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Bacterial illumination

Culturing luminous bacteria

Passengers on board cruise liners are sometimes startled at night by an eerie glow emitted by seawater flush lavatories. The explanation lies in luminous microorganisms, which require oxygen and sometimes physical damage for their light-generating reactions. Such microbes are found in all marine environments [1]. Their presence can be a problem — much of the research on luminescent dinoflagellates has been funded by the US Navy, embarrassed by glowing trails behind ships, and more recently by interference with the laser light used for submarine communications [2].

Luminous bacteria can be very useful, however. They are highly sensitive to pollution and are often used to detect toxins in water, often in place of animal tests [3]. The enzyme and reactants that produce light are also used for analytical work *in vitro* [4].

The bacterium *Photobacterium phosphoreum* (Figure 1) is the brightest of all luminescent bacteria. It is able to thrive at room temperatures (20–25°C or lower), so it does not need specialist incubation facilities. In addition, the bacterium's requirement for saline conditions means that it is unlikely to survive for long if accidentally spilt; hence it is often recommended for elementary school work [5].

P. phosphoreum may simply be cultivated to learn aseptic techniques. Individual McCartney bottles of broth are a convenient means of culturing the organism, although greater volumes in a well-aerated fermenter look particularly spectacular! More challenging, open-ended practical investigations are also possible.

If time and local safety regulations allow, luminous bacteria can be isolated from the skin of seafish or squid using an enriched medium.

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Fig. 1
Photobacterium phosphoreum *streaked onto a Petri dish. This photograph was taken using a long exposure. To the naked eye, glowing cultures of this organism generally appear more green than blue. Strong luminescence like this can only be achieved using a special* Photobacterium *growth medium.*

Fig. 2
Luciferase from Photobacterium phosphoreum. *Data for this computer model was obtained from the Protein Data Bank, ID code 1FVP. See Reference [6].*

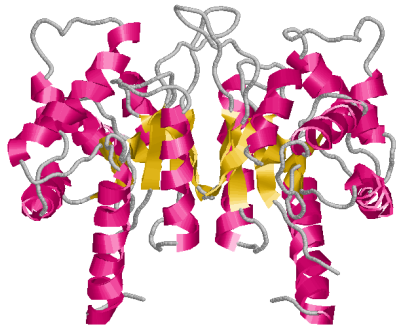


Fig. 3
The structure of bacterial luciferin.

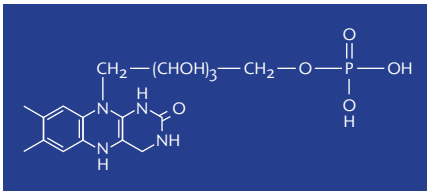
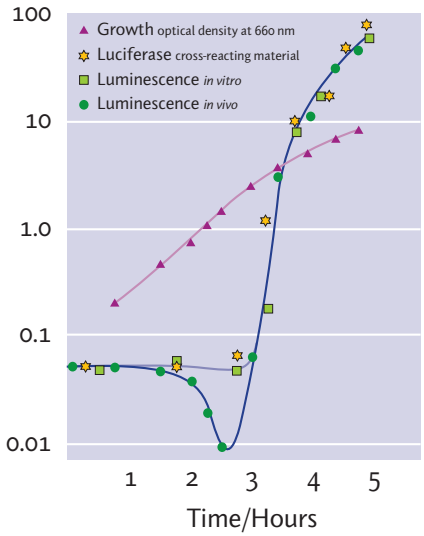


Fig. 4
The development of luminescence and the production of luciferase (measured by its cross-reaction with antibodies) compared with the growth of Vibrio fischeri. *See Reference [7].*



How do bacteria make light?

The light-emitting reaction is a side branch of the electron transport chain of bacterial respiration. Unlike light production in dinoflagellates, which only luminesce in response to physical stress, bacterial luminescence is a continuous process. In the reaction, reduced luciferin ($FMNH_2$) forms a complex with the enzyme luciferase. This complex reacts with molecular oxygen to form oxidised luciferin (oxyluciferin or FMN) and 'excited' luciferase. Long-chain aldehydes are also required in this stage of the reaction. The return of luciferase to its resting state results in the emission of a photon (Figure 5).

Why do bacteria make light?

Although its biochemistry is well-understood, for many years the function of bacterial luminescence remained a mystery. The amount of light generated by an individual cell is too small to be of physiological or ecological significance. It is now known that free-living bacteria in the sea do not produce luciferase and consequently do not emit light. It is only when such cells are packed together, for example, in the specialised light organs of a fish or squid, that they glow brightly. In this symbiotic relationship, the animal provides the bacteria with a nutrient-rich environment for growth. In return, the bacterial light gives the animal a means of attracting prey, communication, or camouflage [1].

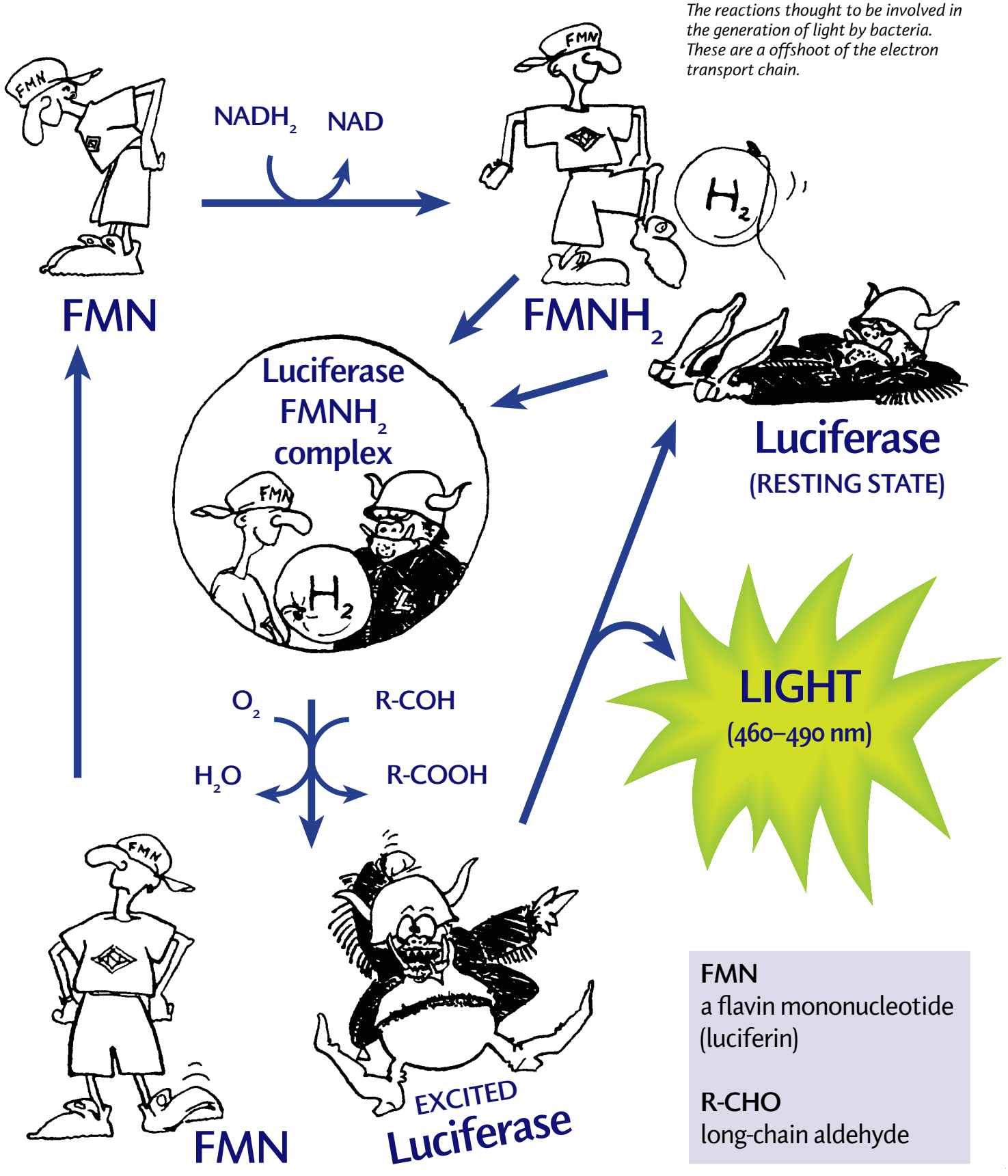
Why do bacteria glow inside light organs and not in the open sea? Laboratory observations in the late 1960s showed that newly-inoculated cultures of *Vibrio fischeri* only begin to emit light once the cell density has reached a certain level (Figure 4). At first it was suggested that this was because the culture media contained an inhibitor of the light-generating reaction, which was removed by the bacteria when greater numbers were present. Later this was shown to be wrong. In fact, the accumulation of an activator molecule or 'autoinducer' at high cell densities is responsible for triggering the production of light. The bacteria are able to sense cell density by detecting not only the presence, but also the *concentration* of the autoinducer. Today this type of cell-to-cell signalling is called *quorum sensing* [for a brief history, see Reference 8].

The basic mechanisms of quorum sensing identified in *V. fischeri* and *Vibrio harveyi* are now known to be similar to those which regulate gene expression in a wide variety of Gram-negative species. Quorum sensing is also found in Gram-positive bacteria and there is even evidence that communication occurs between species ('quorum sensing cross talk'). Bacterial communication like this is now thought to play a major role in the virulence of pathogens and in enzyme and antibiotic production. Research in this field could therefore be of considerable economic and medical importance.

In conjunction with work on quorum sensing, the regulation of bacterial luminescence at the genetic level has been studied [9, 10]. There are seven so-called *lux* genes that are required to produce and regulate luminescence in *V. fischeri*. Two plasmids containing these genes have been constructed for educational use, and their function can be demonstrated by inserting them into *Escherichia coli* [11].

IMPORTANT! Within the European Union, such bacterial transformation experiments may only be undertaken with the permission of the appropriate authorities (in the UK, the HSE [12]).

Fig. 5
The reactions thought to be involved in the generation of light by bacteria. These are a offshoot of the electron transport chain.



Aim

To observe luminescence from *Photobacterium phosphoreum* or *Vibrio fischeri*.

Equipment and materials

Needed by each person or group

- Slope culture of *P. phosphoreum* or *V. fischeri*
- *Photobacterium* or seawater broth (see recipes below). For small-scale cultivation by individuals, dispense the broth in aliquots of 15 ml into McCartney bottles before autoclaving.
- Bunsen burner
- Inoculation loop
- Access to a dark room for viewing the cultures 18–24 hours after inoculation

Procedure

1 Inoculate the broth and incubate overnight at room temperature (20–25 ℃). Aeration is necessary for luminescence. This can be achieved by shaking the McCartney bottles — but they *must* be tightly-capped. **IMPORTANT! Sufficient space must be left above the liquid in the bottles, to permit the production of gas and to allow oxygen to diffuse into the culture.**

Safety

Good microbiological practice must be observed by anyone carrying out this work. The method for isolation of luminous bacteria from fish (see box, page 6) may not be appropriate for use in schools. If this work is undertaken, the precautions described must be followed to ensure that pathogens are not inadvertently isolated and cultivated.

For further guidance, teachers in the United Kingdom should consult *Topics in Safety* [5] and ensure that they abide by any additional guidelines set by their local authority or school governing body.

Media recipes

Photobacterium medium

- Seawater aquarium salt 33 g
- Yeast extract 5 g
- Tryptone 5 g
- Glycerol 3 g
- Tris 6 g
- NH_4Cl 5 g
- Distilled water 1 l

For a solid medium add:

- $CaCO_3$ 1 g
- Agar 15–20 g

Adjust to pH 7.2–7.5.

Seawater medium

- Yeast extract 10 g
- Malt extract 4 g
- Glucose 4 g
- Seawater* 750 ml
- Distilled water 250 ml

For a solid medium add 15–20 g agar. Adjust to pH 7.5.

** Natural sea water must be stored in the dark for at least three weeks to 'age'. If natural sea water is not available use artificial sea water.*

Artificial seawater

- NaCl 28.13 g
- KCl 0.77 g
- $CaCl_2.2H_2O$ 1.60 g
- $MgCl_2.6H_2O$ 4.80 g
- $NaHCO_3$ 0.11 g
- $MgSO_4.7H_2O$ 3.50 g
- Distilled water 1 l

All culture media should be autoclaved at 121℃ for 15–20 minutes before use.

Preparation

Slope cultures of *P. phosphoreum* are able to survive prolonged storage at 4 °C. However, such cultures will need to be 'revived' by transferring them onto fresh slopes or plates before they are suitable use by a class. This should be done 2–3 days before they are required.

McCartney bottles of broth may be prepared and autoclaved in advance and kept until they are needed.

Timing

Only actively-growing cultures will emit light. Maximum luminescence usually develops 18–24 hours after inoculation.

Troubleshooting

Cultures must be grown on *Photobacterium* medium if bright illumination is to be observed. Only cultures in the 'log phase' of growth will produce light (see Figure 4).

Photography

Photographs of plates or flasks inoculated with luminous bacteria can be taken using monochrome 125 ISO film at f/2.0 for 100 seconds. The light produced by small volumes of cultures cannot usually be detected by a digital still or video camera, although it *might* be possible to photograph greater volumes using such equipment.

Further investigations

1 **Effect of temperature on luminescence**

Cultures in McCartney bottles may be placed in water baths at a range of temperatures (*e.g.*, 0–60 °C) to investigate the effect of temperature on generation of light. The proteins required for luminescence in *P. phosphoreum* and *V. fischeri* are denatured at 37 °C, and whereas *P. phosphoreum* will grow at 4 °C, *V. fischeri* will not. Remember that bioluminescence is an enzyme-catalysed process. Unlike fluorescence (*e.g.*, of green fluorescent protein, GFP) a product does not accumulate inside the cells, so luminescence will only be seen in living cultures.

2 **Effect of oxygen concentration**

Oxygen is required for light-generation, hence in a tube of bacterial culture that has been left to stand, luminescence will only be observed at the interface of the air and the liquid.

Methylene blue solution is blue when oxidised and colourless when reduced. It may therefore be used to monitor oxygen concentration within a culture. To do this, add 0.5 ml of 0.01% aqueous methylene blue to a 15 ml culture of luminous bacteria in a McCartney bottle. Allow the tube to stand for about 20 minutes, or until luminescence is only observed at the top of the liquid. It will be noticed that light production is restricted to the blue, oxygenated zone while in the liquid beneath, where bacterial respiration has depleted the oxygen, the methylene blue is colourless.

3 Antibiotic action

Vibrio is Gram-negative and *P. phosphoreum* and *V. fischeri* are sensitive to a range of antibiotics. This sensitivity may be tested by placing paper discs (*e.g., Oxoid Multodiscs*) that have been impregnated with antibiotic onto the surface of plates spread with bacteria. Dark zones around the discs indicate areas where bacteria have not grown.

4 Effect of pollutants

P. phosphoreum is highly-sensitive to environmental pollutants and is often used to detect their presence. The effect of metal ions on luminesence can be investigated. Suitable metal ions and concentrations include: lead (1 mg/l); copper (1 mg/l); cobalt (3 mg/l) or manganese (5 mg/l).

A spectacular demonstration of the effect of chemicals such as bleach may be performed by adding a few drops to a culture of luminescing bacteria. The 'lights go out' almost immediately!

Isolation of luminous bacteria from fish or squid

Fish enrichment medium

- Boil 250 g fish meat in 1 l of water.
- Add 30 g NaCl and sieve to remove solids.
- Add 10 g peptone, 10 ml glycerol and 1 g yeast extract.
- Adjust the pH to 7.
- Autoclave at 121 ℃ for 15–20 minutes.

For a solid medium add 15–20 g of agar to every litre of broth.

Safety note

Several species of *Vibrio* are pathogenic. The chances of inadvertently isolating pathogens in this procedure can be reduced by using at least 3% salt solution and incubating fish and plates at no more than 15 ℃. Human pathogens are unlikely to grow under such conditions.

P. phosphoreum is one of the commonest spoilage organisms of fish such as cod. It is not known to cause disease, but there are reports of people being startled by glowing fish fingers in the fridge!

To isolate glowing bacteria from fish, obtain a freshly-caught seafish or squid. It is very important that the fish has not been frozen or washed in fresh water. It is also better if the fish has not been stored on ice. Place the fish in a container with 3% NaCl solution. The liquid should be deep enough to come half way up the fish.

Cover the container and store the fish for 24 hours at about 12–15 ℃. Note: if this temperature is difficult to achieve, place the fish in a fridge at about 4 ℃ for 48–72 hours.

After incubation, take the container with the fish into a dark room. When your eyes have been adopted to dark, light spots will be visible on the skin of the fish. Use a sterile toothpick or disposable sterile loop or needle to aseptically transfer the brightest spots onto sterile fish enrichment agar plates. Tip: some people find it useful to use a dim red lamp (*e.g.*, a photographic safety lamp) for this step. Turn the fish away from the lamp so that the glowing colonies are in the shade and therefore visible.

Transfer the cultures to new agar plates every second day if you are incubating them at temperatures around 12 ℃ or once a week if you are storing them in a fridge. *P. phosphoreum* will grow at 4 ℃; *V. fischeri* will not. By selecting the brightest colonies when inoculating, it should be possible to isolate a pure culture.

Suppliers

Lyophilised cultures of *Photobacterium phosphoreum* may be obtained from culture collections such as the UK National Collections of Industrial, Marine and Food Bacteria or the German Collection of Microorganisms and Cells. Several school science suppliers (*e.g.*, Blades Biological) provide suitable slope cultures at lower cost. A practical kit for cultivating *P. phosphoreum* is available from Philip Harris Education in the UK ('Bioluminescence kit', Cat. No. A02264); Carolina Biological in the USA sells a kit for cultivating *V. fischeri* ('Bioluminescent bacterium kit', Cat. No. BA-15-4750).

References

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2 US Naval Oceanographic Office. Bioluminescence Survey Systems http://www.navo.navy.mil/biolum/blwebpge.htm

3 Azur Environmental Toxicity Testing http://www.azurenv.com

4 Cambridge Research and Technology Transfer Limited http://www.lumiweb.com

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Further reading

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Salmond, G.P.C. *et al* (1995) The bacterial 'enigma': cracking the code of cell–cell communication. *Molecular Microbiology* 16 (**4**) 615–624.

Losick, R. and Kaiser, D. (1997) Why and how bacteria communicate. *Scientific American* 276 (**2**) 68–73.

Web sites

Luxgene.com — Glow-in-the-dark organisms
http://www.luxgene.com

Luminescent bacteria
http://www.biology.pl/bakterie_sw/index_en.html

HBOI — More about bioluminescence
http://www.biolum.org/

Marine bioluminescence page
http://lifesci.ucsb.edu/~biolum/

Scripps bioluminescence page
http://siobiolum.ucsd.edu/Biolum_intro.html

Acknowledgements

Thanks to Pat C. Hickey of the Fungal Cell Biology Group at the University of Edinburgh for the photograph of *Photobacterium phosphoreum*. The cartoon (Figure 5) was originally drawn by Paul Stevens for the *NCBE Newsletter* in 1990. John Richardson of the Scottish Schools Equipment Research Centre and John Grainger of The University of Reading advised on the safety and practicality of the bacterial isolation procedure. Enevold Fahlsen from Göteborg University also gave expert advice about safety questions.

The Society for General Microbiology kindly permitted the reproduction of C.M. Turley's 1982 educational article on bacterial luminescence (see link in 'Further reading').

