Korean Journal for Food Science of Animal Resources

Korean J. Food Sci. An. 2018 August 38(4):816~822
DOI https://doi.org/10.5851/kosfa.2018.e17

pISSN : 1225-8563 eISSN : 2234-246X
www.kosfa.or.kr

SHORT COMMUNICATION

Construction of a Bioluminescent Labelling Plasmid Vector for Bifidobacteria

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OPEN ACCESS

Received June 11, 2018
Revised July 6, 2018
Accepted July 6, 2018

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Abstract *Bifidobacterium* is recognized as one of the most beneficial microorganisms in our gut. Many researches on bifidobacteria have been done to understand their roles in the gut. The objective of the present study was to develop a bioluminescent labelling plasmid vector for bifidobacteria to facilitate their visualization *in vitro*, *in situ*, and *in vivo*. A plasmid replicon (2.0 kb) of plasmid pFI2576 previously identified from *B. longum* FI10564 was amplified by PCR and cloned into pUC19 plasmid vector (2.68 kb). The cloned replicon was subcloned into pTG262 (*luc*$^+$) recombinant plasmid vector (7.4 kb) where a luciferase gene (*luc*$^+$) from pLuc2 (8.5 kb), an *Escherichia coli* and lactobacilli shuttle vector, was inserted into pTG262 plasmid vector. The final recombinant DNA, pTG262::pFI2576 rep (*luc*$^+$), was transferred into a *B. catenulatum* strain. This recombinant strain showed 3,024 relative luminescence units at OD_{600} value of 0.352. Thus, this recombinant plasmid construct can be broadly used for labelling bifidobacteria.

Keywords *Bifidobacterium*, bioluminescence, luciferase gene, plasmid vector, replicon

Introduction

Research area of human gut microbiota has been booming since gut bacteria are closely related to human physiology including immunity, obesity, diabetes, metabolic syndrome, brain health, and so on (Allaire et al., 2018; Rajani and Jia, 2018; Sun et al., 2018; Tengeler et al., 2018). In particular, human gut bacterial composition is one of the most important factors for maintaining intestinal homeostasis (Zhang et al., 2018). Balance between beneficial bacteria and harmful ones should be considered when measuring the health status of individual gut. Bifidobacteria and lactic acid bacteria (LAB) are among the most beneficial bacteria in our gut (Linares et al., 2017). Bifidobacteria are normally living in the colon. They produce not only lactic acid, but also acetic acid which shows strong antimicrobial activity against pathogenic bacteria (Fukuda et al., 2011; Tejero-Sariñena et al., 2012). These bacteria also possess other

beneficial effects such as immunological, anti-diarrheal, anti-constipation, anti-inflammation, and anti-cancer effects (Alard et al., 2018; Escribano et al., 2018; Kang et al., 2017; Ruiz et al., 2017). For these reasons, bifidobacteria are broadly used as probiotics in functional food market (Kleerebezem and Vaughan, 2009). In particular *Bifidobacterium catenulatum* which belongs to the *B. adolescentis* group has been rarely studied but abundant in feces from humans (Felis and Dellaglio, 2007; Matsuki et al., 2004). Therefore *B. catenulatum* was used as a model for genetic recombination.

Molecular imaging is a powerful tool to investigate phenomena in biological nature. It gives researchers direct evidences via visualization (Ogawa and Takakura, 2018). In general, green fluorescence protein (GFP) and luciferase genes are used for labelling LAB and bifidobacteria. These labelled bacteria have been applied in *in vitro*, *in situ*, and *in vivo* tests to monitor their behaviors (Drouault et al., 1999; Grimm et al., 2014; Karimi et al., 2016; Lee et al., 2007; Moon and Narbad, 2017). The *luxA-luxB* gene cassette of *Vibrio harveyi* and the *gfp* gene of *Aequora victoria* were used for labeling *Lactococcus lactis* strain to investigate its survival, physiology, and lysis in the different compartments of murine digestive tracts (Drouault et al., 1999). The strain orally administered with the diet was resistant to gastric juice (90 to 98% survival), whereas 10-30% was survived in the duodenum. A *Lactobacillus plantarum* strain was labeled with a luciferase gene and monitored in a complex food matrix during fermentation (Moon and Narbad, 2017). The strain was successfully detected in the matrix during the fermentation by measuring bioluminescent signals. In a previous paper for *Bifidobacterium* sp., researchers have shown that a niche environment of a *B. breve* strain in murine intestine is the cecum via molecular imaging technique using a luciferase (Cronin et al., 2008).

In this study, we developed a novel labelling system for bifidobacteria using a luciferase gene based on a replicon of plasmid pFI2576 previously identified from *B. longum* FI10564 (Moon et al., 2009). The recombinant *B. catenulatum* [pTG262::pFI2576 rep (*luc*$^+$)] successfully showed a luminescence signal.

Materials and Methods

Bacterial strains and culture conditions

Escherichia coli DH5α and *E. coli* MC1022 were cultured in LB broth (10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract, pH 7.0) at 37℃ with shaking. *B. catenulatum* #27, which was previously isolated from human fecal samples in our lab and identified by 16S rRNA gene sequence analysis, was cultured in MRS (De Man, Rogosa, and Sharpe) broth at 37℃ under anaerobic condition (Whitley A95 Anaerobic Workstation, Don Whitley Scientific, Bradford, UK). For antibiotic selection of recombinant strains, 100 μg/mL of ampicillin (Sigma, Poole, UK) was used for *E. coli* while 15 and 5 μg/mL of chloramphenicol (Sigma) were used for *E. coli* and *B. catenulatum*, respectively.

Construction of recombinant DNAs

Plasmids and primers used in this study are listed in Table 1. pFI2576 rep (~2.0 kb) PCR product was produced with pFI2576 rep F-1 and R-1 primer set under the following PCR conditions: initial denaturation at 98℃ for 30 s; 30 cycles of 98℃ for 10 s and 72℃ for 1 min; final extension at 72℃ for 5 min. Phusion-HF DNA polymerase (Thermo Scientific, UK) was used for PCR. PCR product was digested with SalI restriction endonuclease (New England Biolabs, Hitchin, UK) and inserted into pUC19 cloning vector digested with the same restriction enzyme. A luciferase gene (*luc*$^+$) was liberated with XbaI and EcoRI restriction endonucleases (New England Biolabs) from pLuc2 recombinant plasmid and inserted into pTG262 vector. The cloned pFI2576 rep was digested with SalI enzyme and subcloned into recombinant pTG262 (*luc*$^+$) digested with the same enzyme. The final recombinant DNA was named pTG262::pFI2576 rep (*luc*$^+$).

Table 1. Plasmids and primers used in this study

Plasmid or primer	Description or sequence (5'→ 3')	Reference
Plasmid		
pFI2576	2.2 kb; A cryptic plasmid isolated from *Bifidobacterium longum* FI10564	Moon et al., 2009
pUC19	2.7 kb; *E. coli* cloning vector, Ap^r	Sambrook and Russell, 2001
pUC19::pFI2576 rep	4.7 kb; pFI2576 replicon (~2.0 kb) PCR product was inserted into pUC19, Ap^r	This study
pTG262	5.6 kb; Shuttle vector, Cm^r	Fernandez et al., 2009
pLuc2	8.5 kb; Shuttle vector harboring a luciferase gene (*luc*$^+$), Ap^r, Em^r	Eom et al., 2015
pTG262 (*luc*$^+$)	7.4 kb; *luc*$^+$ gene was inserted into pTG262, Cm^r	This study
pTG262::pFI2576 rep (*luc*$^+$)	9.4 kb; pFI2576 rep was inserted into pTG262 (*luc*$^+$), Cm^r	This study
Primer		
pFI2576 rep F-1	acgcgtcgacggttgttgctccagcacc	This study
pFI2576 rep R-1	acgcgtcgacctccaatcgtccgtcga	This study

Ap^r, resistant to ampicillin; Cm^r, resistant to chloramphenicol; Em^r, resistant to erythromycin; SalI sites (-gtcgac-) are underlined in primer sequences.

Transformation of *B. catenulatum*

Transformation method was based on a previously published paper (Argnani et al., 1996). For preparation of electro-competent cells, 50 mL of *B. catenulatum* #27 were cultured in MRS broth supplemented with 0.05% L-cysteine and 0.5 M sucrose. After reaching OD_{600} of 0.31, they were set on ice for 20 min. These cells were washed twice with 45 mL of ice-cold electroporation buffer (0.5 M sucrose in 1 mM HEPES buffer, pH 6.0) and then washed with 1 mL of the same buffer twice. Cells were finally resuspended with 200 μL of the same buffer. Recombinant DNA of pTG262::pFI2576 rep (*luc*$^+$) (105 ng) was transferred to 80 μL of electro-competent cells of *B. catenulatum* #27 under electroporation condition of 2.0 kV, 25 μF, and 200 Ω using GenePulser Xcell (Bio-Rad), recovered with 800 μL of MRS broth supplemented with 0.05% L-Cys and 0.5 M sucrose in an anaerobic cabinet (Whitley A95 Anaerobic Workstation), and selected on MRS agar plate supplemented with 5 μg/mL of chloramphenicol (Sigma).

Measurement of bioluminescence

Bioluminescence of recombinant *E. coli* and *B. catenulatum* was measured with a luciferase assay system (E1500; Promega, USA) according to manufacturer's guidance. Cell culture (200 μL) was centrifuged, resuspended with 20 μL of phosphate buffered saline (Sigma), and loaded into a 96-well microplate (Black Costar flat bottom, Corning, USA). Luciferase assay reagent (100 μL) was added to the microplate and bioluminescent signal was measured with a BMG FLUOstar OPTIMA Microplate Reader (BMG LABTECH GmbH, Germany).

Results and Discussion

PCR amplification of pFI2576 replicon

For construction of *E. coli-Bifidobacterium* shuttle vector, a *Bifidobacterium* plasmid replicon from pFI2576 was amplified by PCR. pFI2576 rep F-1 and R-1 primer set (Table 1) successfully amplified a PCR product with expected size of 2,012 bp

(Fig. 1A). Partial nucleotide sequence of the PCR product was 100% identical to original pFI2576 replicon sequence (data not shown), indicating that PCR product was correctly amplified.

Construction of recombinant DNAs

The pFI2576 replicon PCR product was inserted into pUC19 cloning vector. The insert (~2.0 kb) was confirmed by SalI restriction endonuclease digestion (Fig. 1B). The recombinant DNA pTG262 (*luc*$^+$) was also confirmed by restriction digestion with EcoRI and XbaI endonuclease where *luc*$^+$ gene (~1.8 kb) was liberated (Fig. 2A) from pTG262. pTG262::pFI2576 rep (*luc*$^+$) was finally constructed. Its physical genetic map is shown in Fig. 2B.

Bioluminescence of recombinant strains

As mentioned above, pTG262::pFI2576 rep (*luc*$^+$) was successfully constructed for labelling *Bifidobacterium* species. Before transforming *Bifidobacterium* sp., luminescent signal of *E. coli* MC1022 [pTG262::pFI2576 rep (*luc*$^+$)] was measured.

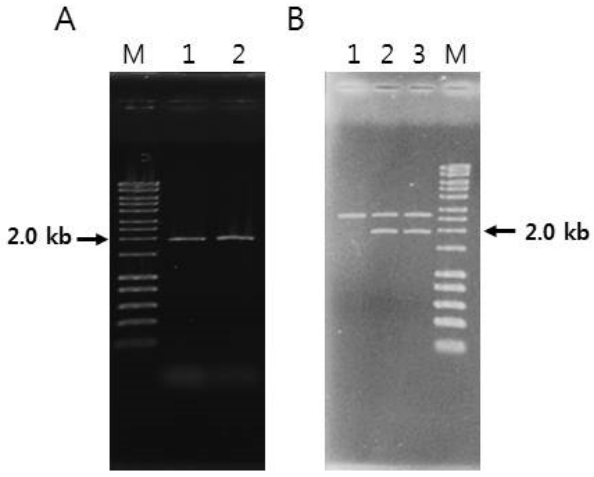

Fig. 1. Amplification of pFI2576 replicon (A) and restriction of pUC19::pFI2576 rep (B). (A) M: HyperLadder™ 1 kb (Bioline Reagents Ltd., London, UK); 1: PCR amplification of the replicon region of pFI2576 (20 µL reaction); 2: PCR amplification of the replicon region of pFI2576 (50 µL reaction). (B) M: HyperLadder™ 1 kb; 1: pUC19 (SalI); 2: pUC19::pFI2576 rep #1 (SalI); 3: pUC19::pFI2576 rep #2 (SalI).

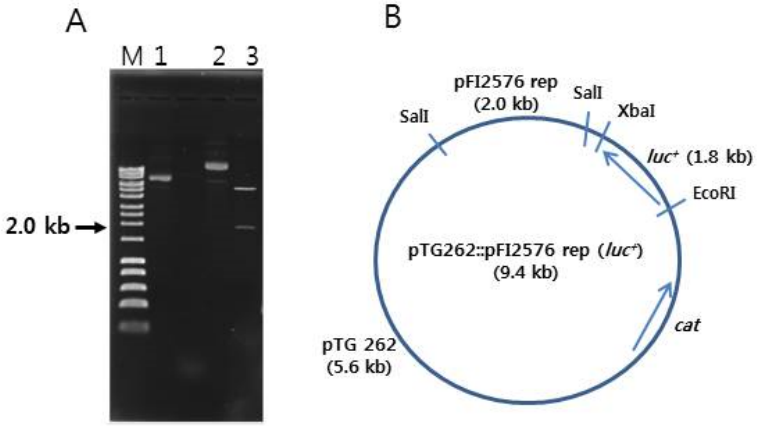

Fig. 2. Restriction of pTG262 (*luc*$^+$) (A) and physical genetic map of pTG262::pFI2576 rep (*luc*$^+$) (B). M: HyperLadder™ 1 kb (Bioline Reagents Ltd.); 1: pTG262 [CCC (covalently closed circular) type]; 2: pTG262 (*luc*$^+$) (CCC type); 3: pTG262 (*luc*$^+$) (EcoRI-XbaI).

E. coli MC1022 [pTG262::pFI2576 rep (*luc*$^+$)] showed very strong luminescent signal (1.0×10^6 RLU), whereas *E. coli* MC1022 (pTG262::pUC19::pFI2576 rep) as a negative control showed only basal signal (13 RLU), indicating that the recombinant DNA was properly constructed for bioluminescent labelling. These two recombinant DNAs were transferred to *B. catenulatum* #27. Transformation efficiencies for pTG262::pUC19::pFI2576 rep and pTG262::pFI2576 rep (*luc*$^+$) were 1.3×10^3 and 4.8×10^1 CFU/μg DNA, respectively. Colonies (two each) were selected and cultured under anaerobic condition and luminescent signals were measured. As shown in Fig. 3, two cultures for *B. catenulatum* #27 (pTG262::pUC19::pFI2576 rep) presented luminescent signals of 2 and 4 RLU, whereas *B. catenulatum* #27 [pTG262::pFI2576 rep (*luc*$^+$)] presented signals of 3,024 and 1,031 RLU. The difference in luminescent signal between the two cultures of *B. catenulatum* #27 [pTG262::pFI2576 rep (*luc*$^+$)] might be due to growth state.

To date, very limited information is available for development of luciferase-based labelling system for bifidobacteria. Cronin et al. (2016) have introduced *in vivo* bioluminescence imaging technique where *E. coli* and *B. breve* are engineered to express *lux* gene cassette. Ninomiya et al. (2013) have used an imaging analyzing method to evaluate *in situ* bioluminescence from a recombinant *B. longum* expressing bacterial luciferase to understand the effect of dissolved oxygen concentration. Guglielmetti et al. (2008) have applied a bioluminescent labelling tool to study the physiological state of *B. longum* strain under various environmental conditions. As described in these papers, bioluminescent labelling system could be broadly used to understand physiological states and behaviors of strictly anaerobic *Bifidobacterium* sp. in *in vitro*, *in situ*, and *in vivo*. In the present study, we developed a luciferase-based labelling system for bifidobacteria. This system successfully worked in a *B. catenulatum* strain as a model. In the near future, the system could be applied to other *Bifidobacterium* species to investigate their physiology and roles under various environments.

Acknowledgements

The research was supported by a grant from the 2015 program for visiting professors overseas in Korea National University of Transportation.

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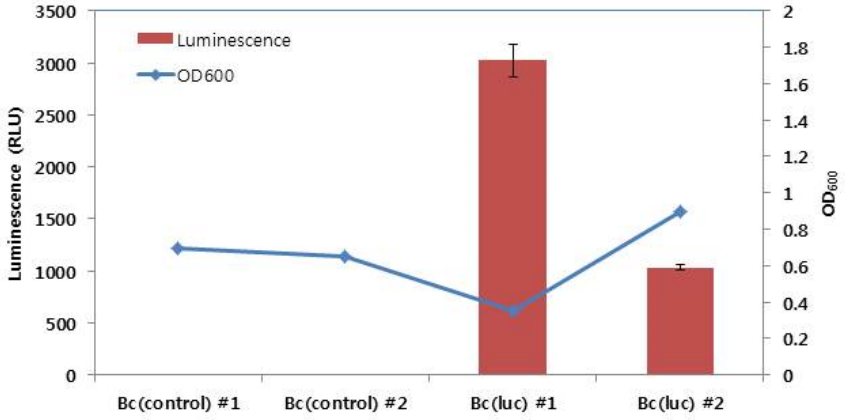

Fig. 3. Bioluminescence of recombinant *Bifidobacterium catenulatum* (Bc) #27. Control: a control vector pTG262::pUC19::pFI2576 rep; Luc: pTG262::pFI2576 rep (*luc*$^+$). Each measurement was done in triplicate and means and standard deviations are presented.

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