Oscillation in bacterial bioluminescence from *Photobacterium kishitanii* liquid culture was examined regarding reproducibility and bacterial cell activities, i.e., dissolved oxygen (DO) consumption, esterase activity, and product production rate. A frequent increase in DO was suspected to be due to a rapid decrease in luminescence, and a simple model describing not only the monotonous decrease in cell activity, but also the luminescence-DO relationship is proposed.

**Key Words:** bioluminescent bacteria; dissolved oxygen; luminescence; oscillation; product

**Introduction**

*Photobacterium kishitanii* is classified as a bioluminescent, gram-negative facultatively aerobic rod-shaped bacterium. This bacterium is commonly found on the surfaces and intestinal contents of marine animals such as cuttlefish, or in the marine environment. Although biological necessity for the bacteria to emit light is under discussion; as long as the light is emitted through the luciferase driven biochemical reaction, the light intensity could reflect biochemical activity of the bacterial cell. As light intensity measurements can generally be performed in a contactless manner, bioluminescence light intensity should be a convenient probe to acquire information on the biochemical activity of the bacterial cells. Bioluminescent bacteria emit blue-green light through the catalytic oxidation of reduced flavin mononucleotides (FMN) with long-chain aliphatic aldehydes, by the action of bacterial luciferases. The maximal emission wavelengths (475 to 486 nm) of some Photobacterium strains are different (blue shifted) from those of purified luciferase (495 nm). A fluorescent accessory protein called lumazine protein (LumP) is responsible for this blue shift. Roles of LumP, i.e., modulation of the emission peak to a shorter wavelength and enhancement of the light intensity, have been investigated from the viewpoint of the protein structure (Sato et al., 2010). In a well-stirred condition, oscillation in the bioluminescence intensity from a luminous bacteria suspension has been observed. The oscillation mode has been observed to change with the liquid broth component. A possibility has been indicated that both diauxic growth and oxygen reaction-consumption resulted with oscillation (Sato and Sasaki, 2008). In well-studied cases, pheromone signaling plays a key role in the regulation of light production in bioluminescent bacteria (Reuter et al., 2016). Certain Photobacterium strains, on the contrary, do not use this regulatory method for controlling luminescence (Dunn et al., 2015). Our preliminary study with bioluminescent bacteria collected from a cuttlefish skin resulted in a similar consequence, where cell free supernatant of a bright liquid culture of the bacteria had no effect on the bioluminescence of another liquid culture of lower density. Quorum sensing has been indicated to have a very small effect on the bacterial luminescence. To date, a search of the initial factors for the oscillation has been performed. The suspension volume, broth-dilution, and repeated stirring effects on the luminescence have been investigated, and, as a result the oscillation mode seems intimately affected by the oxygen supply from the liquid-air interface into the bacterial suspension (Sasaki, 2011). The relationship between the cell growth rate and luminescence intensity has also been investigated by fabricating a system that enables the simultaneous measurement of the luminescence intensity and the optical density of the liquid culture of the bioluminescent bacteria (Sasaki and Kato, 2016). Consequently, luminescence oscillation is observed not only at the log phase, but also at the stationary phase or death phase. In the last phase of growth, the oxygen consumption competition among bacteria is mitigated because of the death of many rival cells. In a low-oxygen culture condition, the death of cells offer a chance for the surviving cells to obtain more oxygen. The
activity of a given cell is affected by the activity of neighboring cells because they share limited dissolved oxygen in one batch of liquid culture. As bioluminescent bacteria consume oxygen for energy production, a lack of dissolved oxygen means a lack of substrate to produce energy inside the cells. Processes, including light emission, cell division and material production, proceed by the consumption of energy. The DO concentration available to a given cell, therefore, affects many metabolic pathways (including bioluminescence) of that cell. An estimation of oxygen demand, luminescence intensity, and material production rate might help to illustrate the cell activity during the luminescence oscillation.

In this study, we have investigated the cell activity of the collected bioluminescent bacteria, namely *Photobacterium kishitanii* KH-2005, during the bioluminescence oscillation, by frequent stirring, by using cell activity marker dye, and by product measurements. Specifically, bioluminescence time courses with varied stirring and on/off periods were examined. Both esterase and respiration activities of the cell were evaluated microscopically using fluorescent labeling dyes. Material product was detected using enzyme assay.

Materials and Methods

**Reproducibility of the oscillation waves.** We were not sure whether the observed luminescence oscillation occurred by accident, because the behavior of bacteria often differs from batch to batch even if the cultures started with the same inoculum. We therefore began by studying the observation of the oscillation reproducibility. One bright colony of KH-2005 on an agar plate (DifcoTM Marine Broth 2216, Beckton Dickinson and Company) with 2% agar (Wako Pure Chemical Co.) was picked up and suspended in 20 mL of liquid broth (2.5 g/L of BactoTM Yeast Extract, Beckton Dickinson and Co., 30 g/L of NaCl, Wako Pure Chemical Co., 5 g/L of BactoTM Peptone, Beckton Dickinson and Co.) in a glass vial (inner diameter 33 mm), regardless of the inoculated cell number. Five different batches of suspensions were thus prepared independently. A solar cell (A-10K, Kenis Ltd.) was connected to a voltage logger (GL220, Graphitec Co.) and the luminescence from each glass vial was recorded intermittently as a voltage at 10-s intervals. The suspensions were stirred using a magnetic stirrer (MC 303, Scinics Co.) at 17°C in an incubator (CN-25A, Mitsubishi Electric Engineering Co., Ltd.). The five sets of measurements were performed independently.

**Relationship between oscillation waves and DO concentration.** The simultaneous measurement of luminescence and the DO concentration was undertaken. A dissolved oxygen probe (DO-5509, Lutron Electronic Enterprises, Co., Ltd.) with a 17-mm diameter was placed inside the bacterial suspension in a glass vial with an inner diameter of 42 mm, thereby realizing an effective liquid-air interface area. All other conditions were the same as in the case of the reproducibility experiments.

**Effect of frequent stirring on oscillation waves.** The luminescence of the bacterial suspension was measured with various stirring on/off periods. The power supply for the magnetic stirrer was controlled using a microcomputer (Arduino UNO, Switch Science) with a self-made “sketch” program. The on/off periods were set at 10, 20, 30 and 60 min. All other conditions were the same as for the reproducibility experiments.

**Bacterial cell activity evaluation.** As *P. kishitanii* shows esterase activity (Yoshizawa et al., 2009), the use of an esterase-active cell staining dye would appear to be appropriate to estimate the cell activity. Five cuvettes (inner diameter 14 mm) with a 2-mL bacterial suspension were kept stirred. 100 µL of the suspension was sampled right after the 3rd luminescence peak (21.75 h), the 4th peak (25.45 h), and a dark (<1% of the peak luminescence) condition (86.76 h). Each sample was mixed with 1.5 µL of CFDA (5(6)-Carboxyfluorescein diacetate, Dojindo Molecular Technologies, Inc.), 1 µL of PI (Propium iodide, Dojindo Molecular Technologies, Inc.), or 2 µL of CTC (5-cyano-2,3-ditolyl tetrazolium chloride, Dojindo Molecular Technologies, Inc.) solution and placed in the cell density calculation room of a bacteria counter (Sunlead Glass Corp.). The cells in the suspension were observed with a fluorescence microscope (DM IRB, Leica Microsystems) under suitable excitation/emission condition. The ratio of esterase active cell density to total cell density was calculated using the transmitted light and fluorescence images of the samples collected from the suspension.

Results

**Reproducibility of the oscillation waves**

Figure 1 shows the results of the time course of luminescence from five independently-prepared liquid cultures of KH-2005. At least 6 apparent peaks (arrowed, defined as local maxima of the luminescence time course curve; i.e., maximum points found during time intervals of about 1 h in the curve) were observed in all five curves, although the time duration of the steep increase in luminescence differed, and the standard deviations of the luminescence intensity were small enough, compared with the oscillation wave amplitude. We also performed similar measurements with different volumes, and, as far as five different vials with different volumes (100 µL suspensions without stirring, or 2 mL suspensions with stirring) were concerned, more than 6 peaks were similarly observed.

**Relationship between oscillation waves and DO concentration**

Figure 2 shows the typical luminescence-DO time course among 4 measurements. After the luminescence reached a first steep peak at around 9 h, the DO level reached less than 2 mg/L. Interestingly, three peaks in DO values were observed (ca. 9, 16 and 24 h), and the DO concentration stayed very low (ca. zero) during the periods (10–14 and 17–22 h) between the peaks. More interestingly, three DO peaks appeared right after a rapid decrease in luminescence intensity (at 9, 15 and 23 h).

**Effect of frequent stirring on oscillation waves**

Luminescence time courses of the bacterial suspension with various stirring on/off periods (0, 10, 20, 30 and 60 min) are shown in Fig. 3. With Figs. 3(a) and (b), where
Cell activity evaluation during bacterial bioluminescence oscillation

Fig. 3(a) no stirring, and Fig. 3(b) 10 min stirring on/off was applied, six apparent peaks were clearly observed, but in other cases (Figs. 3(c), (d) and (e)) with longer on/off periods the peaks were difficult to identify. Rectangular decreases in luminescence curves were observed in Fig. 3(b)–(e) at off periods of stirring. Such rectangular decreases in luminescence appeared constantly in cases Figs. 3(d) and (e), but, interestingly, not constantly in cases Figs. 3(b) and (c). In Fig. 3(b), the decreases emerged after the 3rd (ca. 11–12 h), and before the 5th (17–20 h), luminescence peaks. In Fig. 3(c), similar tendencies as in Fig. 3(b) were observed, although the distribution periods of the rectangular decreases was broad and it was rather difficult to identify a relationship with the luminescence peaks. It seemed worth discussing the coincidence of the low DO level periods in Fig. 2 and the rectangular decrease periods in Fig. 3(b), which commonly emerged after the 3rd, and before the 5th, luminescence peaks. In other words, in Figs. 2 and 3(b), six peaks are indicated by open arrows. The dissolved oxygen concentration seemed very small during the period between the 3rd and 4th peaks, and during the period right before the 5th peak, in Fig. 2. Rapid decreases in the luminescence intensity without stirring were found during the period between the 3rd and 4th peaks, and during the period right before the 5th peak in Fig. 3(b). The DO concentration decrease and the rapid decrease in luminescence might be related in view of the oxygen consumption/diffusion. During the period between the 3rd and 4th peaks, the oxygen supply, as a result of stirring, from the air-liquid interface into the suspension should be necessary to maintain luminescence, because
the DO concentration is quite small. Such a small DO concentration might be due to the large bacterial oxygen consumption. Interestingly, such a condition appeared to be cancelled for some reason at around the 4th peak, and then re-appeared again right before the 5th peak. Such a repetition occurred under an intermittently stirred environment, so this phenomenon is worth discussing from the viewpoint of nonlinear science.

**Bacterial cell activity evaluation**

Figure 4 shows the result of esterase activity of the cell that was evaluated using CFDA, which is a bacterial cell wall and cell membrane permeable dye, and is hydrolyzed by esterase of the cell to stay inside the cell, staining only viable cells. The times of the evaluation, 21.75 h and 25.45 h represent the moments of the 3rd and 4th luminescence peaks, respectively. Both the total and the CFDA active cell densities increased between 21.75 h to 25.45 h, and decreased from 21.75 h to 86.76 h. The ratio (CFDA active cells)/(total cells) simply decreased through the entire evaluated period with values of 0.97 (21.75 h), 0.84 (25.45 h), and 0.44 (86.76 h). This ratio did not seem to decrease linearly with time. We also measured the colony forming unit (CFU) that represented the number of viable bacteria. CFU was therefore counted with the samples harvested at 10 h (brightest) and 87 h (no light) together.

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**Fig. 5.** CTC active cell ratio and luminescence.

A. Total cell and CTC active cell densities with time. Filled and open squares represent total cell and CTC active cell densities, respectively. Error bars represent standard deviations (n = 3). B. Luminescence time course. Spike-like increases indicate the time of sampling.

**Fig. 6.** CTC/PI active cell ratio and luminescence.

A. Total cell, CFDA active cell and PI active cell densities with time (n = 3). Filled triangles, open squares, and open circles represent the total cell, CFDA active cell, and PI active cell densities, respectively. B. Luminescence time course. Spike-like increases indicate the time of sampling.
with the CFDA active cell image and the transmitted image. At 10 h, all three parameters, i.e., total cell density, CFDA active cell density and density from the CFU were identical. At 87 h, on the other hand, the calculated ratios of these two density values were 0.5 and 0.15, respectively, against the total cell density (image data not shown). The respiration activity was also evaluated using fluorescent dye CTC, which has been hypothesized to indicate respiration active cells (Longnecker et al., 2005). As shown in Fig. 5(b), spike-like increases indicate the time of sampling, as a consequence of the incubator door being opened for sampling. The ratio of CTC active cell density to total cell density seemed constant, both at the beginning of growth and at luminescence peak periods. Over a period of days, however, the ratio of CTC active cells was seen to decrease with time. Bacterial cell membrane injury was also evaluated using PI, but no significant PI active cells were observed at the 2nd−4th luminescence peaks. The health condition of the cells was therefore examined using CFDA and PI at the same time (Fig. 6). The ratio of CFDA active cell density to total cell density gradually increased with time, but after 3 days of incubation many cells maintained a CFDA activity. PI activity, on the other hand, seemed very small throughout the experiment.

Discussion

Figure 1 indicated that five independent suspensions showed luminescence profiles with at least six peaks in common, although the time of rapid increase differed (5–16 h). Such a difference probably originated from the cell number difference in the colony inoculated to each suspension. Emergence of these peaks was, therefore, thought to be common among bacterial suspensions with the same broth content, so we focused on the luminescence peaks as an index of cell condition. Therefore, we assumed that discussions on the cell activity difference at different luminescence peaks (or valleys) are rational; i.e., cells showing a 3rd luminescence peak in different suspensions, for example, were suspected to indicate a common cell condition. On such an assumption, we continued the cell activity evaluation.

In Figs. 3(b) and (c), rectangular decreases appeared during two apparent time periods (11 and 18 h with Fig. 3(b), and 11−16 and 20−25 h with Fig. 3(c)). In the cases of stirring on/off periods larger than 30 min, such decreases appeared ceaselessly throughout the tested periods. Both in Figs. 3(b) and (c), the decrease spikes appeared right after the 3rd and before the 5th peaks. These moments were identical with those where the DO concentration remained zero in Fig. 2. Considering that the oxygen supply from the liquid-air interface is minimized when stirring is stopped, luminescence decrease should be due to the lack of oxygen supply. In Figs. 3(b) and (c), therefore, during certain periods cells are not affected by the stirring being stopped; i.e., they could utilize enough oxygen to emit light even without stirring. In other periods, cells could not do this, resulting in a rectangular decrease. Also, 30 min was shown to be too long for the cells to keep on emitting light without additional oxygen supply from the liquid-air interface.

As oxygen is one of the substrates for luminescence reaction, the bacteria consume oxygen both through luminescence and respiration. Both an increase in luminescence intensity and in cell density result in a decrease in DO concentration. This, in turn, should result both in a decrease in luminescence intensity and possibly in cell growth.

In our previous study, after the 3rd luminescence peak no dramatic change in cell density was observed (Sasaki and Kato, 2016). A gradual decrease in the ratio of the actively living cell density against the total cell density was suspected. For example, the ratio of CFDA active cells decreased by 10% from the 3rd to the 4th luminescence peak (Fig. 4). Similar decreasing tendencies were observed in the CFU counting results (not shown). Such a decrease should be responsible for the recovery of luminescence intensity resulting in peaks. For an active cell waiting for oxygen under low DO conditions, the death of neighbor cells should be welcomed in view of sharing oxygen. A temporal recovery of DO in Fig. 2 was similarly suspected to be the result of cell inactivation. Active cells would then emit more light through the consumption of extra oxygen which was not consumed by other inactive cells. A decrease in the total cell density was observed (Fig. 4) from 25.45 h to 86.76 h. As bacteria possess autolytic enzymes (Sturges and Rettger, 1922) and many bacteria lyse when a culture ceased growth (Forsberg and Rogers, 1971), the total cell density is seen to decrease during such a period. In Fig. 5, most of the cells seemed CTC active from the beginning to the oscillation periods. As CTC indicates the microbial respiratory activity, this activity might not be the initial reason for the oscillation in luminescence. As CFDA and PI are markers for esterase active and injured cells, respectively, during the observed luminescence oscillation the cells seemed, therefore, to maintain esterase activity with a small injury ratio (Fig. 6). Inactivation or injury of the bacterial cells might not therefore be the initial reason for the observed oscillation in luminescence.

Gram negative bacterium can grow even during a DO zero condition (Stolper et al., 2010). When stirring ceased for more than 30 min, the cells cannot acquire oxygen for luminescence, and, hence, luminescence dramatically decreased (Figs. 3(d) and (e)).

Why, then, does the luminescence and the DO repeatedly vary? One very simple model can illustrate such behavior, where a DO increase contributes to a luminescence increase because oxygen is the substrate of a luminescence reaction, and a luminescence increase results in a DO decrease because of substrate consumption. Let variables x and y represent the luminescence intensity and the dissolved oxygen concentration, respectively. Then, a simple model can be expressed as

$$\frac{dx}{dt} = a(y - y_0)$$

$$\frac{dy}{dt} = -b(x - x_0)$$
where $x_0$, $y_0$, $a$ and $b$ are constants. A set of points (trajectories) with $(x, y)$ coordinates which satisfy the equations on the $x$-$y$ plane look like clockwise concentric circles (rounds) (Strogatz, 2001) with a center at $(x_0, y_0)$. Such trajectories tell us that both $x$ and $y$ values temporally oscillate in turn with an oscillation center of $x = x_0$ and $y = y_0$, respectively. This model is too simple to illustrate the entire behavior of the bacteria, but is consistent with the characteristics of oxygen and luminescent intensity for a short period of time. Taking the temporal change of the values $a$, $b$, $x_0$ and $y_0$ into consideration, the solution of the equations will look more similar to the results shown. To support this hypothesis, the inhibition of respiratory electron transport was reported to increase the bioluminescence of other bioluminescent bacteria (Karatani et al., 2004) which indicated experimentally the connection of bioluminescence with the respiratory electron transport. By constructing a more accurate model that fits the result, a further understanding of the mechanisms of luminescence oscillation will be realized. Luminescence can be thought of as one aspect among various metabolic characteristics. We assumed that, during the early stage (0–10 h) of growth, all the cells were active enough so that the luminescence intensity was proportional to the total cell number. This assumption seemed rational due to our preliminary experiment using a bacteria counter and a luminous counter. As a result, the cell density of the bacterial suspension and the luminescent intensity linearly increased similarly with time. Then, as the culture process proceeded, the DO reached its lowest level and this reduced the luminescence intensity. Luminescence seemed to have frequently recovered at the expense of other non-active cells. A certain partial pressure (The Pasteur Point), that of oxygen (in equilibrium with a solution) at which facultative aerobic organisms switch to anaerobic metabolisms, has been reported (Stolper et al., 2010). After the switching, some bacteria change the enzymatic activity (Gunsalus, 1992; Stewart, 1988). In cases of bioluminescent bacteria, including relatives of *P. kishitani*, growth and luciferase production preceded similarly, both in cases of aerobic and anaerobic conditions (Eberhard et al., 1979). Mapping of the entire enzymatic activity changes concerning the metabolism related to luminescence in the future would help illustrate the complete picture of luminescence oscillation.

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