Journal of Bioscience and Bioengineering
VOL. 115 No. 2, 196–199, 2013

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TECHNICAL NOTE

Image analyzing method to evaluate *in situ* bioluminescence from an obligate anaerobe cultivated under various dissolved oxygen concentrations

Kazuaki Ninomiya,[1] Ryuji Yamada,[2] Masami Matsumoto,[2] Satoru Fukiya,[3] Takane Katayama,[4] Chiaki Ogino,[5] and Nobuaki Shimizu[1,*]

Institute of Nature and Environmental Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan,[1] Division of Material Engineering, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan,[2] Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan,[3] Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan,[4] and Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Kobe 657-8501, Japan[5]

Received 26 July 2012; accepted 5 September 2012
Available online 4 October 2012

An image analyzing method was developed to evaluate *in situ* bioluminescence expression, without exposing the culture sample to the ambient oxygen atmosphere. Using this method, we investigated the effect of dissolved oxygen concentration on bioluminescence from an obligate anaerobe *Bifidobacterium longum* expressing bacterial luciferase which catalyzes an oxygen-requiring bioluminescent reaction.

[**Key words:** Obligate anaerobe; *Bifidobacterium longum*; Luciferase; Bioluminescence; Dissolved oxygen; Image analysis]

Bioluminescence reporter systems in bacteria have been used as sensor for chemicals in environment (1) and used for imaging bacterial localization in the host (2). The bioluminescence reaction catalyzed by luciferase requires oxygen (3), which is toxic to obligate anaerobes such as Clostridia and Bifidobacteria. Therefore, only few reports have evaluated bioluminescence expression in cultures of the recombinant obligate anaerobes Clostridia (4–6) and Bifidobacteria (7,8). In these reports, the bioluminescent signal from recombinant Clostridia and Bifidobacteria was measured after the anaerobic culture broth was withdrawn and shaken to ensure aeration. Thus, even though the intracellular bioluminescence reaction is limited in the recombinant anaerobe under anaerobic culture conditions, bioluminescence can be detected and measured by oxygen exposure. However, by using such aerobic measurements, it is impossible to evaluate the effect of oxygen concentration on the bioluminescence expression in recombinant anaerobes. Thus, there have as yet been no reports investigating the effects of oxygen concentration on bioluminescence expression in recombinant obligate anaerobes.

In the present study, an image analyzing method was developed to evaluate *in situ* bioluminescence expression, without exposing the culture broth to the ambient oxygen atmosphere. By employing this method, we can investigate the effect of dissolved oxygen concentration on bioluminescence from an obligate anaerobe *Bifidobacterium longum* harboring the luciferase gene operon *luxABCDE*, which can not be achieved by using the conventional luminescent microplate reader.

B. longum 105-A (9) was used throughout the study. *Escherichia coli* DH5α was used as the host for routine genetic manipulations. *B. longum* 105-A cells were cultivated anaerobically at 37°C in Lactobacilli MRS medium (Difco Laboratories, Detroit, MI, USA) supplemented with L-cysteine (0.2 g/L) and L-sodium ascorbate (3.4 g/L) as antioxidants. This medium was named MRScs. Anaerobic conditions were maintained using AnaeroPack®-Anaero (Mitsubishi Gas Chemical Company, Inc., Tokyo). *E. coli* cells were cultured aerobically in Luria–Bertani medium at 37°C, with spectinomycin (75 μg/ml) when necessary. The *E. coli–B. longum* shuttle vector pJW241, which carries the pSC101ori (an *E. coli* low-copy number origin), pTB6ori (the *B. longum* origin), and spectinomycin-resistance (Sp^r) genes (10), was used in this study. Plasmid pJW245 is based on pJW241 and carries the *gltA* gene from *B. longum* JCM1217 encoding the galacto-N-biose/lacto-N-biose I-binding protein (GLBP) and the ORF of the *fliC* gene encoding the *Salmonella typhimurium* flagellin to produce a GLBP-fliC fusion protein (10). Plasmid pSB2025 carries the artificial *luxABCDE* of *Photorhabdus luminescens*, which was modified to enable its expression in Gram-positive bacteria and cloned into the superlinker plasmid pSL1190 (11).

To express *luxABCDE* in *B. longum 105-A*, plasmid pGLBPlux was constructed as follows. pJW245 was used as a template for the amplification of the DNA region including the pSC101ori, pTB6ori, Sp^r gene, and the promoter region of the GLBP gene. The primers F_SacI_pJW245_lux (5′-ACAGGAGCTCCACAGATGAAAACGGTGTAA-3′) and R_SalI_pJW245_lux (5′-CGTAGTCGACTCATCTCAGTGTGA TGACTG-3′) were used for PCR. The amplified fragment was ligated

* Corresponding author. Tel.: +81 76 234 4807; fax: +81 76 234 4829.
E-mail address: nshimizu@t.kanazawa-u.ac.jp (N. Shimizu).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2012.09.006

with *luxABCDE*, which had been excised from pSB2025 using *Sal*I and *Sac*I, to yield pGLBPlux, comprised the pSC101ori, pTB6ori, Sp^r, GLBP promoter, and *luxABCDE*. *B. longum* 105-A was transformed with pGLBPlux by electroporation as described previously (9). The resultant strain was designated *B. longum* 105-A/pGLBPlux. For the control, *B. longum* 105-A was transformed with pJW241 to obtain *B. longum* 105-A/pJW241.

To start the experimental culture of the recombinant *B. longum* 105-A, the pre-culture broth was transferred into a test tube containing 5 ml of the MRScs medium at an initial OD_{600} value of 0.05. The cultures were incubated at 37°C for 15 h under culture conditions with three different dissolved oxygen (DO) concentrations, which are called as anaerobic, microaerobic, and aerobic culture conditions in the present study. For anaerobic and microaerobic culture condition, static test-tube cultures were placed in zippered pouches together with an AnaeroPack®-Anaero and AnaeroPack®-MicroAero oxygen absorber—CO_2 generator, respectively. For aerobic culture conditions, the test-tube culture was shaken at 160 strokes per min. DO concentrations in the culture media were monitored using a combination of a fluorescence quenching-based oxygen sensor tip and a non-contact oxygen sensor (SP-PSt3-YAU-D5 and Fibox3, respectively; TAITEC Co, Ltd., Saitama, Japan). The sensor tip was placed into the test tube containing culture medium, and the fluorescent signal from the sensor tip was detected by the non-contact sensor from outside the test tube. The DO concentrations in the media at 37°C were 0.00 mg/L, 0.51 mg/L, and 5.74 mg/L for the anaerobic, microaerobic, and aerobic cultures, respectively. These DO values were equivalent to oxygen concentrations of 0.0%, 1.6%, and 18.7%, respectively. Cell densities were evaluated by measuring the OD_{600} of the culture broths at the prescribed time points.

Bioluminescence from the recombinant *B. longum* 105-A was evaluated using a luminescence microplate reader or a luminescence imager. For the microplate-reader measurement, culture broth was withdrawn from the test tube and placed in a white microtiter plate. Bioluminescence was measured using a GloMax®-96 Microplate Luminometer (Promega Corporation, Madison, WI, USA) and expressed as relative light units (RLU). For luminescence imaging, the test tube caps were tightly screwed down inside the zippered pouch to maintain anaerobic conditions. The test tubes were placed on the stage of a Lumino image analyzer LAS-3000 (FUJIFILM Corporation, Tokyo, Japan). Luminescent images were acquired with a 5-min exposure time, and the bright-field image was acquired simultaneously. The bioluminescent signal on the luminescent image was evaluated as bioluminescence count (BLC), which was quantified by a gray-image processing series, as shown in Fig. 1. Fig. 1A and B show examples of the bright-field image and the corresponding luminescent image, respectively. First, the test tube region was determined manually on the bright-field image and extracted as the projected test tube region, i.e., the region of interest (ROI) (Fig. 1C). The luminescent image was processed to remove pixels from the ROI to eliminate background noise (Fig. 1D). The digitalized data in the ROI was then examined based on the frequency distribution of the gray-scale levels (Fig. 1E), and the bioluminescence count (BLC) was calculated according to the following equation:

$$\mathrm{BLC} = \sum_{i=0}^{255} (i \cdot f) \qquad (1)$$

where i and f are the gray-scale levels ranging from 0 to 255 and the frequency at a given gray-scale level in the ROI, respectively.

To confirm the expression of *luxABCDE* in *B. longum* 105-A, *B. longum* 105-A/pGLBPlux and the control *B. longum* 105-A/pJW241 were cultured under anaerobic condition of DO = 0.0%, and the bioluminescence of the culture broth was measured using the luminescence microplate reader under ambient oxygen

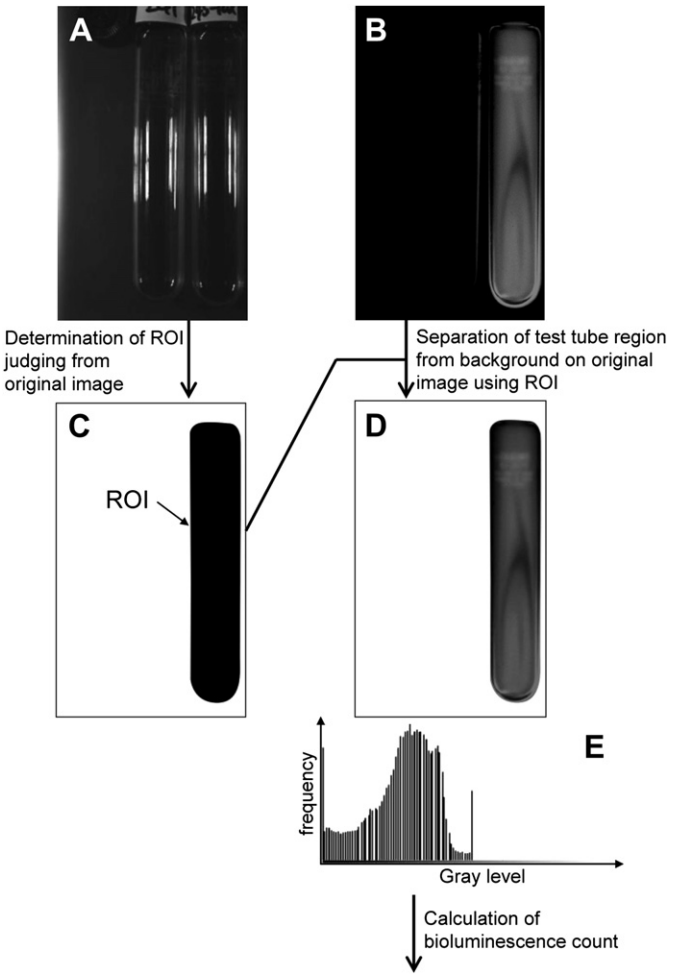

FIG. 1. Diagram of image processing procedure for *in situ* evaluation of bioluminescence from the cultured *B. longum* 105-A. (A) Acquired gray image of the test tube containing culture broth, (B) acquired gray bioluminescence image, (C) binary image of projected test tube region determined as ROI, (D) gray image of bioluminescence separated from background using ROI, and (E) frequency distribution of gray-scale level with respect to the gray image of bioluminescence processed by using the ROI.

atmosphere. There were no differences in the growth profiles between the two recombinant *B. longum* 105-A cultures, irrespective *luxABCDE* expression. No bioluminescence signal was detected from the control strain throughout the culture period. With respect to *B. longum* 105-A/pGLBPlux, a bioluminescence signal was detected at 5 h, during the exponential growth phase. The signal diminished after 10 h, during the late log phase (data not shown). These results confirmed that *luxABCDE* was functionally expressed in *B. longum* 105-A.

To examine the influence of oxygen exposure during measurements on the bioluminescence of the broth, recombinant *B. longum* 105-A was cultured under anaerobic condition of DO = 0.0% and then the bioluminescence signal was measured using the luminescence imager with the culture broth kept under anaerobic or aerobic conditions. Fig. 2A–C show the pseudo-color luminescence images of test tubes (Fig. 2A and B) and a microplate well (Fig. 2C) containing *B. longum* 105-A/pGLBPlux cultured anaerobically for 5 h. When the luminescence imaging was performed while maintaining anaerobic conditions in the test tube by tightly screwing the cap within the zippered pouch, no bioluminescent signal was observed (Fig. 2A). However, when the test tube was opened and mixed under ambient oxygen atmosphere, a bioluminescent signal was detected (Fig. 2B). Further bioluminescent