Proposal of a Utilization of a Luminous Bacterium in the Teaching and Learning of Radiation Safety

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We isolated the luminous bacterium Vibrio phosphoreum H1 as a tool for education in radiation safety. It emits strong and steady luminescence. It is nonpathogenic, cannot be grown under normal low-salt conditions, and can be handled without any special equipment or reagents. We can cultivate it on a desk at room temperature, and can use a home-made broth containing a high salt concentration. Heat treatment at 37°C kills the bacterium, leading to its loss of luminescence. Although X-ray irradiation clearly kills it as the exposure dose increases, luminescence remains intact for some time, suggesting a delayed appearance of the biological effect of radiation exposure. We showed that the luminous bacterium Vibrio phosphoreum H1 can be used as a tool for teaching and learning about the effects of radiation. We proposed a practical plan that can be employed at high schools as well as universities.

Key words: radiation education, luminous bacteria, Vibrio phosphoreum, X-ray irradiation, DNA damage

1. Introduction

Education in radiation safety is important to understand the appropriate handling of radiation and radioisotopes, which have been used in various fields1). This has become even more important for the public after the Fukushima Daiichi nuclear plant accident. There are a number of ways to learn the health effects of radiation based on bacterial systems2–5). However, there is some difficulty for students to benefit from them, because they must master techniques for the handling of cells and actual experimentation. Bioluminescence provides the broadest opportunities for teaching biology at high schools as well as universities. Light-emitting bacteria are the most abundant and widespread luminescent organisms found in marine environments6). Luminous bacteria require oxygen and ATP as energy to produce light. Thus, the luminescence of bacteria can be used as an index of life activity7). In the field of toxicity assessment, they are used as biosensors to assess environmental pollutants in water, sediment, and soil8). Most luminous marine bacteria are non-pathogenic, and they require a high salt concentration to grow and exhibit bioluminescence9). These characteristics show that luminous bacteria may be an ideal tool for education. We need no special equipment or techniques of microbiology to handle the bacteria. In this study, we aimed to show the use of luminous marine bacteria as an educational tool to learn about the biological effects of radiation. We propose a practical course that can be incorporated at high schools as well as universities.

2. Materials and Methods

2.1 Bacterial strains and a plasmid

Vibrio fischeri sp. was isolated from the surface of Loligo beka, V. leiognathi sp. was isolated from the light organ of Doryteuthis kensaki, and V. phosphoreum H1 was isolated from the surface of Todarodes pacificus. A non-luminescent strain of V. phosphoreum L4 was isolated as a spontaneously emerging non-glowing clone from V. phosphoreum H1. Species identification of cloned marine bacteria was determined based on the 16S ribosomal RNA sequence9). The plasmid pVIB that carries the V. fischeri lux operon was obtained from the Carolina...
Biological Supply Company (North Carolina, USA). E. coli JM109 was used for the transformation assay of plasmid DNA.

2.2 Media and cultivation

Marine Broth 2216 (BD Difco, Sparks, MD 2115, USA) and LB3 (Bacto trypton: 1 g, Bacto yeast extract: 0.5 g, NaCl: 3.0 g, per 100 ml of H2O) was used to grow marine bacteria. LB (Bacto trypton: 1 g, Bacto yeast extract: 0.5 g, NaCl: 0.5 g, per 100 ml of H2O) was used to grow E. coli JM109. Marine bacteria were cultured at room temperature (around 22°C). E. coli JM109 was incubated at 37°C. BS3 (3.0% NaCl and 66 mM NaPO4 buffer at pH 7.0) was used for the dilution of marine bacteria. Soy sauce broth: soy sauce at 10 ml, baking soda at 0.1 g, and 100 ml of H2O were also used for marine bacteria.

2.3 Measurement of luminescence

To examine the relative strength of luminescence, the bacterial strains were grown to the late log phase. This corresponds to an optical density of the culture medium of about 0.8 (OD600 = 0.8). The optical density was measured with a spectrophotometer (U1100, HITACH Ltd., Tokyo, Japan). The culture medium of bacteria was diluted to 1:100 with BS3 to halt the growth, and the relative strength of luminescence was measured with a lumino-meter (Lumat LB 9507, Berthold Technologies GmbH & Co., KG, Bad Wildbad, Germany). The luminescence spectrum of V. phosphoreum H1 was measured with a fluorescence spectrophotometer (F1000, HITACH Ltd., Tokyo, Japan) in the 400 to 600 nm regions. Each measurement was performed two to three times, respectively.

2.4 Heat treatment of V. phosphoreum H1

Two ml of overnight bacterial cultures were heat treated in a 37°C water bath. At the times indicated, the test tubes were removed from the bath, and 10 µl of the culture medium were diluted to 1:100 with BS3 at room temperature. Luminescence was measured immediately.

2.5 X-ray exposure

X-ray irradiation of bacteria was carried out using an irradiator (MBR-2520R (200 kV, 20 mA, HITACH Ltd., Tokyo, Japan). Two ml of overnight bacterial cultures were irradiated at about 10 Gy/min without filters. After irradiation, 10 µl of irradiated cultures were diluted to 1:100 with BS3 and kept at room temperature for 1 to 24 h. The luminescence was measured as described above. The irradiated bacteria were serially diluted and plated to LB3 agar plates overnight at room temperature. Their viabilities were estimated in colony-forming units (CFU). CFU/ml is derived from the following equation.

\[
\text{CFU/ml} = \frac{\text{number of colonies}}{\text{dilution factor} \times \text{ml of inoculum}}
\]

Each measurement was performed two to three times.

2.6 Agarose gel electrophoresis and transformation assay

X-ray irradiated plasmid DNA was electrophoresed in 0.8% agarose gel in 1 × TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) for 60 min at 50 V. The gel was then stained with ethidium bromide (0.5 mg/l, Sigma-Aldrich Japan Co., Tokyo, Japan), and the images were recorded.

Chemically competent E. coli JM109 cells (TaKaRa Biomedicals, Tokyo, Japan) were transformed with 100 ng of plasmid DNA (pVIB) following the manufacturer’s protocol. Transformed bacteria were diluted and plated on LB agar with ampicillin (100 µg/ml) and incubated at 37°C overnight to detect colony formation.

3. Results and Discussion

3.1 Comparison of the relative luminescence

The relative strength of luminescence was determined using the cultures of each bacterial strain at 18°C. The luminescence of V. phosphoreum H1 was the highest among them. It showed about 60 times stronger luminescence compared to the V. fischeri strain, and that 6 times stronger than the E. coli strain carrying the plasmid of the lux operon (Fig. 1). No luminescence was detected from the pJE202/JM109 strain. This strain showed no luminescence in the late log phase and no bioluminescent activity in marine bacteria. The growth of marine bacteria was also observed, and it was found that the growth of marine bacteria was inhibited by the presence of bioluminescent bacteria. The growth of E. coli JM109 was not inhibited by the presence of bioluminescent bacteria.

![Graph showing relative luminescence of different bacterial strains](image)

Fig. 1 Measurement of bacterial luminescence. The luminescence of each bacterial culture in the late logarithmic growth phase (OD600 = 0.8) was measured. Luminescence relative to V. fischeri is indicated. Standard deviations are also included.
observed in the *V. phosphoreum* L4 strain. As a strong luminescence is desirable for our purpose, we used *V. phosphoreum* H1 in the following experiments.

### 3.2 Growth curve of *V. phosphoreum* H1

The proliferation of *V. phosphoreum* H1 in LB3 at 18 and 27°C was examined (Fig.2A). The doubling time was estimated to be about 135 min at 18°C and 72 min at 27°C. Bacterial cultures in the late log phase of growth (OD$_{600}$ = 0.8) were obtained and used for the experiment.

### 3.3 Luminescence spectrum of *V. phosphoreum* H1

Fig.2B shows the luminescence spectrum of *V. phosphoreum* H1 at 2 different temperatures in the 400–600 nm region. The bacterium showed steady luminescence at an ambient temperature. The peak spectrum shifted slightly with the temperature: 475 nm at 18°C and 485 nm at 27°C, showing favorable consistency with a previous report\(^{10}\). There was no notable influence on the luminescence intensity measurement in this temperature range.

### 3.4 Effect of heat treatment

To observe the damage of proteins on luminescence and the cell viability, we performed the heat treatment of bacteria. When the *V. phosphoreum* H1 culture was heat-treated at 37°C in a water bath, the luminescence disappeared rapidly up to 60 min (Fig. 3A). The viability of bacteria was analyzed in colony-forming units. It showed a similar curve to that of the luminescence intensity, except for the initial slope (Fig. 3B). The results indicated the important role of proteins in the functions of luminescence and cellular viability.

### 3.5 Effect of X-ray irradiation

The delayed appearance of a biological effect is a feature of the acute as well as late effect of radiation. To observe DNA damage caused by radiation, we analyzed the changes in luminescence emitted from *V. phosphoreum* H1 on X-ray irradiation.
The luminescence intensity was measured as a function of the exposure dose (0 to 1,000 Gy). The luminescence slightly decreased 1 h after 1,000 Gy exposure (Fig.4). However, it decreased with an increasing exposure dose, showing a background level at 24 h after 1,000 Gy exposure. The 50% lethal dose was estimated as 150 Gy. Fig.4B shows the viability of the bacteria, exhibiting a rapid decrease in the range of 100–1,000 Gy exposure. This observation yielded a good example of the biological effect of radiation exposure for the educational purposes.

### 3.6 X-ray irradiated plasmid DNA

We analyzed the effect of X-ray irradiation on plasmid DNA as a model of luminous bacterial DNA. Plasmid DNA carrying the *V. fischeri lux* operon (pVIB, 13 kb) was irradiated and used to transform *E. coli* JM109 cells. As a broken plasmid cannot transform *E. coli*, damage caused by X-ray irradiation can be seen as the number of transformants after irradiation. X-ray irradiation caused a decrease in transformants (Table 1). A few transformants were observed after 1,000 Gy irradiation. Irradiated plasmids were analyzed with agarose gel electrophoresis. The supercoiled intact plasmids were damaged with X-ray irradiation. We observed the decrease in the supercoiled form, and the increase of open circular and linear forms with X-ray dose increases (Fig. 5). From these observations, we could understand that the biological effect of X-ray irradiation is correlated with the DNA damage.

#### 3.7 Presentation for students

In this report, we propose the use of luminous bacteria as an educational tool for understanding the effect of radiation on human health. The luminous bacteria are nonpathogenic and cause no hazard to the environment. Students can learn to handle the bacteria and have a varied experience of the biological and molecular methodologies, including fluorescence spectroscopy, bacterial transformation, polymerase chain reaction, and agarose gel electrophoresis.
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The practical course consists of five main parts:

1. **The mechanism of bacterial bioluminescence.** Students grow luminous bacteria and learn the mechanism of the bacterial bioluminescence. Living status of the cells (energy and oxygen consumption) can be easily identified as they emit luminescence.

2. **Heat treatment of bacteria.** Students treat the bacteria with heat or protein degradation reagents, and measure luminescence and viability. The loss of cellular function can be seen as a loss of luminescence. The lag phase of viability shown in Fig. 3 was explained as recovery mediated by the newly synthesized proteins from intact genomic DNA. Heat inactivation of proteins provides a direct demonstration of the cellular functions mediated by such proteins.

3. **Irradiation of bacteria.** Students irradiate bacterial cultures and measure luminescence and viability. X-ray irradiation clearly killed the bacteria as the exposure dose increased; however, the cellular functions remained intact for a while. This is a good example of the delayed appearance of the biological effects of exposure. The difference with heat treatment can be explained by the target size and number. When viewing DNA as one large molecule, and proteins as multiple small molecules, we can understand that one hit to DNA kills the bacteria, but proteins receiving multiple hits still retain some functions.

4. **DNA analysis.** Students isolate the DNA from bacteria (heat-treated and X-ray irradiated), and examine it with agarose gel electrophoresis. The heat-treated DNA is intact, but X-ray irradiated DNA is degraded as the dose increases. Normal supercoiled plasmid DNA changes its conformation from open circular to linear forms following a single strand break (Fig. 5). Such a single strand break (nick introduction) of plasmids is the most sensitive method to see DNA damage and can be detected with agarose gel electrophoresis based on the large mobility shift that occurs[11]. We can also use a comet assay[12].

5. **Transformation.** Students transform *E. coli* with irradiated plasmid DNA. Each transformant (bacteria carrying the introduced plasmid DNA) is observed as a glowing colony. When mutations occur in *lux* genes, dark colonies are observed. The number of transformants correlates with DNA degradation.

The students can learn that DNA is the final target of irradiation with this practice. The advantages of this method are the simplicity of the handling and effect of luminescence as a visual aid. However, over 100 Gy irradiation is needed to see a clear difference in the experiments using luminous bacteria. As this is an extremely large dose, we need to exercise special caution to avoid radiation exposure to students.

4. **Conclusions**

In this study, we showed that the luminous marine bacterium *V. phosphoreum* H1 isolated from *Todarodes pacificus* was useful as an educational tool to understand radiation safety, especially the health effects of radiation. We proposed a practical presentation plan for students in high schools as well as universities.

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