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Fluoresceination of Lactobacillus *rhamnosus* through the expression of green fluorescent protein

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ABSTRACT: *Lactobacillus rhamnosus has been gained interest as probiotics in recent years. We attempted to label a strainof L. rhamnosuswith green fluorescent protein (GFP) as a tool to elucidateits mechanism of action.*
GFPmut2 gene was subcloned onto the Lactobacillus-E. coli shuttle vector pRN14. We produced pRNemgfp, which consists of the gfpmut2 gene downstream of the erythromycin resistance gene, erm on pRN14.Culturing in MRS culture medium at 37°C produced L. rhamnosus with a green fluorescence in the exponential growth period.This fluorescence rate reduced to nearly zero during thestationary phase, with the decrease of pH in the medium. The pH regulation on the green fluorescence signal was indicatedby artificial control of the pH in culture medium. Byco-culturing GFP-labeled L. rhamnosus with mammalian cell lines, live L. rhamnosus was observed with GE1 and MC3T3-E1until 4 h.
These results suggest thatpRNemgfp/L. rhamnosuscan facilitatelive analysis of the mechanism of the probiotic action of L.rhamnosus at neutral–weak acid pH ranges.

Key words: *gene expression, green fluorescent protein, Lactobacillus-E. coli shuttle vector, Lactobacillus rhamnosus, pH*

I. INTRODUCTION

The Food Agricultural Organization (FAO)/World Health Organization (WHO) defined probiotics as live microorganisms, which contribute a health benefit on the host when appropriate amounts wereadministered from food or supplement[1], [2]. The representative strains of probioticsare Lactobacillus and Bifidobacterium[3], which are frequently found in the oral cavity[4]. Although Lactobacillus is known as a cariogenic bacteria, several reports have shown that oral isolates of lactobacilli from caries-free persons have the potential to inhibit the growth of mutans streptococci[5], [6]. We have previously shown that *Lactobacillus rhamnosus* L8020 isolated from a caries-free volunteer inhibited the growth of periodontal and cariogenic bacteria, and Candida[7].We also found that bovine milk fermented with L8020 reduced the oral carriage of mutans streptococci and four periodontal pathogens[7].Reports are accumulating on the use of probioticsfor treating periodontal disease[8], [9], [10]. The search for effective probiotic microorganisms appear to be a promising to reduce the risk of both caries and periodontal disease. There is, however, little data available on the direct observation between probiotic bacteria and both cariogenic and periodontal pathogens.

Green fluorescent protein (GFP) is a 27-kDa protein with a pI of 5.6 purified from *Aequorea victoria* that is excited by light at a wavelength of around 390 nm to produce green fluorescence of around 500 nm[11]. From research into wild-type GFP, the active siteswere found on the amino acids S65, Y66, and G67.In another fluorescent substance, luciferase, in fireflies, fluorescence requires an ATP cofactor for the oxidation of luciferin; in contrast, GFP has the advantage of not requiring a cofactor[12].GFPmut2 is a GFP mutant with three amino acid substitutions: S65A, V68L, and S72A. It has stronger fluorescence and folds effectively into its tertiary structure at 37°C[13], [14].

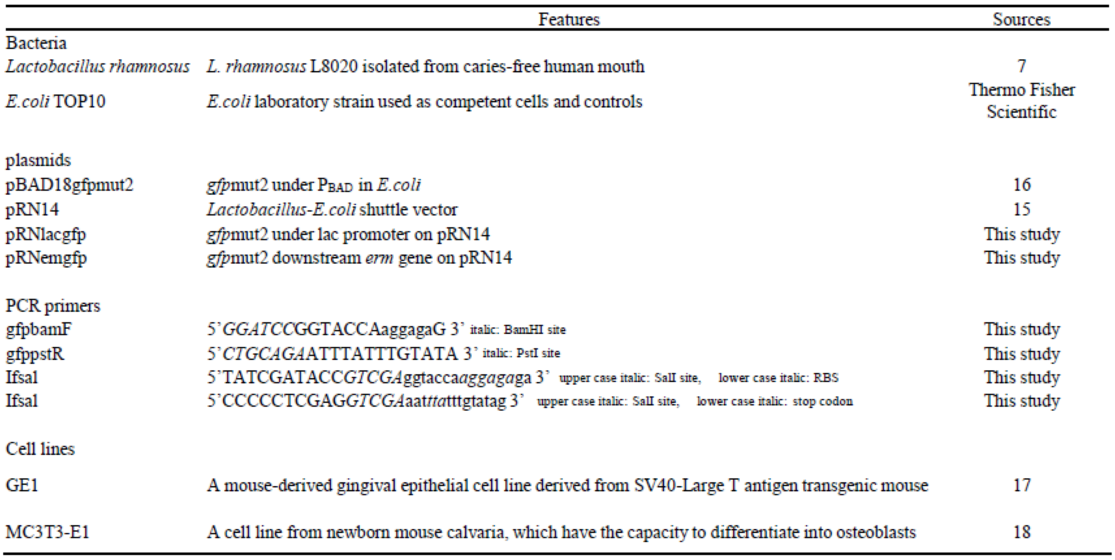
A lot of research currently centers on the probiotic action of *L. rhamnosus*; however, most of them are biochemical studies. Morphological observations are also useful in elucidating the mechanism of this action.While the usual method for observing bacteria is gram staining, discrimination becomes difficult when observing other bacilli or bacteria mixed directly with host cells.Immunostaining using antibodies to *L. rhamnosus* is effective for making morphological observations of the relationship between *L. rhamnosus* and other cells and tissues.The only disadvantage of this method is that it cannot be used to observe living *L. rhamnosus*.In this study, we report the creation of recombinant *L. rhamnosus* expressing the GFPmut2 gene to make live observations of the relationship between living *L. rhamnosus* and the host organism, as well as an investigation of the GFP expression conditions.

II.MATERIALS ANDMETHODS

2.1. Bacteria, plasmids, and culture conditions

The L8020 strain of *L. rhamnosus*was cultured using MRS medium (Difco BD, Tokyo, Japan) at 37°C under aerobic conditions[7].*Escherichia coli*TOP10 (Thermo Fisher SCIENTIFIC, Osaka, Japan) were cultured using LB medium (Difco BD) at 37°C under aerobic conditions (Table 1). If necessary, 100 µg/mL ampicillin sodium (Sigma-Aldrich Japan, Tokyo) or 5µg/mL erythromycin (Sigma-Aldrich) were added to the culture medium.The pH was measured using a Double Junction Waterproof pH meter (Scientific Instrument Services, Inc. NJ, US).The OD_{600} of suspensions of the bacterial cultures was measured using a BioPhotometer Eppendorf (Eppendorf Japan, Tokyo, Japan).

Table 1. Bacteria, Plasmids, PCR primers, and cell lines



	Features	Sources
Bacteria		
Lactobacillus rhamnosus	*L. rhamnosus* L8020 isolated from caries-free human mouth	7
E.coli TOP10	*E.coli* laboratory strain used as competent cells and controls	Thermo Fisher Scientific
plasmids		
pBAD18gfpmut2	*gfp*mut2 under P_{BAD} in *E.coli*	16
pRN14	*Lactobacillus-E.coli* shuttle vector	15
pRNlacgfp	*gfp*mut2 under lac promoter on pRN14	This study
pRNemgfp	*gfp*mut2 downstream *erm* gene on pRN14	This study
PCR primers		
gfpbamF	5'*GGATCC*GGTACCAaggagaG 3' italic: BamHI site	This study
gfppstR	5'*CTGCAGA*ATTTATTTGTATA 3' italic: PstI site	This study
Ifsal	5'TATCGATACC*GTCGA*ggtacca*aggaga*ga 3' upper case italic: SalI site, lower case italic: RBS	This study
Ifsal	5'CCCCCTCGAG*GTCGA*aat*tta*tttgtatag 3' upper case italic: SalI site, lower case italic: stop codon	This study
Cell lines		
GE1	A mouse-derived gingival epithelial cell line derived from SV40-Large T antigen transgenic mouse	17
MC3T3-E1	A cell line from newborn mouse calvaria, which have the capacity to differentiate into osteoblasts	18

2.2. Microscopic observation of GFPexpressed from the recombinant organisms

Genetic manipulation ofthe *gfp*mut2 gene into the *Lactobacillus-E. coli* shuttle vector, pRN14, was followed the method described else [15], [16].A fluorescence microscope (NIKON E1000M, Tokyo, Japan) was used to observe the GFP expression of recombinants grown in MRS culture at a magnification power of 100x and the 10x magnification of the ocular lens. Fluorescence signals and phase contrast images were captured using cellSens Standard 1.11 software (OLYMPUS, Tokyo, Japan).

Relative %GFP was calculated as the percent ofNo. of bacteria fluorescing at certain condition, divided by No. of fluorescing bacteria at theinitial exponential phase (maximum fluorescing in individual experiment).The number of GFP-expressing cells was counted at three or more random locations to derive %GFP.The experiments were performed in triplicate, and the means and standard deviations were calculated.

2.3. Culturing of GFP-labeled *L. rhamnosus* with mammalian cells

GE1 was amouse-derived gingival epithelial cell line derived from SV40-Large T antigen transgenic mouse[17]. MC3T3-E1was a cell line from newborn mouse calvaria, which have the capacity to differentiate into osteoblasts and osteocytes[18]. GE1 or MC3T3-E1 was cultured on the microscopic cover glasses (18 x 18 mm) put in the tissue culture dishes and GFP-labeled *L. rhamnosus* was added into the medium.After 2-4h, cover glasses were recovered and put on the microscopic slides. Fluorescence observation was performed using the microscope (NIKON E1000M) described above.

III. RESULTS AND DISCUSSION

3.1. Expression of GFP in *E. coli* or*L.rhamnosus*

In order to express the *gfp*mut2 gene in this vector, we conceived twoplasmids.One was pRNlacgfp which carried the *gfp*mut2 gene downstream ofthe *E. coli* Lac promoter, P_{lac}(Fig. 1).The other one was the pRNemgfp recombinant, which had the *gfp*mut2 gene inserted downstream of the *erm* gene. pRNlacgfp and pRNemgfpwere expressed in *E. coli* TOP10. Fluorescence microscopic observation revealed that both pRNlacgfp/*E. coli* and pRNemgfp/*E. coli* showed strong GFP signals as shown in Figures 1a-d.

*L. rhamnosus*carrying pRNlacgfp were cultured at 37°C in MRS culture medium with 5 µg/mL erythromycin. In this condition, no fluorescence signal was observed in pRNlacgfp/*L. rhamnosus* (Fig. 1e,f).In contrast, *L. rhamnosus*cellscarrying pRNemgfp exhibited fluorescence atthe initial exponential growth phase (Fig. 1g, h).

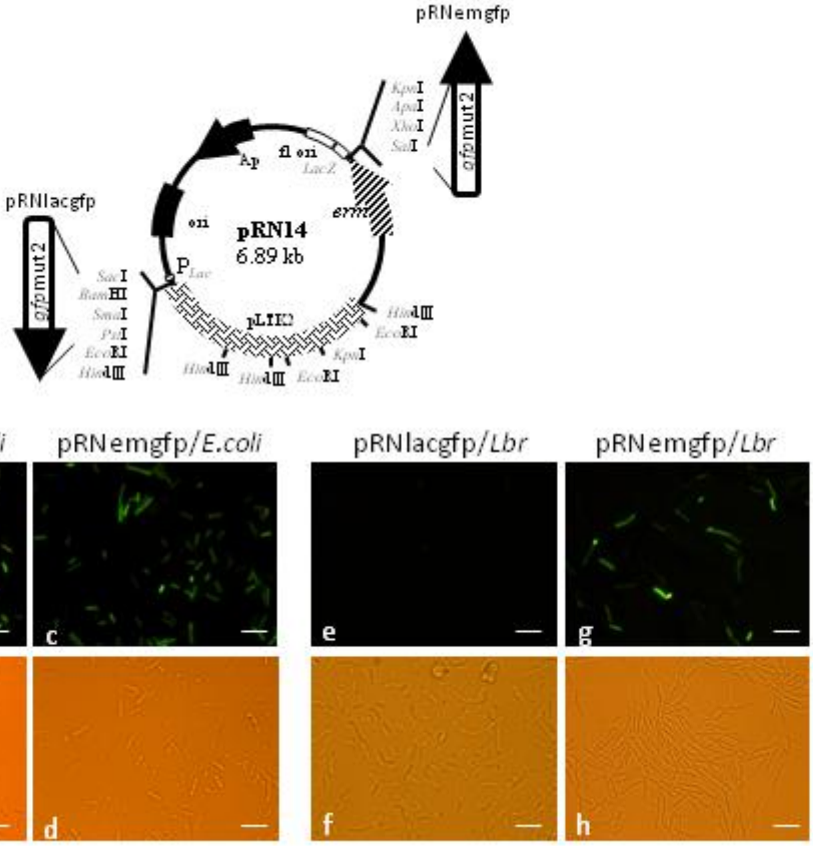


Fig. 1. Cloning strategies and microscopic observation of GFP.

Insertion sites of GFPmut2 gene on the Lactobacillus-E.coli shuttle vector pRN14 (upper panel) and the expression of GFP in E.coli (a-d)andL. rhamnosus (e-h). Fluorescence microscope imaging (a,e) and phase-contrast microscopic imaging (b,f) of pRNlacgfp/E.coli or L. rhamnosus (Lbr). Fluorescence microscope imaging (c,g) and phase-contrast microscopic imaging (d,h) of pRNemgfp/E.coli or L. rhamnosus. The bar represents 5 µm.

To observe a relationship between the growth curve and GFP signal, the relative %GFP was measured as a time series under 37°Caerobic culture conditions (Fig. 2a).*L. rhamnosus* carrying pRNemgfp showed a peak fluorescence rate at the initial exponential growth phase (3 h after starting the culture). Relative %GFP declinedat the late exponential and stationary phase, as the pH of the culture medium reduced (Fig. 2a). The expression of GFP in lactic acid bacteria was first reported in 2003, when Pérez-Arellano et al. used the lactic acid bacteria Lac promoter to express the *gfp* gene in *L. casei*[12]. Since then, the expression of GFP in *L. reuteri* using the promoter of the erythromycin ribosome methylase gene, has been reported[19]. Researchers in Japan have also successfully expressed the GFP derivative, cyan fluorescence protein (CFP), in *L. reuteri*, by inserting CFP gene directly downstream of the erythromycin promoter[20]. In the case of *L. rhamnosus*,there is one report of the expression of GFP using the *Lactococcus lactis* Nisin promoter[21]. The method used in that study efficiently induced fluorescence in *L. rhamnosus*; however, it had the disadvantage that, in order to make Nisin promoter functional, the *nis*RK gene was introduced into the chromosomal genes of *L. rhamnosus*, and that GFP could only be expressed in such recombinant mutant strains. The plasmid pRNemgfp that we produced in the present study can be easily applied to any strain of *L. rhamnosus* by only one step of introducing pRNemgfp into them.

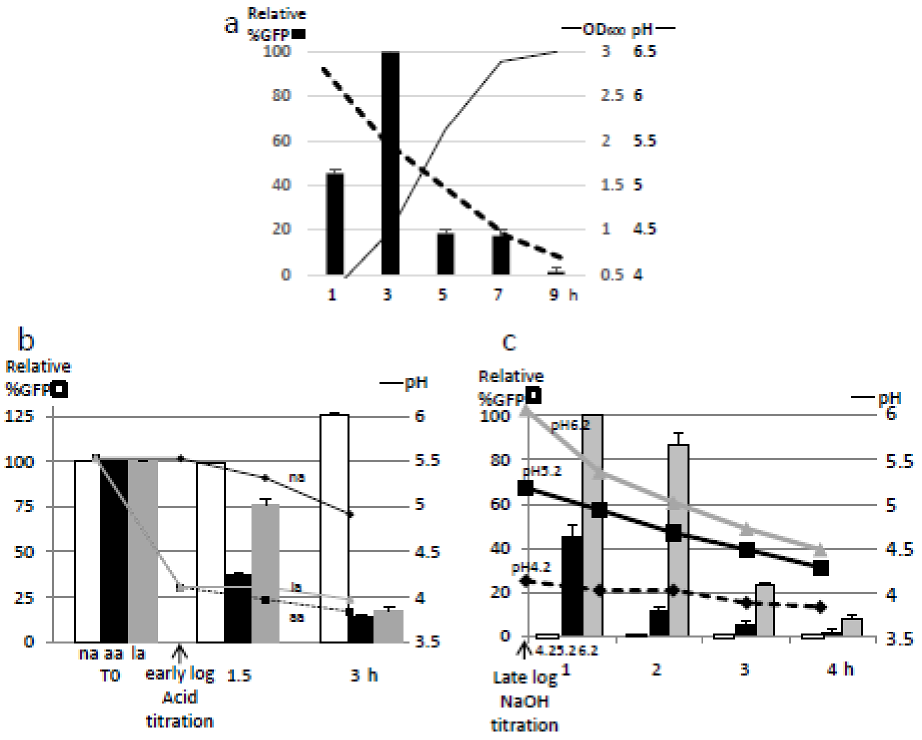


Fig. 2. Growth curve, pH, and relative %GFP of Lactobacillus rhamnosus/pRNemgfp.
Panel a: Growth curve, pH, and relative %GFP were observed in over time. Panel b and c: Artificial pH control at the initial exponential growth phase of pRNemgfp/L. rhamnosus. Abbreviations: acetic acid (aa), lactic acid (la), not titrated (na: not added).

3.2. Culture pH and GFPsignal

Since there appeared to be a relationship between the reduction in the pH of the pRNemgfp/*L. rhamnosus* culture solution and %GFP, two experiments were performed.In the first experiment, the culture solution from the initial exponential growth phase, where the %GFP was high, was titrated with either lactic acid or acetic acid to artificially reduce the pH.In the second experiment, the culture medium from the stationary phase (OD_{600}=nearly 3, in which the %GFP was almost zero) was titrated with sodium hydroxide to raise the pH.

The pH of the culture solution in the initial exponential growth phase of pRNemgfp/*L. rhamnosus* was approx. 5.5 (Fig.2b).After confirming GFP fluorescence, cultures were divided into three aliquots. Tube1 was titrated with acetic acid (aa), and tube 2 with lactic acid (la), to a pH of 4.2.Tube 3 was not titrated (na: not added), and culturing resumed as-is.Observation of the growth curves, pH, and %GFP revealed that samples titrated with acetic acid (aa) had a reduced relative %GFP (36.9%) after 1.5 h.The relative %GFP was further reduced after 3 h following both titration with acetic acid (aa: 13.8%) and lactic acid (la: 17.9%).In contrast, %GFP increased in the samples (na: 126%) where pH was not reduced.

Next, we investigated whether %GFP would be restored in pRNemgfp/*L. rhamnosus* in the late stationary growth phase(in which %GFP was nearly zero) by increasing the pH of the culture solution during culturing.The pH of the culture solution was 4.5–3.5 during the late stationary growth phase.This culture was divided into three aliquots and titrated with NaOH to increase the pH to 6.2 in tube 1 and 5.2 in tube 2.Tube 3 was not titrated and culturing continued at a pH of 4.2 (Fig.2c).The growth curves, pH, and %GFP were observed over time.After 1 h, the samples at pH 4.2 showed no fluorescence, while a slight recovery in relative %GFP (45.1%) occurred in the samples at pH 5.2.For pH=6.2, a high %GFP was observed between 1 and 2 h.%GFP was reduced after 3-4 hours, as the pH of the culture declined from 6.2 to 5-4. The results in this study strongly imply that low pH below around 4–4.5 contributes to reductions in %GFP. Several researchers reported that GFP with some mutations were low pH resistance[22], [23]. [24]. On investigating GFP fluorescence in low-pH environments, Kneen et al. observed that fluorescence occurred even at pH 4.5 for T203I mutations in the primary structure of GFP, and showed that GFP can be used as a reporter of mammalian cell pH[22]. Patterson et al. reported thatfluorescence at pH 4 reduced by approximately 20% of the fluorescence of wild-type GFP at pH 6–9, and EGFP with the mutations F64L and S65T exhibited a fluorescence of 50% at low pH[25]. Meanwhile, Ehrmann et al. observed that rsGFP, which bears the F64L, S65C, and R168T mutations, exhibited fluorescence even at pH 3.7[24]. Llopis et al. showed that enhanced cyan fluorescent protein (ECFP), which carries the K26R, Y66W, N146I, M153T, V163A, and N164H mutations, fluoresced even at pH 4, and could be used as a reporter for the Golgi apparatus and mitochondria[23]. Additionally, Cameleon, a chimeric protein of calmodulin with GFP carrying the L68V and Q69K mutations, fluoresces in a Ca^{2+} ion-dependent manner even at low pH, and has been used as an intracellular Ca^{2+} indicator in mammalian cells [26]. According to these reports, it can be possible to use *L. rhamnosus/*pRNemgfp as intracellular pH indicator of this bacterium in various conditions.

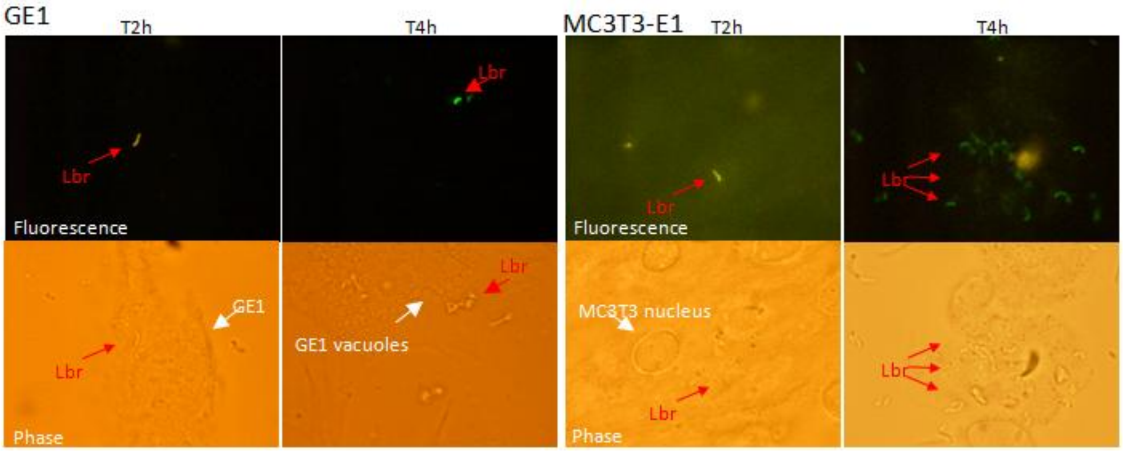


Fig. 3. Visualization of live L. rhamnosus in mammalian cell culture.
GFP-labeled L. rhamnosus (pRNemgfp/L. rhamnosus) was co-cultured with mammalian cells.

3.3. Visualization of live*L. rhamnosus* in mammalian cell culture

To mimic the probiotic effect of *L.rhamnosus* in the mouth, GFP-labeled *L. rhamnosus* (pRNemgfp/*L. rhamnosus*) was co-cultured with mammalian cells, GE1 or MC3T3-E1. Co-culturing of *L.rhamnosus* and GE1 mimicked the situation of *L. rhamnosus* living on gingival sulcus. Fig. 3showed the GFP-labeled *L. rhamnosus*with GE1 cell grown in SFM-101 medium with 1%FBS at pH=7.1-7.4 in an atmosphere of 5% CO_2 in air at 0-4h. At 4h, GFP signal wasobserved beside the GE1 cell in which some vacuoles appeared in the cytoplasm.The pHs in the culture medium did not change during this experiment.

Co-culturing of *L.rhamnosus* and MC3T3-E1 mimicked the situation of *L. rhamnosus* living in the periodontal pockets. If periodontitis became severe, *L. rhamnosus* may interact to osteoblast and osteocyte as probiotics in the deep site of the periodontal pocket. At 2-4h, GFP-labeled *L. rhamnosus* was found on the MC3T3-E1 cells (Fig. 3).The pH (6.9-7.2) in this alpha-modification of Eagle's minimal essential medium did not change for 4h.

In this study, we attempted to make fluorescent *L. rhamnosus*, with a view to study probiotics for the oral cavity and periodontal disease. The pH of the oral cavity is usually maintained at about 5-9 by buffer action of the saliva[27]. In severe inflammation, the pH in inflamed tissue became 5.4[28]. In the periodontal pocket, the lowest pH measured was 6.35 among human subjects[29] and 5.2 at the deep pockets of the rats[30]. In these pH ranges, GFP-labeled *L. rhamnosus* is enough to use in the further experiments on the periodontitis models. In the caries model, the use of mutant GFP with resistance to low pH may be necessary when making live observations of *L. rhamnosus* at lower pH in severe dental caries, because the lowest pH in extremely active region was 4.5[31].

IV. CONCLUSION

We attempted to label a strain of *L. rhamnosus* with green fluorescent protein (GFP) as a tool to elucidate its mechanism of action. We produced pRNemgfp, carrying the *gfp*mut2 gene downstream of the erythromycin resistance gene, *erm* on pRN14. Culturing in MRS culture medium at 37°C produced *L. rhamnosus* with a green fluorescence in the exponential growth period. The pH regulation on the green fluorescence signal was indicated by artificial control of the pH in culture medium. By co-culturing GFP-labeled *L. rhamnosus* with mammalian cell lines, live *L. rhamnosus* was observed with GE1 and MC3T3-E1 until 4 h.Although we can successfully observed live *L. rhamnosus* in the mammalian culture, mammalian culture cells become fatigue to appear vacuoles in their cytoplasm, especially in GE1, by co-culturing them for 4h. We have to seek the best condition, such as bacterial dose, pH, and preculture medium, to add GFP-labeled *L. rhamnosus* into the culture medium of mammalian cells.

These results suggest that pRNemgfp/*L. rhamnosus* can facilitate live analysis of the mechanism of the probiotic action of *L. rhamnosus* at neutral–weak acid pH ranges.

V. Acknowledgements

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