Invited paper

How do marine bacteria produce light, why are they luminescent, and can we employ bacterial bioluminescence in aquatic biotechnology?*

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Grzegorz Węgrzyn[1,2]
Agata Czyż[3]

[1] Department of Molecular Biology,
University of Gdańsk,
Kładki 24, PL–80–822 Gdańsk, Poland;
e-mail: wegrzyn@biotech.univ.gda.pl

[2] Institute of Oceanology,
Polish Academy of Sciences,
Św. Wojciecha 5, PL–81–347 Gdynia, Poland

[3] Laboratory of Molecular Biology (affiliated with the University of Gdańsk),
Institute of Biochemistry and Biophysics,
Polish Academy of Sciences,
Kładki 24, PL–80–822 Gdańsk, Poland;
e-mail: czyz@biotech.univ.gda.pl

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Abstract

Bioluminescence, the phenomenon of light production by living organisms, occurs in forms of life as various as bacteria, fungi and animals. Nevertheless, light-emitting

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bacteria are the most abundant and widespread of luminescent organisms. Interestingly, most species of such bacteria live in marine environments. In this article, the biochemical mechanism of bacterial luminescence and its genetic regulation are summarized. Although the biochemistry and genetics of light emission by cells have been investigated in detail, the biological role of bacterial luminescence has remained obscure. Here, we discuss recent discoveries that shed new light on this problem. Finally, we provide examples of how bacterial luminescence can be employed in marine biotechnology, especially in the detection of toxic and mutagenic pollution in aquatic environments.

1. Introduction

Bioluminescence is the process by which living organisms emit light. This phenomenon occurs in many species of bacteria, fungi and animals (both invertebrates and vertebrates). The mechanisms of luminescence in all of these groups of organisms are generally similar, though some details (like the substrates for the chemical reaction) may vary considerably among different species. Interestingly, it seems that bioluminescence has appeared several times independently during the evolution of life forms (Rees et al. 1998).

Bacterial bioluminescence occurs mainly (though not exclusively) in species living in marine environments (Nealson 1978). Importantly, light-emitting bacteria are the most abundant and widespread of luminescent organisms (Meighen 1994). Two bacterial species able to emit light, *Vibrio fischeri* and *Vibrio harveyi*, have been investigated intensively, and most of this article will concern these bacteria. *V. fischeri* is a symbiotic bacterium living in the light organs of fish of the family Monocentridae and of the cephalopods *Sepiola* and *Euprymna* (Fitzgerald 1977, Ruby 1996). *V. harveyi* is a free-living bacterium, though it may also be found occasionally on the surface of marine animals or in their gut (Baumann et al. 1973, Ruby & Morin 1979).

Since the biochemical mechanism of bacterial luminescence and the genetic regulation of this process have recently been reviewed by others (Bassler & Silverman 1995, Rees et al. 1998, Swift et al. 1998, Winans & Bassler 2002), in this article we will summarize briefly our current knowledge about the biochemistry and genetics of light emission by bacteria. We will then focus on the biological role of bacterial luminescence and possible evolutionary drives in the early stages of its development. Finally, we will discuss possible applications of luminescent bacteria in aquatic biotechnology.

2. Biochemical mechanism of bacterial bioluminescence

The reaction of bacterial luminescence is catalyzed by luciferase, an enzyme composed of two subunits, called α and β (Belas et al. 1982). The luciferase substrates are long-chain aldehydes and $FMNH_2$. The reaction leads to the oxidation of $FMNH_2$ to FMN and the oxidation of the aldehydes to organic (fatty) acids. A quantum of light is an additional product of this reaction, which can be summarized as follows:

$$RCHO + FMNH_2 + O_2 \rightarrow RCOOH + FMN + H_2O + h\nu$$

The fatty acids produced in the reaction catalyzed by luciferase are subsequently reduced to aldehydes by a specific reductase. In the same reaction, $NADPH + H^+$ is converted to $NADP^+$ and ATP is hydrolyzed to ADP (Ziegler & Baldwin 1981). $FMNH_2$, which is necessary for the luminescence reaction (see above), is generated from FMN by NAD(P)H–FMN oxidoreductase (Jabłoński & DeLuca 1978).

As can be deduced from the above description, bioluminescence is an energy-consuming reaction. In fact, for light emission bacteria may use up to 20% of the total cellular energy (Nealson & Hastings 1979, Bassler & Silverman 1995).

3. Genetic regulation of bacterial bioluminescence

There are several bacterial genes involved in bioluminescence and its regulation. The two subunits of luciferase, α and β, are encoded by genes *luxA* and *luxB*, respectively (Belas et al. 1982). In both *V. fischeri* and *V. harveyi* these genes are organized in an operon together with other genes involved in the bioluminescence reaction. In *V. fischeri* this operon consists of the *luxI*, *luxC*, *luxD*, *luxA*, *luxB*, *luxE* and *luxG* genes (the *luxI* is the most proximal gene to the promoter, *luxG* the most distal). In *V. harveyi* the *lux* operon is organized in a similar way to that of *V. fischeri*, but *luxI* is absent and *luxG* is followed by the *luxH* gene. *LuxC*, *luxD* and *luxE* code for proteins that form a complex of the fatty acid reductase. The products of genes *luxG* and *luxH* are responsible for the synthesis of the reduced flavine (Meighen 1994).

Expression of the *lux* operon in both *V. fischeri* and *V. harveyi* undergoes a specific regulation called quorum sensing (for detailed reviews see Swift et al. 1998, Winans & Bassler 2002). As a result of this mechanism, expression of the *lux* genes, and thus the efficiency of light emission, depends on the concentration of cells in the environment, that is, bacterial luminescence is effective when cells occur at a high density, whereas light emission is negligible in diluted cultures.

There are different mechanisms of the *lux* operon expression regulation by quorum sensing of *V. fischeri* and *V. harveyi*. In *V. fischeri*, the *luxI* gene codes for the enzyme responsible for the synthesis of N–(3–oxyhexanol)–L–homoserine lactone, which acts as an autoinducer interacting with the *luxR* gene product. The LuxR protein is a repressor of the promoter of the *lux* operon, and interaction of this protein with the autoinducer results in de-repression of this promoter (Meighen 1994, Bassler & Silverman 1995). Since the autoinducer is excreted from cells, it acts on other bacteria in the culture. Therefore, the denser the culture, the more efficient the expression of the *lux* operon, and the bacteria produce more light.

The *lux* regulatory system in *V. harveyi* seems to be more complicated than in *V. fischeri*, though recent studies have indicated that in the latter species additional regulatory mechanisms may also occur (Miyamoto et al. 2000). Apart from the *luxCDABEGH* operon, *V. harveyi* contains several additional genes involved in the regulation of bioluminescence. These are the regulatory genes *luxR*, *luxO* and *luxU*, genes coding for two autoinducer synthetases (*luxL* and *luxM* coding for the synthetase of an autoinducer called AI–1, *luxS* coding for the synthetase of the autoinducer AI–2), and genes coding for sensors of the autoinducers *luxN* (AI–1 sensor) and *luxP* and *luxQ* (AI–2 sensor) (Bassler et al. 1994, Freeman & Bassler 1999). The *luxR* gene product is an activator of the *luxCDABEGH* operon (note that this protein reveals no homology to LuxR of *V. fischeri*) (Chatterjee et al. 1996, Miyamoto et al. 1996). The negative regulator of this operon is the LuxO protein (Bassler et al. 1994). The sensory proteins LuxN and luxPQ are responsible for detecting autoinducers AI–1 and AI–2, respectively, and the subsequent signal transduction, mediated by the LuxU protein and based on phosphorylation and dephosphorylation reactions, leads to the inactivation of LuxO (Freeman & Bassler 1999, Freeman et al. 2000) and the subsequent stimulation of the expression of the *luxCDABEGH* operon. This leads to the efficient production of luciferase and other enzymes necessary for the luminescence reaction.

4. Why do bacteria emit light?

There are many bacterial species that are able to emit light. However, although the genetics and biochemistry of bacterial luminescence have been investigated extensively, the biological role of this phenomenon has until recently remained unclear. Luminescence occurs in symbiotic, saprophytic, parasitic, as well as in free-living bacteria (Meighen 1994). The ecological benefit for a fish or squid living in a symbiotic association with luminescent bacteria has been established (Morin et al. 1975, Nealson & Hastings 1979).

The host organism can use the light emitted by bacteria to attract prey, escape from predators or for communication. However, it is not understood what specific benefit symbiotic bacteria derive from producing light. Although one could imagine some advantages for bacteria living in the light organs of animals, it seems unlikely that the establishment of such a symbiosis could have been the main evolutionary drive to develop very complicated light-emitting systems. The biological role of luminescence in free-living bacteria remains even more mysterious. This is because such bacteria are able to produce light but cannot sense this signal. On the other hand, it is obvious that luminescence must have a positive selective value, since about 20 percent of the bacterial cell energy is consumed by this process (Makemson 1986, Bassler & Silverman 1995).

Recent studies of *V. harveyi* have led to possible explanations of the mystery of the biological function of bacterial luminescence (Czyż et al. 2000b). The starting point of these studies was the random mutagenesis of *V. harveyi* and the isolation of many UV-sensitive mutants. Surprisingly, most of these mutants have also lost the ability to emit light. It seemed unlikely that these two phenotypes of randomly isolated mutants were simply coincidental.

One of the possible interpretations of these results, indicating an unexpectedly large proportion of non-luminescent mutants among UV-sensitive bacteria, was that dark mutants of *V. harveyi* may be defective in repairing DNA lesions caused by UV light. To test this hypothesis, the survival of UV-irradiated *V. harveyi* cells subsequently grown in the dark or in the presence of external light was investigated (this type of experiment is commonly used to investigate the efficiency of photoreactivation, a process of DNA repair by photolyase). It was found that UV-mediated killing of luminescent *V. harveyi* cells was somewhat more effective when bacteria were cultivated in the dark following irradiation than when the cultivation was performed in the presence of external light. However, cell survival was significantly less efficient when UV-irradiated *luxA* or *luxB* mutants were cultivated in the dark. These results suggest that luminescence may serve as an internal source of light used in a photoreactivation-type reaction when bacteria grow in the dark.

To test the hypothesis regarding the stimulation of DNA repair by bioluminescence, plasmids bearing the *V. harveyi luxCDABE* operon and *luxR* gene were introduced into cells of the non-luminescent bacterium *Escherichia coli*. It was then demonstrated that the survival efficiency of UV-irradiated *E. coli* wild-type (non-luminescent) cells is lower when bacteria are subsequently cultivated in the dark as against bacteria exposed to external light, whereas the survival efficiency of UV-irradiated *E. coli*

luminescent cells did not depend on the conditions of subsequent cultivation, in the dark or in the presence of external light. Moreover, luminescent *E. coli* cells were significantly less sensitive to UV irradiation than their non-luminescent counterparts. These results supported the hypothesis that luminescence may be an internal source of light used in DNA repair by photoreactivation.

It is interesting that the luminescent bacterium *V. harveyi* is generally more sensitive to UV-irradiation than *E. coli* (Czyż et al. 2000b). This suggests that DNA repair systems other than photoreactivation are less effective in *V. harveyi*. Therefore, one might speculate that this bacterium (and possibly other luminescent bacteria) had to develop a mechanism ensuring that at least one DNA repair system (e.g. photoreactivation) is efficient irrespective of the external conditions.

It was suspected that the quorum sensing mechanism could render acceptance of the above hypothesis problematic. Namely, if *V. harveyi* were able to emit light only at a high cell density irrespective of other environmental conditions, the hypothesis would seem rather unlikely, as mechanisms ensuring efficient DNA repair should also operate at a low cell density. However, it was found that while light emission by *V. harveyi* cells growing at a low density is negligible relative to high cell density conditions or immediately after dilution of a culture, UV irradiation of cells at a low density caused transient but efficient induction of light emission (Czyż et al. 2000b). Most probably, this was caused by inactivation of the LexA repressor and the resultant induction of the SOS response. In fact, when studied in *E. coli* cells, repression of the *lux* operons from *V. fischeri* and *V. harveyi* by the product of the *lexA* gene has been reported (Ulitzur 1989, Shadel et al. 1990, Czyż et al. 2000b).

Very recent studies have demonstrated that luminescence of six strains of marine bacteria (belonging to four species: *Photobacterium leiognathi*, *P. phosphoreum*, *V. fischeri* and *V. harveyi*) is significantly increased by UV irradiation relatively soon after dilution of cultures (Czyż et al. 2002a). Such stimulation of luminescence was suppressed in cells treated with chloramphenicol before UV irradiation, indicating that effective gene expression is necessary for UV-mediated induction of light emission. These results suggest that stimulation of luminescence in UV-irradiated bacterial cells may operate independently of the quorum sensing regulation. Such a double regulation of the expression of bacterial *lux* genes may arise from the presence of two independent promoters, one being regulated by quorum sensing and the other belonging to the SOS regulon. In fact, the presence of two promoters in the *lux* operon of *V. fischeri* has already been demonstrated

(Ulitzur et al. 1997, Ulitzur 1998a). Of these two promoters, only one is regulated by the quorum sensing mechanism (Ulitzur 1998b).

A significant induction of luminescence was also observed upon treatment of diluted cultures of *P. leiognathi*, *P. phosphoreum*, *V. fischeri* and *V. harveyi* strains with chemical mutagens (Czyż et al. 2002a). These results support the proposal that the genes involved in bioluminescence belong to the SOS regulon.

The question remains whether quorum sensing regulation of bacterial luminescence is a kind of a 'social behaviour' in which cells 'predict' the possibility of increased mutagenesis where conditions allow for efficient metabolism leading to extensive bacterial growth. Such conditions may cause the appearance of large quantities of metabolites, including mutagenic agents. Thus, one could speculate that enhanced light emission at a high cell density may ensure more efficient DNA repair in response to potentially increased concentrations of mutagens. Alternatively, quorum sensing may be needed for another role of bacterial bioluminescence, besides stimulation of photoreactivation.

Recent research has suggested that bacterial luminescence systems play a biological part unconnected with the visual behaviour of organisms. Studies of the structures and biochemical activities of various luciferases have led to the proposal that a primary role of luminescent systems could be to detoxify deleterious oxygen derivatives (Rees et al. 1998). If this hypothesis were true, one could assume that bacterial luciferase precursors and perhaps also present-day enzymes could be involved in the detoxification of the toxic metabolites (e.g. H_2O_2, aldehydes) generated when cells are subjected to oxidative stress. Therefore, one could ask whether *V. harveyi* luciferase is involved in the protection of bacterial cells against oxidative stress. To address this problem, the sensitivity of wild-type bacteria and otherwise isogenic *luxA* and *luxB* mutants to H_2O_2 was investigated. Preliminary experiments demonstrated that *V. harveyi* *luxA* and *luxB* mutants are more sensitive to H_2O_2 than the wild-type, luminescent, bacteria (Czyż & Węgrzyn 2001a).

To answer the question whether the increased sensitivity of the *luxA* and *luxB* mutants to H_2O_2 results from the loss of luminescence or the loss of the luciferase enzyme itself, the experiments described above were repeated using the *luxD* mutant and an otherwise isogenic wild-type strain. The *luxD* gene codes for the acetyltransferase enzyme producing fatty acids for the luminescence reaction, thus *luxD* mutants are dark without affecting luciferase. No significant differences in the sensitivity of lux^+ and *luxD* cells to H_2O_2 were found (Czyż & Węgrzyn 2001a). Therefore, it seems that *V. harveyi* luciferase is involved in the detoxification of H_2O_2, thus playing

a part in protecting cells against oxidative stress. Nevertheless, it is clear that more detailed studies are necessary to confirm the hypothesis regarding the part played by bacterial luciferases in the detoxification of deleterious oxygen derivatives.

5. The problem of the early evolution of bioluminescence systems

The origin of bioluminescence is regarded as a problematic aspect of Darwin's theory of evolution (for recent discussions see Czyż & Węgrzyn 2001a, Labas et al. 2001). The problem is that the function of bioluminescence is generally believed to be directly associated with the visual behaviour of organisms. If this were true, there should be no benefit to organisms possessing weakly luminescent systems, i.e. emitting low amounts of light. Quite simply, for the development of an efficient system, there has to be positive environmental pressure to select individuals bearing more and more effective systems among population of organisms. However, if we consider that luminescence is advantageous to glowing organisms only when the emitted light is sufficiently intens to be visible, then the development of luminescence during evolution remains obscure, as it is hard to believe that early luminescent systems were as efficient as currently existing ones, or that they appeared suddenly in their present forms. Thus, early luminescence systems of low efficiency had to evolve in order to produce enzymes and substrates allowing highly efficient reactions that produce light easily detectable by the optic organs of other organisms. But how to imagine an evolutionary process based on the selection of organisms producing light more effectively than other individuals at a stage when the intensity of light emitted during bioluminescence was far below that capable of being seen by the naked eye? The question is, therefore, what was the evolutionary drive that led to the establishment of weakly luminescent systems and their further improvement?

Recent studies of the biological role of bacterial luminescence, discussed in the preceding section, have shed new light on the problem of the early evolution of luminescence systems. As described above, bioluminescence can stimulate DNA repair by activating the photoreactivation reaction (Czyż et al. 2000b). Moreover, this stimulation is effective even when luminescence is several hundred times less intense than that observed in wild-type *V. harveyi*, as demonstrated in experiments with *E. coli* cells bearing the *luxCDABE* operon and the *luxR* gene (Czyż et al. 2000b). Such *E. coli* cells produce light invisible to the naked eye, but this luminescence is of an intensity that is still sufficient to stimulate effective DNA repair. Therefore, it seems plausible that stimulation of DNA repair could have been

an evolutionary drive for bacterial luminescence. When the light emission of the early luminescent bacteria was very weak, more luminescent cells could repair DNA more effectively. This mechanism could have operated even when the emitted light was still invisible to the naked eye. Thus, weakly luminescent bacteria might prevail in competition with cells producing light less effectively in environments endangered by mutagenic factors like UV irradiation.

Very recent results from our laboratory (Czyż & Węgrzyn, submitted) suggest that bioluminescence provides no advantage to bacteria under non-stress conditions. When wild-type luminescent bacteria (a wild-type *V. harveyi* strain) were cultured together with otherwise isogenic *luxA* mutants under standard laboratory conditions, the *luxA* mutants became absolutely predominant over wild-type cells in such mixed cultures. This predominance might be due to the consumption of a significant portion of cell energy for light emission by wild-type bacteria that resulted in their slower growth relative to dark mutants, thus *luxA* cells prevailed. However, when the mixed cultures were irradiated with UV light, luminescent bacteria dominated over dark mutants. This may well have been because wild-type cells were able to repair DNA more efficiently in the absence of external light and compete more effectively with the *luxA* mutants.

The results described above (Czyż & Węgrzyn, submitted) may support the hypothesis that stimulation of DNA repair could have been an early evolutionary drive of bacterial bioluminescence. This mechanism could have operated, especially at stages when the efficiency of luminescent systems was yet too weak to produce light detectable by the naked eye but good enough to stimulate photoreactivation. It should also be noted that a similar mechanism could govern another process stimulated by the activity of luciferase, i.e. the detoxification of deleterious oxygen derivatives (see the preceding section). Finally, one might speculate that after the appearance of improved luminescent systems capable of producing light sensed by animals, other evolutionary drives started to operate which led to the establishment of the symbiosis between luminescent bacteria and fish or cephalopods. Nevertheless, stimulation of DNA repair and detoxification of deleterious oxygen derivatives may still be important roles played by bioluminescence in present-day free-living bacteria.

6. Using luminescent marine bacteria in aquatic biotechnology

As mentioned above, light-emitting bacteria are the most abundant and widespread of luminescent organisms, and most luminescent bacterial species live in marine habitats. We can, therefore, ask whether bacterial

luminescence is just an interesting phenomenon, or whether it has potential biotechnological applications.

The use of bioluminescent bacteria in the detection of toxic chemicals was proposed over 20 years ago (Ulitzur et al. 1980, Bulich & Isenberg 1981), and various assays have been described since then (see for example: Thomulka & Lange 1995, 1996, Lange & Thomulka 1997). In those studies, the toxicity of different chemicals was determined by a relatively simple method based on measuring the decrease in bioluminescence following the addition of toxic compounds. These bioluminescence assays are simple and can be useful in the detection of toxic substances. However, mutagens occurring in natural habitats at concentrations too low to provoke serious toxic effects in bacterial cells cannot be detected using this technique.

In several mutagenicity tests, fusions consisting of *lux* operons under the control of one of the LexA-repressed promoters are used (Bar & Ulitzur 1994, Ptitsyn et al. 1997, van der Lelie et al. 1997, Ben-Israel et al. 1998, Min et al. 1999, Verschaeve et al. 1999). Strains containing such fusions emit light upon contact with SOS response-inducing agents (due to cleavage of the LexA repressor), which is a sensitive and quick indication of the presence of mutagenic compounds in the tested sample. However, these fusions were constructed in *Escherichia coli* or *Salmonella enterica* serovar Typhimurium, which may be a disadvantage in direct testing of samples from certain marine habitats. These bacteria normally live in completely different environments and the addition of sea water samples to their cultures might induce a stress response *per se*. Moreover, the survival of *E. coli* and *S. enterica* in sea water is severely impaired relative to that of marine bacteria (Czyż et al. 2002b). Therefore, for monitoring marine habitats, an organism that naturally lives in these habitats should be more useful.

A very useful group of genotoxicity tests employing light emission by marine bacteria is that based on the detection of luminescence restoration in dark mutants, including the commercially available Mutatox test (Ulitzur et al. 1980, Ben-Itzhak et al. 1985, Levi et al. 1986, Ulitzur & Barak 1988, Sun & Stahr 1993). These assays are often very sensitive, and their usefulness has been demonstrated in environmental studies (Brenner et al. 1993a, b, 1994, Belkin et al. 1994).

Recently, a sensitive mutagenicity assay based on the marine luminescent bacterium *V. harveyi* (though not employing light emission in measurements) has been developed (Czyż et al. 2000a). In this assay, a series of genetically modified strains of *V. harveyi* is used (these strains have been characterized recently; see Czyż et al. 2001b, Dutkiewicz et al. 2002, Sikora-Borgula et al. 2002, Słomińska et al. 2002). The assay is simple, as

it is based on the positive selection for neomycin-resistant mutants that appear frequently upon contact with chemical mutagens. Recent analysis has shown, as expected, that *V. harveyi* survives well in sea water samples taken from different geographical regions. This, together with the fact that the assay is of a very high sensitivity (the assay was found to be at least several times more sensitive than the commonly used Ames test; Czyż et al. 2002b), makes it potentially useful in the detection of mutagenic pollution in marine environments.

Finally, since the heat shock response of *V. harveyi* has been characterized, it has been suggested that the heat shock genes of this bacterium, or their regulatory elements, might be used to design assays for detecting different stress conditions in marine habitats (Klein et al. 1995, 1998). Although no such assays have yet been developed, this idea could turn out to be useful in the near future.

7. Concluding remarks

Marine luminescent bacteria make up the largest fraction of light-emitting organisms. The biochemical reactions leading to light emission by bacteria have been examined in great detail. The genetic regulation of the efficiency of bacterial luminescence is a fascinating process, and provides an example of how bacterial cells can communicate with each other (the quorum sensing mechanism). Recent studies have shed new light on a problem that has long remained unsolved, i.e. the biological role of bacterial luminescence. It seems that bacteria emit light to stimulate DNA repair. Moreover, luciferase activity may be important in the detoxification of deleterious oxygen derivatives. The discovery of the biological roles of luminescence may, in turn, help us to understand the early stages in the evolution of this process, a problematic aspect of the Darwinian theory. Finally, apart from being models in basic research, luminescent marine bacteria have potential biotechnological applications, mainly in the detection of mutagenic and toxic compounds in marine environments.

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