBFH78, pBFH80, and pBFK85, respectively. pBFK86, in which T$^{clpP}$ is inserted upstream of the promoterless Cre gene, was constructed as follows. The T$^{clpP}$ stem-loop, BglIII site, RBS$^{h3}$, and Cre ORF were amplified by PCR in this order and inserted into the BglIII site of pBS63 using an In-Fusion® HD cloning kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The 5$'$-protruding end of BglII-digested pBFS63 was removed by mung bean nuclease (New England Biolabs, Inc., Ipswich, MA, USA). The primers, DNA templates, and cloning strategies are listed in Table 2 nos. 9–12.

2.4.3. Insertion of a Promoter

P$^{cscBA}$, the promoter of a putative operon including sucrose permease and β-fructofuranosidase genes [29], was amplified by PCR from B. longum 105-A genomic DNA and inserted into the BglIII site (upstream of the promoterless Cre gene) of pBFK86, resulting in pBFK94. The primers and cloning methods are indicated in Table 2 no. 13.
<table>
<thead>
<tr>
<th>No.</th>
<th>PCR Product 1</th>
<th>DNA Template</th>
<th>Cloning Strategy 2</th>
<th>Primer</th>
<th>Nucleotide Sequence (5'-3') 3</th>
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<td>1</td>
<td>Sp(^R) gene</td>
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<tr>
<td>23</td>
<td>BL105A_1894</td>
<td>B. longum 105-A genomic DNA</td>
<td>NA</td>
<td>Pr-Ble0430</td>
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<tr>
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<td></td>
<td>Pr-Ble0373</td>
<td>ACCCGTAAATACGGTGAACGCTAG</td>
</tr>
</tbody>
</table>

¹ SpR: spectinomycin resistance, CmR: chloramphenicol resistance; ² NA: not applied; ³ Bold single underlines indicate restriction sites, while normal single underlines represent the sequences for In-Fusion cloning. Double underlines indicate the ribosome-binding site (RBS) and spacer region. Lowercase letters represent the sequences for the modified T_{clpP} stem-loop.
2.5. Evaluation of Basal Cre Expression Levels in Promoterless Cre Plasmids

Basal expression levels of Cre from plasmids harboring the promoterless Cre gene were evaluated by measuring the retention ability of the SpR gene in the loxP-Sp strain. A plasmid lacking the Cre gene (pBFS63) was used as a negative control. Each plasmid was introduced into the loxP-Sp strain by electrotransformation as described previously [27]. After electroporation of the plasmid, the cells were anaerobically incubated in 1/2MRSCS-Sp broth for 3 h and then spread on 1/2MRSCS-Cm agar plates. Two colonies of the transformants were picked up and independently transferred to 1/2MRSCS-Cm broth. After overnight incubation, the cultures were spread on 1/2MRSCS-Cm agar plates and the obtained colonies were replicated on 1/2MRSCS-Cm agar plates with or without Sp. The proportion of SpR strains was determined by dividing the number of SpR strains by that of the tested strains. Retention of the SpR gene was confirmed by colony PCR using the primer pair Pr-Blo0099/Pr-Blo0100 (Table 2 no. 14).

The loxP-Sp strain harboring pBFK94 (Cre expression plasmid under the control of PcscBA) was also used for the evaluation as follows. Competent cells of the loxP-Sp strain were electroporated with pBFK94, resuspended in 1/2MRSCS-Sp broth, and then incubated for 3 h. The culture was further incubated on 1/2MRSCS-Cm agar plates containing 1% (w/v) glucose (uninduced condition) or 1% raffinose (induced condition) as a sole carbohydrate source. The proportion of SpR strains was determined as described above.

2.6. Construction of the Genomic DNA Library

A plasmid library was constructed by inserting a bifidobacterial genomic DNA fragment into the BglII site of pBFK86 (Figure 1). Genomic DNA (480 µg) from B. longum 105-A was partially digested with Sau3AI, and then 500–1500-bp DNA fragments were collected using a MinElute gel extraction kit (Qiagen, Hilden, Germany) after agarose gel electrophoresis. The DNA fragments were ligated into BglII-digested pBFK86, and the ligation products were cloned in E. coli DH5α. Plasmids were extracted from ~80,000 E. coli transformants and introduced into the loxP-Sp strain by electrotransformation. After electroporation, the cells were anaerobically incubated for 1 h in 1/2MRSCS-Sp broth and then spread on 1/2MRSCS-Cm agar plates. After 2 days of incubation, ~120,000 Bifidobacterium transformants were anaerobically suspended in 1/2MRSCS-Broth supplemented with 10% glycerol and the resulting R-IVET library was dispensed into aliquots and frozen at −80 ºC until use. Colony PCR was carried out using a primer pair Pr-Blo0277/Pr-Blo0318 (Table 2 no. 15), and the resulting PCR products were used for Sanger sequencing to determine the size and nucleotide sequence of the DNA fragments inserted upstream of the Cre gene.

2.7. Screening for In Vivo-Induced Genes in B. longum

2.7.1. First and Second Trials

An overview of the screening is shown in Figure 1. The R-IVET library was thawed on ice, and then ~10⁷ cells (100 µL) were inoculated into 5 mL 1/2MRSCS-SpCm broth and cultured anaerobically overnight. The culture was transferred to 40 mL fresh 1/2MRSCS-SpCm to an initial OD₆₆₀ of 0.05 and cultured anaerobically to OD₆₆₀ of 0.8–1.0. The cells (2 mL culture) were washed once with anaerobically stored phosphate-buffered saline (PBS) and then resuspended in 200 µL of the same buffer. The inoculum (approximately 10⁸ cells) was then administered orally to a 6-week-old mouse (n = 4 for first and second trials, respectively) that was fed with MR stock. At 3 and 12 h after oral administration, fresh feces were collected from the mice, homogenized with PBS, and plated on a 1/2MRSCS agar plate supplemented with Cm (10 µg/mL). After 60 h of incubation, the colonies had replicated on 1/2MRSCS-Cm and 1/2MRSCS-SpCm agar plates. Subsequently, Sp-sensitive (SpS) clones were used for colony PCR and Sanger sequencing. The colony PCR was conducted using the primer pair Pr-Blo0099/Pr-Blo0100 (Table 2 no. 14) to confirm excision of the SpR gene, whereas Sanger
sequencing following colony PCR using a primer pair Pr-Blo0277/Pr-Blo0318 (Table 2 no. 15) was carried out to determine the nucleotide sequence of the DNA fragments inserted upstream of the Cre gene. The complete genome sequence of *B. longum* 105-A (GenBank accession no. AP014658.1) [36] was used as a reference. Consequently, inserted DNA fragments containing an intergenic region(s) were identified as in vivo-induced promoters. Genes located downstream of the identified promoters were subjected to blastp analysis (BLAST + v2.2.25) against the database of Clusters of Orthologous Groups (COG) [37] and assigned to COG categories.

2.7.2. Third and Fourth Trials

The third and fourth trials of the screening were conducted as described in Section 2.7.1 except for differences in the animal rearing conditions and timing for collection of fecal samples after administration of the R-IVET library. The inoculum (approximately $10^9$ cells) of the R-IVET library was prepared as described above and orally administered to 7-week-old mice ($n = 2$ for third and fourth trials, respectively) that were fed the AIN-93G control diet. After the administration, the diet was changed to the AIN-93G-based, 1-kestose containing diet, enabling *B. longum* 105-A to colonize the mouse intestines (see Section 2.2). Fresh feces were collected from two mice in each trial at 4 days after the administration and used for screening in vivo-induced genes as described in Section 2.7.1.

2.8. RNA Extraction and qRT-PCR Analysis to Verify Specific In Vivo Gene Expression

2.8.1. Administration of *B. longum* 105-A Harboring pBFS63

BALB/c mice ($n = 6$), which were acclimatized and reared with the AIN-93G control diet, were used for this experiment. *B. longum* 105-A harboring pBFS63 (Figure 2) was cultured in 1/2MRSCS-Cm broth, and the cells were collected for administration as described in Section 2.7.1. Inoculum containing approximately $10^9$ cells was administrated orally to the mice once per day for 3 days to ensure a population of *B. longum* 105-A cells in the cecal microbiota. After the first administration, the diet was changed to the AIN-93G-based, 1-kestose containing diet, enabling *B. longum* 105-A to colonize the mouse intestines (see Section 2.2). Fresh feces were collected from two mice in each trial at 4 days after the administration and used for screening in vivo-induced genes as described in Section 2.7.1.

2.8.2. RNA Extraction and qRT-PCR Analysis

*B. longum* 105-A harboring pBFS63 was cultured anaerobically in 1/2MRSCS-Cm broth until the mid-log phase (OD$_{660} = 0.5–0.7$). The cells were pretreated with RNAProtect bacteria reagent (Qiagen) and total RNA was extracted as described in our previous study using enzymatic cell wall digestion and mechanical cell disruption with zirconia beads [38]. Total RNA was also extracted from pretreated mouse cecal contents (see Section 2.8.1) using the same protocol. cDNA synthesis and qRT-PCR analysis were conducted as described previously [38]. A relative standard curve method was used to calculate the relative expression of the target gene against the reference gene. Data obtained from the *B. longum* 105-A in vitro culture ($n = 4$) and the same strain-administered mice ($n = 6$) were statistically compared by Student’s or Welch’s two-tailed *t*-tests after testing the equality of variance by the *F*-test. *p*-values of less than 0.05 were considered as significantly different.
Figure 2. Proportion of Sp$_R$ strains when each Cre expression plasmid was independently introduced into the loxP-Sp strain. Detailed methods are described in Section 2.5 of the Materials and Methods. The proportion of Sp$_R$ strains in the tested strains is shown as a percentage. The values in parenthesis indicate the number of Sp$_R$ strains among the tested strains. Sp$_R$, spectinomycin resistance; Cm$_R$, chloramphenicol resistance. Green box with an arrow, terminator; yellow box, ribosome-binding site (RBS); pink box, promoter.

Candidate genes identified by the R-IVET analysis were subjected to qRT-PCR analysis to verify in vivo-specific expression: BL105A_0130 (presumable pilin subunit for Tad-pili), BL105A_0467 (putative adhesin), BL105A_0547 (ATPase of the ABC transporter), BL105A_1291 (serine protease inhibitor), BL105A_1293 (galactoside transport protein), BL105A_1294 (glycoside hydrolase family 32 β-fructofuranosidase), BL105A_1798 (putative glycosyltransferase), and BL105A_1894 (raffinose
transport system permease protein). Among them, BL105A_1294 was not identified in the R-IVET analysis, but this gene was used as an expected positive control gene for in vivo-specific expression because the β-fructofuranosidase encoded by this gene is necessary to degrade 1-kestose in the mouse diet [39]. BL105A_1946 (rnpA encoding the RNase P protein component) was used as a reference gene [17,38]. Gene-specific primers for qRT-PCR analysis are shown in Table 2 nos. 16–24. The inserted fragments of the R-IVET clones corresponding to the genes subjected to qRT-PCR analysis were subjected to promoter prediction. First, genomic positions of the inserted fragments were verified in the B. longum 105-A genome. Subsequently, bacterial vegetative promoters were predicted in the inserted fragments using Genetyx ver.12 (GENETYX corp., Tokyo, Japan) with the consensus sequence (5′-TATAAT-3′ as the −10 region and 5′-TTGACA-3′ for the −35 region).

3. Results

3.1. Development of the R-IVET System for B. longum 105-A

We generated the loxP-Sp strain in which loxP-SpR-loxP was inserted into the intergenic region between BL105A_1451 and BL105A_1452 on the chromosome of B. longum 105-A (Figure 1). When grown in 1/2MRSCS liquid medium, the growth abilities were indistinguishable between loxP-Sp and wild-type strains (generation time: 73.2 min for former and 72.8 min for latter (an average of biological duplicates)). After 41 generations of culture without Sp, all 307 clones of the loxP-Sp strain retained the phenotype of SpR. Colony PCR also showed that all 36 tested clones harbored the SpR gene. These results indicated that the SpR gene was stably maintained in the chromosome of B. longum 105-A without exerting negative effects on their in vitro growth.

Next, we constructed a promoterless Cre plasmid suitable for the R-IVET system. The R-IVET system evaluates the promoter activity of a DNA fragment inserted upstream of the promoterless Cre gene based on phenotypic examination of the SpR ability. Therefore, basal Cre expression should be suppressed in the absence of the inserted DNA fragment. First, to determine a suitable RBS, three plasmids, pBFH65-5, pBFH65-4, and pBFK71, carrying promoterless Cre genes with different RBSs were used for the evaluation (Figure 2). When these plasmids were independently introduced into the loxP-Sp strain, 96.7% of pBFK71-carrying transformants showed the SpR phenotype. This result was comparable with that of transformants of pBFS63 lacking promoterless Cre genes (Figure 2). In contrast, the pBFH65-5- or pBFH65-4-carrying transformants retained the SpR phenotype at only 17.5% and 85.4%, respectively. These results indicated that pBFK71 strongly suppressed basal expression of the promoterless Cre gene (Figure 2).

Subsequently, transcriptional terminators were inserted upstream of the promoterless Cre gene in pBFK71 to construct the plasmids pBFH78, pBFH80, pBFK85, and pBFK86 to further suppress the basal Cre expression. Evaluation was then conducted as described above (Figure 2). The insertion of transcriptional terminators Tlas, Tps9, and TreuB unexpectedly facilitated excision of the SpR gene in the transformants. In contrast, a modified stem-loop of TclpP (pBFK86) increased the proportion of SpR transformants to 98.9%, indicating the ability of the potent transcriptional terminator TclpP to decrease expression of the promoterless Cre gene. Even after 41 generations of culture without Sp, 97.5% of pBFK86-carrying clones (731/750 clones) retained SpR. Furthermore, 99.7% of the clones (395/396 clones) showed the SpR phenotype when this strain was grown in Sp-containing medium, administered orally to mice fed a standard chow diet, and then recovered from feces at 12 h after administration.

We next analyzed whether insertion of the raffinose-inducible promoter PcscBA into pBFK86 increased the excision rate of the SpR gene (Figure 2). Generated plasmid pBFK94 was introduced into the loxP-Sp strain and used for the analysis. When the transformants were incubated on 1/2 MRSCS-Cm agar plates containing glucose or raffinose as the sole carbohydrate source, 86.6% of clones (175/202 clones) showed SpR on glucose (uninduced condition). In contrast, only 10.1% of clones (28/278 clones) retained SpR on raffinose (induced condition). These results strongly suggested that pBFK86 was suitable as the promoterless Cre plasmid of the R-IVET system for B. longum 105-A.
3.2. Construction of the Genomic DNA Library

The R-IVET library consisting of ~120,000 clones was constructed by inserting the genomic DNA fragment from B. longum 105-A upstream of the promoterless Cre gene in pBFK86 and introducing them into theloxP-Sp strain. Colony PCR showed that the 78 tested strains harbored different sizes of the inserted DNA fragments. DNA sequencing further revealed that (i) 77.5% of clones had a single DNA fragment, (ii) all DNA fragments were unique, and (iii) the average fragment length was 787 bp. Based on the average size of the inserted fragment, it was estimated that 20,098 clones were necessary to cover 99.9% of the 2.3 Mbp of B. longum 105-A genomic DNA (Clarke and Carbon formula [40]). These results indicated that the quality and coverage of the library were sufficient for further analyses. It should be noted that only 60.0% of clones in the library retained the Sp<sup>R</sup> gene, indicating that various inserted DNA fragments were functional as a promoter under in vitro conditions.

3.3. Screening of In Vivo-Induced Genes

3.3.1. First and Second Trials

The first and second trials were carried out using mice fed a standard chow diet (see Section 2.7.1) (Figure 3). Abundant clones were recovered from feces at concentrations of 10<sup>8–9</sup> cfu/g feces at 3 h after administration. In contrast, the concentration was rapidly decreased to 10<sup>6</sup> cfu/g feces at 12 h after administration, indicating a low persistence of B. longum 105-A in the intestinal tract of mice. The B. longum 105-A clones collected from mice feces at 3 and 12 h after administration showed the Sp<sup>S</sup> phenotype at proportions of 2.3% (128/5615 clones) and 12.8% (20/156 clones), respectively, at the first trial and 3.2% (39/1207 clones) and 5.8% (30/518 clones) at the second trial. Then, 155 clones (86 clones from the first trial and 69 clones from the second trial) were used for further analyses. Colony PCR analysis revealed excision of the Sp<sup>R</sup> gene in 84 out of 86 clones in the first trial and 66 out of 69 clones in the second trial. Sanger sequencing showed insertion of a single and unique DNA fragment upstream of the Cre gene in 70 strains in the first trial and 60 strains in the second trial. Among them, 24 and 11 strains, respectively, harbored DNA fragments containing an intergenic region(s) (candidate in vivo-induced gene promoter) located in the same direction as the Cre gene (Table 3). Genes located downstream of the candidate promoter in B. longum 105-A genome were selected as in vivo-induced genes in these two trials.

<table>
<thead>
<tr>
<th>Trial</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet for mice</td>
<td>Standard diet (MR stock)</td>
<td>1-Kestose-containing diet (AIN-90G)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time after administration</td>
<td>3 h</td>
<td>12 h</td>
<td>3 h</td>
<td>12 h</td>
</tr>
<tr>
<td>No. of recovered clones (cfu/g feces) (average)</td>
<td>1.2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>8.7 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>4.8 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proportion of Sp&lt;sup&gt;S&lt;/sup&gt; clones (Sp&lt;sup&gt;S&lt;/sup&gt; clones / total clones)</td>
<td>2.3% (128 / 5615)</td>
<td>12.8% (20 / 156)</td>
<td>3.2% (39 / 1207)</td>
<td>5.8% (30 / 518)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trial</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp&lt;sup&gt;+&lt;/sup&gt; clones verified by colony PCR</td>
<td>97.7% (94 / 96)</td>
<td>95.7% (66 / 69)</td>
<td>99.0% (98 / 99)</td>
<td>97.2% (175 / 180)</td>
</tr>
<tr>
<td>Clones harboring a single DNA fragment</td>
<td>83.3% (70 / 84)</td>
<td>90.9% (60 / 66)</td>
<td>95.9% (94 / 98)</td>
<td>83.4% (146 / 175)</td>
</tr>
<tr>
<td>Clones harboring the DNA fragment containing an intergenic region(s) (in vivo-induced gene promoter)</td>
<td>34.3% (24 / 70)</td>
<td>18.3% (11 / 60)</td>
<td>22.3% (21 / 94)</td>
<td>16.4% (24 / 146)</td>
</tr>
</tbody>
</table>

Figure 3. Summary of the results leading to identification of candidate in vivo-induced genes in the four trials of the R-IVET analysis. First and second trials were performed with four mice, respectively. Third and fourth trials were carried out with two mice, respectively. See Sections 2.2 and 2.7 of the Materials and Methods for detailed procedures.
Table 3. In vivo-induced genes identified by R-IVET using *B. longum* 105-A.

<table>
<thead>
<tr>
<th>No.</th>
<th>In Vivo Induced Genes 1</th>
<th>Annotation 1</th>
<th>Identified Round</th>
<th>COG Category 2, 3</th>
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<td>3rd</td>
<td>S</td>
</tr>
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<td>BL105A_0117</td>
<td>GpcP protein</td>
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<td>O</td>
</tr>
<tr>
<td>4</td>
<td>BL105A_0130</td>
<td>Presumably pilin subunit for the Tad-pili</td>
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<td>5</td>
<td>BL105A_0136</td>
<td>Recombination protein RecR</td>
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<td>BL105A_0138</td>
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<td>BL105A_1419</td>
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<td>BL105A_1885</td>
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Table 3. Cont.

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<th>Annotation</th>
<th>Identified Round</th>
<th>COG Category</th>
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</table>

1 The complete genome sequence of *B. longum* 105-A (GenBank accession no. AP014658.1) [36] was used as a reference.


3 Non-assigned into COG categories.

3.3.2. Third and Fourth Trials

The third and fourth trials were conducted using mice fed the 1-kestose-containing diet to promote intestinal colonization of *B. longum* 105-A (see Section 2.7.2). In these trials, the clones were recovered at high concentrations (10⁹–10¹⁰ cfu/g feces) even at 4 days after administration (Figure 3), indicating the increased persistence of *B. longum* 105-A in the mouse intestines by feeding the 1-kestose-containing diet. The proportion of Sp⁵ clones in the recovered colonies at 4 days after administration was increased to 31.7% (99/312 clones) and 71.2% (2937/4125 clones), respectively. An obvious increase in the proportions of Sp⁵ clones compared with the first and second trials (Section 3.3.1) also reflects the prolonged colonization of the administrated strain in the mouse intestines. Next, 99 clones from the third trial and 180 clones from the fourth trial were used for further analysis. Colony PCR analysis revealed excision of the Sp⁵ gene in 98 out of 99 clones of the third trial and 175 out of 180 clones in the fourth trial. Sanger sequencing showed insertion of a single and unique DNA fragment upstream of the Cre gene in 94 strains in the third trial and 146 strains in the fourth trial. Among them, 21 and 24 strains, respectively, harbored DNA fragments containing an intergenic region(s) (candidate in vivo-induced gene promoter) located in the same direction as the Cre gene (Table 3). Considering redundant detection of candidate genes in the four rounds of administration experiments, 73 different genes, which were assigned to various COG categories, were finally identified as in vivo-induced genes (Table 3).

3.4. Verification of In Vivo-Induced Gene Expression in the Cecum

Among the 73 genes identified by R-IVET, in vivo-induced expression of selected genes was verified by comparing the gene expression of *B. longum* 105-A in vitro (cultured in 1/2MRSCS-Cm) and in vivo (in mouse cecal contents) using qRT-PCR (Figure 4). In vivo-induced expression of the positive control gene, BL105A_1294 (β-fructofuranosidase), indicated that the qRT-PCR analysis conducted in this study was rational for the verification. The qRT-PCR analysis verified in vivo-induced expression of four out of seven tested genes in the cecum. In vivo-induced expression of BL105A_0467 (putative adhesin) and BL105A_1291 (serine protease inhibitor) was verified as observed in the transcriptome analysis of *B. breve* UCC2003 colonized in conventionally raised BALB/c mice [17]. BL105A_0130 (presumptive pilin subunit for Tad pili) was also significantly induced in vivo as inferred by the induced expression of the Tad pilus-encoding gene cluster in *B. breve* UCC2003 colonized in BALB/c mice [17]. BL105A_1293 (galactoside transport protein) was also confirmed as an in vivo-induced gene. Collectively, these results indicated that R-IVET is a rational strategy to identify in vivo-induced genes of *B. longum*. 
with cDNA from closely related bacteria. In contrast to the advantages, R-IVET disadvantageous procedures, (ii) application of R-IVET is limited to genetically amenable strains, and (iii) further analyses partially attributed to the distinct principles of R-IVET and DNA microarray approaches. R-IVET because (i) comprehensive gene identification is not feasible owing to the tedious and complicated data in the presence of the gut microbiota irrespective of the persistence ability of the bacterial strains multiple cells of the bacteria in a given environment. R-IVET also generates in vivo gene expression RNA-sequencing, in principle because these technologies evaluate average gene expression levels of these genes are di...

...BL105A_0130 (encoding β-fructofuranosidase (glycoside hydrolase family 32)) was used as a positive control (PC) in qRT-PCR analysis, while the other genes were used to verify in vivo-induced expression in the cecum. BL105A_1946 (rnpA) was used as a reference gene. Data obtained from in vitro (n = 4) and in vivo (n = 6) conditions are expressed as the mean ± standard deviation together with each data plot. After testing the equality of variance by the F-test, Student’s or Welch two-tailed t-tests were used to evaluate statistical significance. p-values of the t-tests are also indicated in each panel and p < 0.05 was considered as statistically significant.

4. Discussion

This study demonstrated the novel application of R-IVET to genus Bifidobacterium and identified 73 in vivo-induced genes in B. longum 105-A. As shown in Section 3.3, several genes were commonly identified to be induced in the digestive tract of the conventionally raised BALB/c mice colonized with B. longum 105-A (R-IVET) or B. breve UCC2003 (DNA microarray) (Table 3) [17]. However, the obtained gene dataset showed overlapping yet different contents compared with the transcriptome dataset of B. breve UCC2003 [17]. Although they are not appropriately comparable because different Bifidobacterium species/strains were used in these studies, the observed difference in the gene datasets appears to be partially attributed to the distinct principles of R-IVET and DNA microarray approaches. R-IVET evaluates in vivo expression in each single cell by an irreversible recombination reaction. Therefore, this technology enables identification of transiently and site-specifically expressed genes. In contrast, these genes are difficult to detect by other transcriptome approaches, such as DNA microarray and RNA-sequencing, in principle because these technologies evaluate average gene expression levels of multiple cells of the bacteria in a given environment. R-IVET also generates in vivo gene expression data in the presence of the gut microbiota irrespective of the persistence ability of the bacterial strains in certain environments. RNA-sequencing is not suitable to analyze the in vivo gene expression of low-persistent strains because huge numbers of reads are required to obtain sufficient information on the transcripts of target strains. DNA microarray may be hindered by unexpected cross-hybridization with cDNA from closely related bacteria. In contrast to the advantages, R-IVET disadvantageous because (i) comprehensive gene identification is not feasible owing to the tedious and complicated procedures, (ii) application of R-IVET is limited to genetically amenable strains, and (iii) further analyses are required to identify sites of in vivo gene expression. Taken together, the R-IVET data obtained in this study provide valuable information for comprehensive understanding of the in vivo commensal mechanisms of B. longum, especially when integrated with other types of transcriptomic data.
In this study, we verified the in vivo-induced expression of seven genes identified by the R-IVET analysis using qRT-PCR. Four genes (BL105A_0130, BL105A_0467, BL105A_1291, and BL105A_1293) showed significantly increased expression in the cecum, but the other three genes (BL105A_0547, BL105A_1798, and BL105A_1894) did not (Figure 4). Although this inconsistency might be attributed to that different mice were used between these two assays, there are some other possible reasons. The first possible reason is that the in vivo-induction does not necessarily occur in the cecum. Indeed, several in vivo-induced genes in *Lactobacillus plantarum* WCFS1, which was identified by R-IVET as conducted in this study, are reported to be expressed transiently or locally in the gastrointestinal tract other than in the cecum [41]. This finding suggests that the three genes detected in our R-IVET analysis might be also expressed in the similar pattern.

The second possible reason is the occurrence of false-positive clones lacking the promoter region (discussed later) due to the high sensitivity of the Cre/loxP system used in this study. The sensitivity of the Cre/loxP system in *B. longum* 105-A was so high that it induced excision of the Sp^{5} gene even by a slight level of Cre expression from the promoterless Cre expression vectors (Figure 2). The high sensitivity was also indicated by comparing with the reported results of R-IVET in *L. plantarum* WCFS1, which adopted the almost same strategy as this study [19]. It is evident from the rates of the loss of the antibiotic-resistance gene in the libraries from the two studies: (i) 40% in *B. longum* and 10% in *L. plantarum* during preparation of the genomic DNA library; (ii) 2.8% at 3 h and 9.3% at 12 h after administration of *B. longum* (average data of the first and second trials) compared with 3.3% even at 24 h after administration of *L. plantarum*, when the library was recovered from feces (Figure 3). High sensitivity of the Cre/loxP system in *B. longum* 105-A is advantageous for detection of genes that are not expressed in vitro, but induced in vivo at a low level although it may be concurrently disadvantageous because of the detection of the false-positive clones.

Our R-IVET used a random genomic library constructed by partial digestion of the genomic DNA of *B. longum* 105-A with Sau3AI. Therefore, the inserted sequence was not always incorporated into the clone as the promoter exists to allow expression of Cre. In other words, clones harboring a truncated promoter region (digested within the promoter by Sau3AI) or without harboring a promoter region would be generated. We examined the putative vegetative promoter sequence in the inserted sequence of R-IVET clones corresponding to the seven genes (Supplementary Figure S1). As expected, clones for four genes, in which in vivo-induced expression were validated by qRT-PCR analysis, harbored the 5′ region of the ORF and its upstream region in the inserted sequences. Furthermore, the promoter regions deduced from the consensus sequence of the bacterial vegetative promoter (5′-TATAAT-3′ as the −10 region and 5′-TTGACA-3′ as the −35 region), which was detected in the genome of *B. breve* UCC2003 [42], were predicted at the 20–249 bp upstream position from the 5′ end of the ORF (Supplementary Figure S1). The predicted positions of these promoters from the ORF were consistent with the length of the 5′ untranslated region (7–240 bp) of the genes in the *B. breve* UCC2003 genome [42]. Consequently, consistent results of the expression of the four genes in R-IVET and qRT-PCR analyses were thought to be caused by possible Cre expression in the clones as occurred in the *B. longum* 105-A genome. By contrast, the corresponding clones of two out of three genes, which showed inconsistent results regarding the gene expression R-IVET and qRT-PCR analyses, were not harbored the predictable promoter sequence (Supplementary Figure S1). These genes were possibly detected in R-IVET as a false positive due to the high sensitivity of our Cre/loxP system. The inserted sequence of the clone for BL105A_1798 showed a similar DNA structure to those observed in the four genes showing consistent expression (Supplementary Figure S1). Transient expression or local expression in other than the cecum might have occurred as mentioned above (the first possible reason).

Detection of the false-positive clones is an issue in the current R-IVET system in *B. longum* 105-A. Further repeated R-IVET trials together with the use of the increased numbers of mice will improve the reliability of the in vivo-induced genes identified by R-IVET. Additional verification of the in vivo-induction of the identified genes by qRT-PCR will be also necessary. From another perspective, further improvement of the current R-IVET system will be effective to obtain more reliable
data. One effective solution to reduce the false-positive clones is decreasing the sensitivity of Cre/loxP by modifying the loxP sequence. In fact, in the study of Enterococcus faecalis, fewer genes were identified by R-IVET using mutated loxP than that using native loxP [22].

Although there remains some future issues, R-IVET adopted in this study is an attractive approach to identify B. longum genes induced in the gastrointestinal tract of mice. Combining R-IVET with other transcriptome analyses based on the different principles may lead to more in-depth understanding the strategies of B. longum for survival and colonization in the intestinal tract. As a further step, we are aiming to reveal the functions and physiological roles of in vivo-induced genes by gene disruption approaches. Such analyses may provide further significant information to reveal the in vivo commensal mechanisms of B. longum.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/8/3/410/s1, Figure S1: The structure and genomic position of the inserted DNA fragment in representative R-IVET clones, Table S1: Diet compositions for the third and fourth R-IVET experiments.

Author Contributions: Conceptualization, S.F., K.S., and A.Y.; formal analysis, H.K. and K.Y.; funding acquisition, S.F.; investigation, H.K., N.I., M.S. (Mikiyasu Sakanaka), K.Y., and M.S. (Mina Shimada); methodology, H.K., M.S. (Mikiyasu Sakanaka), and S.H.; project administration, S.F.; resources, S.F. and K.S.; supervision, S.F.; validation, H.K., N.I., and K.Y.; visualization, H.K., M.S. (Mikiyasu Sakanaka), and K.Y.; writing—review and editing, N.I., K.Y., S.H., M.S. (Mina Shimada), S.F., K.S., and A.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported in part by JSPS-KAKENHI (23780072 and 16H04893 to S.F.).

Acknowledgments: We acknowledge B Food Science Co., Ltd. (Tokyo, Japan) for the generous gift of 1-kestose and the National BioResource Project (NIG, Mishima, Japan) for providing us with E. coli DH5α. We also thank Shin Nakajima (Hokkaido University, Sapporo, Japan) for technical assistance to identify COG categories and Mitchell Arico from Edanz Group (www.edanzediting.com) for editing a draft of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References


