Control of the Bioluminescence Starting Time by Inoculated Cell Density

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In this study, we attempted to control the timing of light-emission from bioluminescent bacteria, by changed cell numbers inoculated into medium. Luminous bacteria express bioluminescence when the number of cells reached a threshold. Inoculated cell density had an effect on the time of bioluminescence starting. Samples were prepared by varying cell density of inoculation. In the results, all the vials showed different luminescence profiles in the order of inoculated cell population.

(Received April 13, 2006; Accepted June 26, 2006)

Many methods concerning environmental pollution are studied, and among them microbial methods are reported to be effective because they give direct information about the effect of pollutants on life.¹⁻⁶ The growth of a microbe is affected by the incubation conditions, including several factors, such as the temperature, nutrition concentration, amount of oxygen, or the pollutant concentration. The generation time (doubling time) of cell growth should be affected by the incubation condition; doubling time must be longer when the cell culture is polluted. Changes in the time course of microbial population will, therefore, provide information about the influence of a pollutant on microbial growth.

Bioluminescent bacteria are known to emit blue-green light with a maximum wavelength of 485–495 nm. This bioluminescent reaction is catalyzed by the enzyme luciferase, and requires reduced flavin mononucleotide (FMNH₂), long-chain aliphatic aldehyde, and O₂. Typical bacteria are classified in the genus *Photobacterium* or *Vibrio*. Most of them are marine organism, and are found in association with fish.⁷⁻⁹ Bioluminescent bacteria do not express bioluminescence when they are free-living in the ocean because of low cell population. The synthesis of the enzyme luciferase is regulated by the cell density, *i.e.* when the bacterial growth reaches a sufficiently high density the enzyme is synthesized. Such a mechanism is called quorum sensing.¹⁰ It is clear that the bacteria tell us by luminescence that they have reached a certain growth density, because the luminescence can be seen by the naked eye.

The application of luminous marine bacteria for toxicity testing in a reagent format has been elucidated during past two decades. The intensity of bioluminescent emission provides an instantaneous measure of the organism's metabolic state. In addition, applications of luminous bacteria on environmental monitoring have also been reported.¹¹⁻¹³ All of these previous reports are based on the traditional biosensor system, *i.e.* the combination of microbial cells with transducers, such as photo detectors. The measurement system is based on an analog signal treatment, *i.e.* to know the target material concentration by the quantity of the signal.

Our point of view, on the other hand, is based on the time difference of the bioluminescence starting. When a bacterial cell is inoculated into a medium, the number of cells doubles in the generation time. Light-emitting begins when the cell number reaches a threshold. After the generation time, the number of cells becomes twice that before. Light emission, therefore, starts earlier when the culture contains more cells. If the generation time is affected by the pollutants, the time to start bioluminescence will be delayed. We can then see how the bacterial growth is affected by measuring the time to see blue light.

In this study, we tried to control the timing of light-emission of these bacteria, by the changed cell numbers inoculated into the medium. The dependence of the time to start light emission was examined.

Experimental

Bioluminescent bacteria were collected from a cuttlefish, *Todarodes pacificus*, purchased at a local supermarket, and were cultivated on agar plates with marine broth (Marine Broth 2216, Difco Laboratories, USA) for purification at 18°C. Luminescent colonies were chosen and cultivated in the liquid broth (Marine Broth 2216, Difco Laboratories, USA). Harvesting was performed at O.D. 600 of about 0.40. The cultures were diluted by the same broth to have optical density values of 0.2, 0.1, 0.05, and 0.025. Then, 100 µl of cultures with different cell densities were inoculated into 20 ml of marine broth liquid medium in 50 ml glass vials. The sample glass vials were incubated at 18°C during the measurement without stirring. The luminescence of the glass vials was recorded every 20 min using a digital still camera (Camedia E-20, Olympus, Japan) with an exposure time of 8 min. The light intensity at 470 nm from the glass vials was measured by an optical power meter (Model 3664, Hioki E.E. Co., Japan). Light detectors were attached to the glass vials to detect light from the culture medium.

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Fig. 1 Photographs of glass vials with different starting cell density. Photographs were taken every 20 min. A 100- μ l volume of cell suspensions with absorbance of 0.4 (a), 0.2 (b), 0.1 (c), 0.05 (d), and 0.025 (e) were added to the vials. Firstly, the traveling bands were observed (i), whereas later the luminescence intensity increased in all parts of the vials (ii).

Results and Discussion

Five glass vials with different starting cell numbers were arranged in a line in a dark room. As can be seen in Fig. 1, bioluminescence started earlier in the vial with a greater starting cell number. In the first ten pictures (i), the luminescent band traveled from the bottom to the top in each vial. This might have been due to precipitation of the broth contents. Presumably, the bacterial cell density exceeded the threshold first around the precipitation, and the cell density wave traveled from the bottom to the top. It was suggested that after luminescence the dissolved oxygen was consumed, and no luminescence was observed. The traveling density wave and oxygen consumption are possible reasons for the spatial movement of the luminescent bands in all five vials. The traveling luminescent band was observed earlier in the vial with more bacterial cells at the moment of inoculation, *i.e.* the vial (a) in Fig. 1 showed the earliest movement of luminescent band, and vials (b), then (c), (d) and (e) followed afterwards. This

Fig. 2 Time courses of bioluminescence intensity with different starting cell density. The absorbencies of the cell suspensions added to the vials were 0.4 (a), 0.2 (b), 0.1 (c), 0.05 (d), and 0.025 (e).

Fig. 3 Bioluminescence starting time plotted against the logarithm of cell suspension absorbance ($\log_2 A$) added to the vials.

may have been due to the difference in the time required for the cell density to exceed the threshold, because of the difference in the starting cell density. In the pictures after the eleventh (ii), the bright part seemed to expand from the top to the bottom in each vial. The top parts, *i.e.* the air/liquid interfaces, were the brightest in all of the vials. This may have been due to the richness of dissolved oxygen at the interface. This brightest part was thought to be the light source. Light emission from the top seemed to increase with time because of cell growth. The light from inside of the liquid media, which was thought to be the scattered light by bacteria or nutrient particles in the suspension, also increased. In pictures of (ii), the cell density increased with time and the luminescence was generally higher in top part of the liquid. Together with the long exposure time of 8 min, all of the liquids seemed to be brighter compared to those in pictures in (i). Figure 2 shows the time course of the bioluminescence intensity. The first increase in the luminescence intensity was observed from the vial with the highest inoculated cell density at 500 min after inoculation. All of the vials showed different luminescence profiles in the order of the starting cell density; increases in the bioluminescence intensity above the baseline were observed first from vial (a), then (b), (c), (d) and finally (e). As is clear from Fig. 2, the differences in the luminescence rising time were approximately 30 min, which was equivalent to the typical generation time for the bioluminescent bacteria.¹⁴ The shoulders found in all of the profiles at 500–700 min might have resulted from the above-mentioned traveling bands. The light detectors were attached at the middle of the vial and the luminescence from the bottom part of the vial was thought to be observed as the shoulders. After the shoulders, the main peaks appeared in all of the

profiles at 800 – 1500 min. These were presumably the result of a general increase in the bacterial cell population in the middle of the vials, as in (ii) in Fig. 1. Figure 3 shows the relationship between the logarithm of the inoculated bacterial cell density and the luminescence starting time. The luminescence starting time was defined as the time when the luminescence intensity (measured in nanowatt by the photometer) jumped up from the baseline. A linear relationship was clearly seen, as was predicted from the exponential growth property of bacteria.

We have shown in this study that the bioluminescence starting time could be controlled by the inoculation cell density. The result provides an important clue concerning the development of a novel bioluminescence-based analytical method. For instance, a cell suspension that contained any kind of growth-inhibition material would show delayed bioluminescence. The luminescence could be recognized by the naked eye, and by comparing two vials, one containing a sample material and the other a control, one could easily tell whether the former contained a growth inhibitor or not. When the bacterial suspension was provided at a density just below the threshold for the bioluminescence, the time required to see the light become much less, resulting in a shorter measurement time.

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