A Simple Instrument for Luminescence/Growth Measurement for Bioluminescent Bacteria

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The present study examines the relationship between the cell growth rate and luminescence intensity by fabricating a system that enables the simultaneous measurement of the luminescence intensity and optical density of the liquid culture of *Photobacterium kishitani*, in order to investigate a possible mechanism for the luminescence oscillation. An instrument is fabricated using a microcomputer-controlled LED and a photo IC diode for the measurements. Two signals, cell density and bacterial bioluminescence were distinguished from one data set. A possible hypothesis for the oscillation in bacterial bioluminescence from oxygen level viewpoint has been created.

Introduction

Photobacterium kishitanii is known to be coccoid, Gram-negative, catalase-positive, and weakly oxidase-positive or oxidase-negative bacterium that emits blue luminescence (Ast *et al.*, 2007). Its draft genome has already been read (Bjornsdottir-Butler *et al.*, 2015). Oscillatory behavior in the bioluminescent intensity from the bacterial suspension has been observed (Sato and Sasaki, 2008). The reason for the oscillation has been investigated experimentally on account of cell variety, i.e., motility difference (Sasaki *et al.*, 2009), surface attachment ability (Sasaki and Mochizuki, 2012), or oxygen supply orientation (Sasaki *et al.*, 2010). There should be an intercellular communication to realize any sort of synchronization. Typical communication among bioluminescent bacteria studied so far is quorum sensing (O'Toole, 2016). Such communication is proved experimentally in the case of *E. coli*. For example, cell-free supernatants from of liquid culture activate the lux reporter (Milton *et al.*, 1997). We found, on the other hand, that cell-free supernatant of bright liquid culture of the bacteria had no effect on the bioluminescence of another liquid culture of lower density, even when the supernatant was thoroughly concentrated using an evaporator. Quorum sensing, at least at our experimental conditions, had a very small effect on the bacterial luminescence. Search for other factors, therefore, are necessary to understand the initial mechanism of the oscillation. In our preliminary experiments based on real-time PCR (polymerase chain reaction), induction of luciferase was probed to have no effect on the luminescence oscillation, i.e. luciferase gene transcription occurs even at low cell density. Luciferase expression of *P. kishitanii* might, therefore, not depend on the quorum sensing. In fact, some *Photobacterium* species are reported to show no luciferase regulation/induction subject to cell division (Watanabe and Nakamura, 1980; Dunn *et al.*, 2015). Interestingly, the addition of substrates such as oxygen or dodecanal often results in the increase in bioluminescence. The rate determining step of the bacterial bioluminescence is thus imagined to be the substrate-supplying step. As the synthesis of linear alkyl aldehyde within the bacterial cell is performed at the cost of ATP, any process other than bioluminescence that consumes ATP should be the bioluminescence competitor. Roughly speaking, with a constant supply of oxygen into the liquid culture, the bacteria might consume ATP competitively by the bioluminescence and other cellular metabolic reactions that lead to cell division. Time course measurement of both cell density and bioluminescence might be, therefore, interesting to check as to whether the cell growth have some effect on bioluminescence oscillation. For this purpose, a measurement system is desired that enables the simultaneous measurement of both luminescence intensity and cell density of the liquid culture. Quantitative analysis, however, has not been performed because of the lack of a suitable system for the measurement. Commercially available systems that measure the optical density during cultivation use near infrared light continuously irradiated on the culture vial. A simple combination of this system with a sensitive bioluminescence detector always failed to measure cell density and luminescence intensity simultaneously, mainly because the light source signal overwhelmed the bioluminescence signal. Use of a liquid culture with larger volume helps to gain larger bioluminescence signal, although the bioluminescence oscillation wave becomes more unclear. A system that measures bioluminescence from, and the optical density of, ca. 2 mL of liquid culture, from which an obvious oscillatory wave is usually obtained, is desired. Cell growth rate can be calculated as the time derivative of cell number. As cell number can be calculated by multiplication of cell

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density and liquid culture volume, cell density time course measurement is necessary and sufficient as well. A typical method for the cell density is often based on transmittance decrease measurement (Junker *et al.*, 1994) or dynamic light scattering (Loske *et al.*, 2014). As for *P. kishitanii*, both cell density and bioluminescence intensity can be measured, in principle, by the combination of an optical sensor and a small light source. Both bacterial bioluminescence intensity and cell density can be measured using an optical sensor. A merit of using *P. kishitanii* is that this bacterium is relatively brighter among bioluminescent bacteria, and the maximal emission wavelength is near the most sensitive one of a photo IC diode. Commercially available silicon chips (LED and photo IC diode) were therefore suggested. Use of such devices should allow for smaller measurement systems. The oscillation mode in the bacterial luminescence changes with the suspension volume of the shape of the cell. A luminescence/growth measurement system is, therefore, highly desired to be flexible for the cell size. In view of adjusting the light source intensity, a light emission diode (LED) is suitable because the intensity can be controlled simply by the applied voltage or the frequency of the voltage pulse. LED generates very little heat compared to other light sources. This factor is favorable for the temperature control using the cooling incubator, especially for the psychrophilic bacteria such as *Photobacterium kishitanii*. The cell growth rate of *P. kishitanii* is not constant during luminescent growth (Sasaki, 2011). Thus, we focused on the relationship between cell density and luminescence intensity. A small module that can measure both the cell density and bioluminescence intensity will enable characterization of *P. kishitanii* at various experimental conditions. Especially, a smaller sized module allows for realizing spatially high resolution characterization of the bacterial suspension of interest. The purpose of this study was to fabricate a system for the simultaneous measurement of the luminescence intensity and optical density of the bacterial suspension of *Photobacterium kishitani* to evaluate the relationship between the cell growth rate and luminescence intensity.

1. Experimental

1.1 Materials

A luminescent bacterium (*Photobacterium kishitanii*) was collected and isolated from a cuttlefish, *Todarodes pacificus*, purchased at a local supermarket. For the agar plates, 2% w/w of agar powder (Wako Pure Chemical Industries, Ltd.) and Marine Broth (MarineBroth 2216, Becton, Dickinson and Company) were used. For the liquid broth preparation, Marine Broth without agar was used. As the chemical composition of the marine broth was unknown, a broth (mini broth) was also prepared with KH_2PO_4 (1 g), K_2HPO_4 (4.5 g), $(NH_4)_2SO_4$ (0.5 g), $MgSO_4$ (0.096 g), NaCl (30 g), glycerol (2 mL), and one liter of ultrapure water (slightly modified after the report of Eberhard (1972)). *P. kishitanii* was grown in two kinds of liquid broth. Bacterial growth and measurements were performed in an incubator (VS401, VERSOS) at 16°C. A liquid culture of the bacteria was kept stirred with a stirrer bar using a magnetic stirrer (MC303 and MS101, Scinics Corp.) inside the optical cell (12.5 mm×12.5 mm×45 mm). A yellow LED (HK-LED5H(Y)) was used as a light source, and a photo IC diode (S9648-100, Hamamatsu Photonics) was used as an optical sensor. The switching on/off of the LED was controlled using a microcomputer (Arduino UNO, Switch Science Inc.), and the signal from the optical sensor was measured and recorded using voltage loggers (GL220-UM-801, Graphtec Corp.). Cell count was performed using a bacteria counter and an optical microscope (DM IRB, Leica).

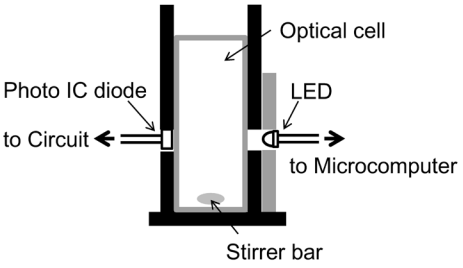

Fig. 1 Schematic illustration of optical cell with light source (LED) and optical sensor (photo IC diode)

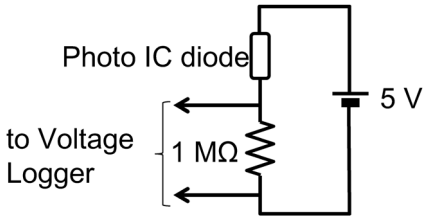

Fig. 2 Circuit for the photo IC diode

As illustrated in **Figure 1**, the LED and an optical sensor were placed in a cell holder made of black acrylate board (3 mm thick). As for the circuit, an optical sensor and a resistor were connected in series. DC 5 V generated using an AC/DC voltage converter was applied to both ends of the connected parts. A data logger was connected to both ends of the resistor (**Figure 2**). The final size of the cell-sensor-light source system resulted in 30 mm in length, 75 mm in width, and 50 mm in height, that enabled two systems to be set in one incubator. When the door is completely closed, the sensor signal appeared to be in the range from −0.001 to 0.002 mV. Considering that the voltage logger lower detection limit is 0.001 mV, the measured "dark signal", which could be regarded as "dark noise", appeared to be negligible. The analog voltage signal from the photo IC diode was sampled at 1 Hz frequency. The LED was turned on for 30 s for every 300 s, because in our preliminary study irradiation of yellow light, 30 s showed no effect on bacterial bioluminescence. Bioluminescence was, therefore, measured for 270 s, followed by the measurement of LED+bioluminescence for 30 s. This sequence was repeated. As for the data processing algorithm, bioluminescence signal (weak) and LED+bioluminescence signal (strong) were distinguished by setting a threshold voltage value, and aver-

ages of the strong signal (LED+bioluminescence) for 30 s were calculated, using a macro of the spreadsheet application (Microsoft Excel, Microsoft Co.). The threshold level was installed manually according to the maximal value of bacterial bioluminescence.

1.2 Bacterial suspension preparation and measurement

A liquid culture of *P. kishitanii* was diluted by the fresh marine broth to give a final cell density of ca. 1×10^6 cells mL^{-1}. The suspension (liquid culture) was constantly stirred throughout the measurement for up to 5 d inside the incubator at 16°C. The LED signal at the beginning (no bioluminescence was detected) was tuned to ca. 170 mV. Measurements were performed both for the marine broth culture and mini broth culture, to check the system as to whether it could distinguish the two profiles with different broth.

1.3 Cell density calibration

Relationship between the cell density and the observed LED signal was checked for the calibration using bacterial suspensions with known cell density. The cell density of one bacterial suspension (master suspension) was calculated by counting the cells through a microscope using a bacteria counter. Then suspensions with 75, 50, and 25% of the cell density relative to the master suspension were prepared through dilution by the fresh marine broth. LED-irradiated signals from the five suspensions with different cell densities, including the master suspension and a fresh marine broth, were measured to obtain the calibration curve. Five data points were fitted with a function using a Microsoft Excel Command.

2. Results and Discussion

2.1 Cell density calibration

The cell density of the master suspension was calculated to be 16,750 cells μL^{-1}. Five diluted suspensions with different cell densities were poured into the optical cell and the sensor signals (without bioluminescence) were measured. Curve fitting for the five data points (sensor signal vs. cell density) was performed. A calibration curve in cubic function seemed a better fit compared to a simple linear one in that it agreed better with microscopic cell density value at ca. 7–30 h, a period when three major bioluminescence peaks were observed.

2.2 Cell density time course

The sensor signal after subtraction of the bioluminescence during the cell growth was plotted vs. time (**Figure 3(a)**). The signal was translated into cell density using the calibration function (**Figure 3(b)**).

2.3 Cell growth rate and bioluminescence time courses

The time derivative of the cell density curve was calculated by time division of subtracted cell density-data using spreadsheet application. The results were plotted as seen in **Figure 4(a)**. Bacterial bioluminescence intensity was plotted

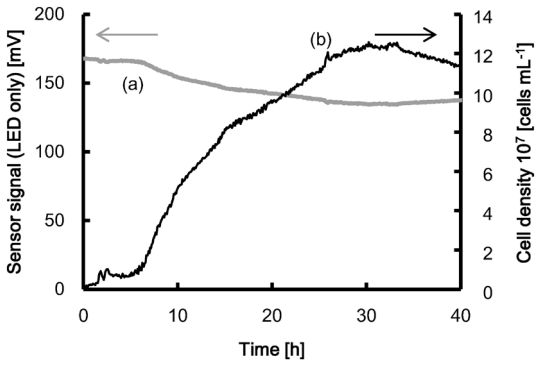

Fig. 3 Time courses of cell density (a), and bacterial bioluminescence (b)

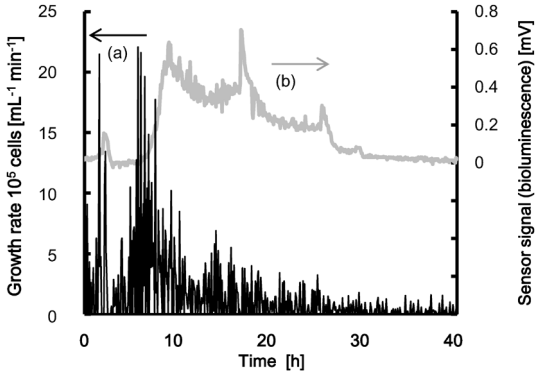

Fig. 4 Time courses of cell growth rate (a), and bacterial bioluminescence (b)

in mV unit (**Figure 4(b)**). Four definite peaks were identified in the bioluminescent profile.

In Figure 4(a), at times of 2, 5, 14, and 22–25 h, the maximum values in growth rate curve were seen. These times were obviously in good agreement with those when bioluminescence started to increase in Figure 4(b).

It has been known that the growth of bioluminescent bacteria is inhibited at low oxygen concentrations, although the synthesis of the luminescent system is not inhibited in some species (Nealson and Hastings, 1977). Several experiments have been performed concerning the bacterial bioluminescence and ATP or oxygen. Firstly, the oxygen and ATP effects on bioluminescence have been studied. ATP concentration was measured to be constant during the *P. phosphoreum* cell growth period under nutrition rich environment (Aleskerova *et al.*, 2014). In this work, data was acquired in several hours of frequency. In our study, on the other hand, the signal was acquired every 5 min. Our data, with higher temporal resolution, showed fluctuations in the bioluminescence and cell density.

The luminescent bacteria separated according to their motility using a microfluidic device have shown different bioluminescent intensities per cell (Sasaki *et al.*, 2009). This work proved that cells in a suspension vary in their ability to emit light. Intercellular signal material other than autoinducer is therefore suspected to work for the synchroniza-

tion of the bioluminesence. Experimentally, cell growth and luminescence do not increase at the same time (Watanabe *et al.*, 1991). With lower oxygen concentrations, cell growth is inhibited while the luminescence goes on (Nealson and Hastings, 1977). Two biochemical processes are reported in *P. phosphoreum*, i.e., oxygen consumption and luminescence. These two processes were chosen according to the oxygen concentration (Bourgois *et al.*, 2001). This means that oxygen level affects the bacterial decision whether to emit light or not (Bourgois *et al.*, 2001). A cat-and-mouse game played by cell growth and bioluminescence could, therefore, possibly describe the oscillatory behavior. In the well-stirred condition all the bacterial cells enjoy the same oxygen flux. In the rich broth cells grew in a rate larger than that in the case with poor broth. We often found that the oxygen concentration inside the luminous bacterial suspension to be almost zero (Sato and Sasaki, 2006). This might be the result of higher level of oxygen consumption with the cell growth. Substrates for the bioluminescence such as long-chain aliphatic aldehydes are produced inside the cell by the use of ATP (Bourgois *et al.*, 2001), soon the luminescence should decline because of the lack of substrates produced through the ATP consuming reaction chain. Under no bioluminescence condition, no oxygen consumption through the luminescence should occur, and this should result in a more or less increase of oxygen inside the cell. At this stage, bacterial cells might choose cell growth, rather than luminescence. This hypothesis is consistent with our results of continuous cell growth in Figure 3. Continuous cell growth, toghether with the simultaneous long-chain aliphatic aldehydes synthesis, was therefore thought to keep the oxygen level inside the cells lower than a thereshold, a level at which bioluminescence was allowed to occur. Finally, oxygen slowly diffused; probably because of higher cell density than before, through the cell walls and the oxygen level might exceeded the threshold, and luminescence might have had restarted. No oscillatory behavior was observed with mini-broth based suspension. Smaller growth rate, therefore, were thought to cause no oscillation. Measurements of the rates of substrate consumption (Abu-Soud *et al.*, 1992) and synthesis (Meighen, 1979) were performed. Further *in vivo* measurement might, if possible, to illustrate the whole image of oscillatory behavior.

Conclusion

Bioluminescence bacterium is a good model of a gram-negative bacterium that enables a noncontact metabolism evaluation. Our simple device showed a possible relationship between the cell growth rate and the bioluminescence with a small amount of liquid culture.

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