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# ISOLATION AND CHARACTERIZATION OF BIOLUMINESCENT BACTERIA ASSOCIATED WITH *UROTEUTHIS DUVAUCELLI*, AN INDIAN SQUID FROM ANDAMAN WATERS

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**ABSTRACT**
Bioluminescence is a natural phenomenon of light emission produced by metabolic enzymatic reactions of the living organisms. Luminous bacteria, found either as free-living or symbiont in host, are the major light emitters widely distributed in the oceanic realms. These bacterial species emit blue-green light at 490 nm by the activity of the enzyme luciferase and regulated by the lux gene operon. Some of the key and vital applications of the bioluminescence are as genetic reporters, markers and biosensors. Bioluminescent bacteria from the squid, *Uroteuthis duvaucelli* were isolated by following standard microbiological techniques. Characterization of 7 isolates was conducted by routine biochemical tests and further analysis was carried out at molecular level using lux gene and multiplex PCR for species confirmation.

**KEY WORDS:** Squids, *Uroteuthis duvaucelli*, Bioluminescence, lux gene, Multiplex PCR.

## INTRODUCTION

Oceans are the largest ecosystems covering two third of the earth's surface, a multifactorial driven ecosystem consisting of 80% earth life forms, remains largely unexplored (Das *et al.,* 2006). The oceanic waters comprises dominant marine microbiota with marked diversities of various group of organisms such as bacteria, fungi, protozoa and viruses involved in recycling and transfer of nutrients among the different tropic levels. Directly or indirectly, these microbes influence and shape the biosphere regulating the ecological and biogeochemical mechanisms (Aylward *et al.,* 2015). Significantly, marine bacterial community possesses huge potentiality to produce diversely amazing traits such as production of pigments, secondary metabolites, emission of fluorescence and bioluminescence.

Bioluminescence is the ability of organisms to emit light at visible range that is produced by natural chemical reaction of enzymatic activity and molecular biochemicals of living organisms. The word "bioluminescence" is derived from the Greek word (*bios* = living) and Latin word (*lumen* = light) meaning "living light". It is attributed that it serves as primary source of light beyond the photic oceanic zone (Haddock *et al.,* 2010). The biological significance of bioluminescence mainly underlines to support interspecific and intraspecific cell signaling, attracting prey, camouflage, deterring predators and in aiding hunting (Dunlap and Urbanczyk, 2013). Bacterial bioluminescence has been widely studied and applied in several biotechnological applications. In the entire bacterial kingdom, this phenomenon is observed only in class Gammaproteobacteria which are luminescent heterotrophs, dominant in abundance than biomass in comparison with other luminous organisms (Widder, 2010). All luminescent bacteria, are grouped into 5 genera- *Aliivibrio, Photobacterium, Photorhabdus, Shewanella* and *Vibrio* which are Gram negative, facultative anaerobes.

The principle of bioluminescence involves oxidation of long chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$), catalyzed by the enzyme luciferase, in the presence of oxygen. The mechanism involves liberation and emission of excess energy as luminescent blue-green light at 490 nm, as depicted in the following reaction:

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{Luciferase}} FMN + H_2O + RCOOH + h\nu\ (490\ nm)$$

The light emission process is mediated and guided by a group of genes known as *lux* operon which consists of LuxA, LuxB and LuxCDE. Gene LuxA and LuxB codes for α and β subunits, respectively while LuxCDE is responsible for enzyme fatty acid reductase. Also, the emission of luminescence also depends on a phenomenon called "Quorum sensing" regulated by five core genes LuxCDABE on the lux operon (Dunlap and Urbanczyk, 2013). These genes are being widely used as "reporter genes" in practical applications as they possess extensive biological importance as biosensors to detect several toxins, metals and organic compounds and biomolecules at micro levels, especially in the detection of infectious diseases by in-vivo bioluminescence scanning and imaging (Baban *et al.,* 2012 and Roura *et al.,* 2013).

In the present study, an attempt to isolate and identify bioluminescent bacteria associated with the deep water squid (*Uroteuthis duvaucelli*) from the deeper waters of Andaman archipelagoes has been attempted.

## MATERIALS AND METHODS

### Isolation of bacteria

Squid (*Uroteuthis duvaucelli*) was collected from the Junglighat fish landing centre a major fish landing center (Fig. 1) and was transferred to an ice box and transported to the laboratory for further analysis. They were washed with sterile sea water to remove loosely bounded organisms. The body surface was swabbed, inoculated on to freshly prepared Zobell Marine Agar, Photobacterium Agar, Luminous Agar plates and incubated at 20°C for 48 hours (Reichelt *et al*., 1973). For isolation of gut microflora, the intestine was dissected and homogenized with 1ml Phosphate Buffer Saline (PBS) in a mortar pestle. The homogenized tissue was serially diluted in sterile sea water and 100 µl from each dilution was plated on to freshly prepared media and incubated at 20°C for 48 hours. After 48 hours, the plates were observed in the dark room for the growth of luminescent colonies. Identified luminescent colonies were isolated and subcultured to obtain pure colonies and to observe luminescence. The working cultures of isolates were maintained at 4°C for further characterization.

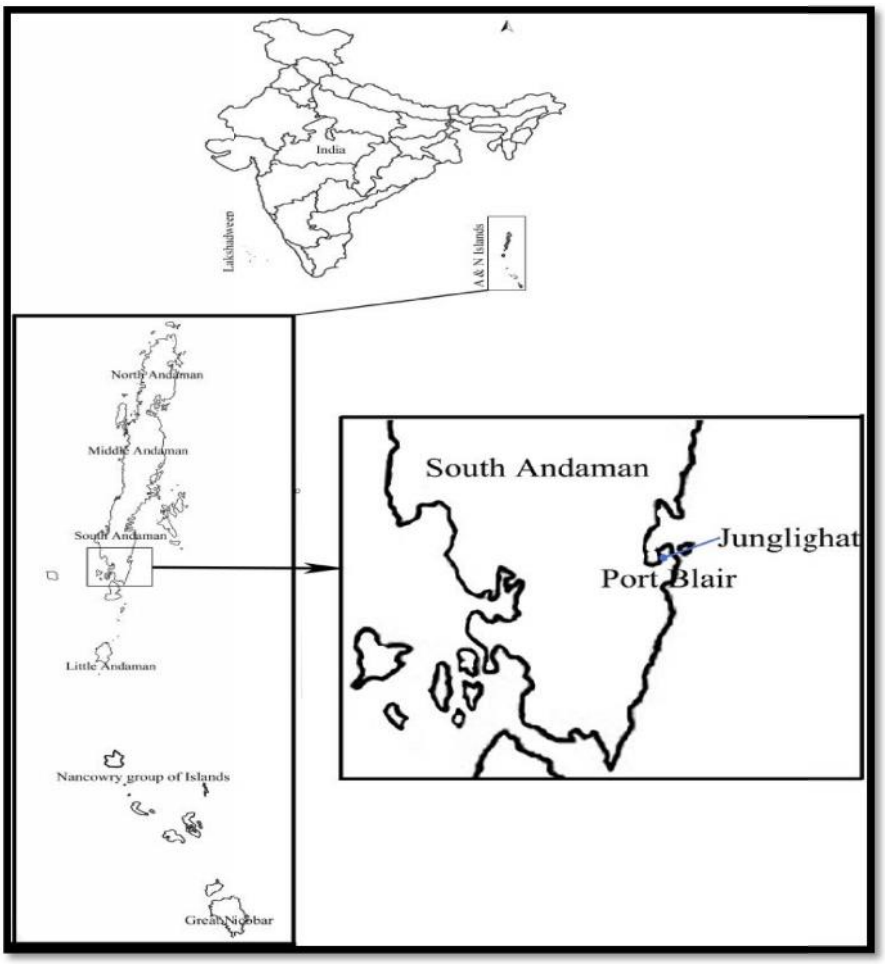



**FIGURE 1**: Map showing study area Junglighat (11°39'33.6"N, 92°43'41.8"E)

### Phenotypic characterization

On the basis of various biochemical tests, phenotypic characterization was employed for the identification of bacteria. Isolates obtained from the squid were subjected to various biochemical substrates. These biochemical tests were performed according to the procedures described by Whitman (2004) and Benson (2001). The tests includes Gram staining, oxidase test, catalase test, production of indole and Hydrogen sulphide, Motility test, Amino acid decarboxylation for lysine, ornithine and arginine, gelatinase test, Citrate utilization test, Voges – Proskauer test. Sugar fermentation Tests for the acid production from sugars such as sucrose, glucose, D-sorbitol, mannitol, mannose, lactose, inositol, maltose, galactose, trehalose, rhemnose, melibiose, arabinose and xylose. Salt Tolerance were done in Nutrient broth supplemented with 0%, 1%, 3%, 6%, 8% and 10% sodium chloride (NaCl) Temperature tolerance was carried out by incubating the culture broth at 16°C, 18°C, 20°C, 25°C, 30°C, 35°C, 38°C and 40°C. Results were interpreted using the software Identax Bacterial Identifier Version 1.2 (Flores *et al*., 2009) and ABIS online software.

### Genotypic characterization

Isolation of Genomic DNA was carried out according to CTAB method as described by Nishiguchi *et al*. (2002). Overnight grown bacterial cultures in Marine Broth were centrifuged at 8000 rpm for 10 min at 4°C in an Eppendorf Centrifuge 5427R (Germany). Approximately 500 mg of

the pellet was re-suspended in 1.5 ml extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 1 M NaCl and 1% CTAB). The extraction buffer was preheated at 60ºC for 60 min prior to treatment of the samples. The samples were well mixed and incubated at 65ºC for 60 min and centrifuged at 12,000 rpm for 10 min. The resulting supernatant was collected and 500 µl of phenol: chloroform: iso-amyl alcohol mixture (25:24:1) was added to it. The samples were centrifuged at 12000 rpm for 10 min. This step was repeated and the supernatant was collected to which 500µl chloroform was added and centrifuged at 12000 rpm for 10 min. Further, 100% chilled ethyl alcohol was added, centrifuged and washed with 70% ethyl alcohol and dried. The dried pellets were re-suspended in sterile double distilled water. The isolated DNA was run on 0.8% (w/v) agarose gel electrophoresis and visualized in a Gel Documentation System (BIOTOP Fluor Shot Ver. 1.5).

**Lux gene PCR amplification**

For Lux gene amplification, molecular analysis of LuxA 745 bp gene was carried out in accordance with Wimpee *et al*. (1991). PCR was carried out by using the corresponding forward [5'-CTA CTG GAT CAA ATG TCA AAA GGA CG-3'] and reverse [5'-TCA GAA CCG TTT GCT TCA AAA CC-3'] primers. The reaction mixture (total volume 25 µl) consists of 10X PCR buffer, 50 mM of $MgCl_2$, 10 µM DNTPs, 1 U of Taq polymerase, 10 µM of each forward and reverse oligonucleotide primers and 20 ng of genomic DNA. PCR amplification was performed using GeneAmp PCR system 2720-ThermoCycler (Applied Biosystems, CA, USA). The amplification conditions consists of an initial denaturation of 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 58°C for 30 sec, 72°C for 30 sec and final extension step of 72°C for 7 min. PCR products were further resolved and visualized in 1% agarose gel for 40 min at 90V. The gel was stained with Ethidium bromide and loaded with 10 µl of each sample, negative control (DNA of non-luminescent bacteria) and 1 kb ladder. The band pattern was observed and photographed in Gel documentation system.

**Multiplex PCR assay to identify *Vibrio campbelli, Vibrio harveyi* and *Vibrio parahaemolyticus***

To confirm the presence of *Vibrio* sp. *i.e. V. campbelli*, *V. harveyi* and *V. parahaemolyticus*, the isolates were subjected to PCR assay targeting hly genes of *V. campbelli* and *V. harveyi* and tlh gene of *V. parahaemolyticus* as described by Haldar *et al.* (2010). The assay was performed with total volume of 30 µl consisting of 3 µl of 10X buffer, 50 mM $MgCl_2$, 10 µM dNTPs mix, 5U/µl Taq polymerase and 10 µM of each of forward and reverse primers and 50 ng of sample DNA. Amplification conditions were: an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec and a final elongation step of 72°C for 5 min. The amplification corresponding to 328 bp, 454 bp and 695 bp are positive for *V. campbelli*, *V. harveyi* and *V. parahaemolyticus*, respectively.

## RESULTS

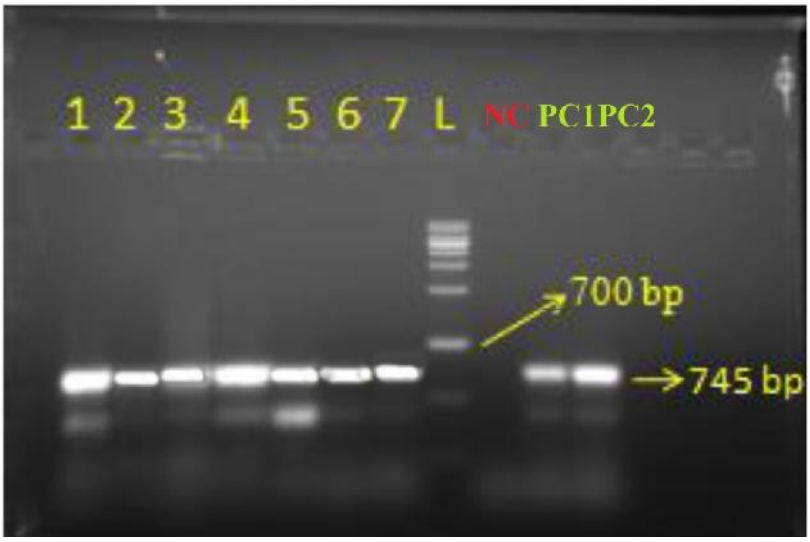
A total of 520 colonies (Zobell Marine Agar plates (420) followed by Photobacterium plates (96) and Luminous Agar (4)) were observed in all the plates together, out of which, 7 isolates showed bioluminescence. These 7 isolates were selected and subjected to various phenotypic and genotypic characterizations for their identification. All 7 strains were subjected to standard biochemical tests in which all were Gram negative, small comma shape except strain 2 which were small rods. Strains 2, 5, 6 and 7 were yellow in colour on TCBS agar while 1, 3 and 4 were green in colour. One of the major observations was that all strains showed growth at different range of temperatures from 16°C to 38°C. All 7 isolates exhibited luminescence in TCBS, Zobell Marine Agar and Photobacterium Agar, at both 20°C and room temperature (32°C). Based on these biochemical tests, all strains were identified upto the genus level as *Vibrio* and *Photobacterium* (Table 1).

**TABLE 1:** Biochemical test results

| **Morphology** | **S-1** | **S-2** | **S-3** | **S-4** | **S-5** | **S-6** | **S-7** |
|---|---|---|---|---|---|---|---|
| Gram staining | - | - | - | - | - | - | - |
| Shape | Comma | Rod | Comma | Comma | Comma | Comma | Comma |
| Motility | + | + | - | + | + | + | + |
| Growth in TCBS (Colony colour) | Green | Yellow | Green | Green | Yellow | Yellow | Yellow |
| **Biochemical Tests** | | | | | | | |
| Catalase | + | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + | + |
| Citrate | - | + | + | + | + | - | + |
| Urease | + | + | - | - | + | - | - |
| Indole | + | + | + | + | - | - | + |
| $H_2S$ | + | + | + | + | - | - | - |
| Methyl red | + | + | + | + | + | + | + |
| Vogus-Proskauer | - | + | - | - | - | - | - |
| Gelatinase | + | + | + | + | + | + | + |
| Starch hydrolysis | + | + | + | + | + | + | + |
| Arginine dihydrolysis | + | + | + | + | - | - | - |
| Lysine decarboxylase | + | + | - | - | - | - | - |
| Ornithine decarboxylase | + | - | - | + | + | + | + |
| Growth at 0% NaCl | + | + | + | + | + | + | + |
| Growth at 1% NaCl | + | + | + | + | + | + | + |
| Growth at 3% NaCl | + | + | + | + | + | + | + |
| Growth at 6% NaCl | + | + | + | + | - | - | + |
| Growth at 8% NaCl | + | + | + | + | - | - | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Growth at 10% NaCl | + | - | + | + | - | - | - |
| Growth at 16°C | + | + | + | + | + | + | + |
| Growth at 38°C | + | + | + | + | + | + | + |
| Luminescence | + | + | + | + | + | + | + |
| **Sugar fermentation** | | | | | | | |
| Sucrose | + | + | + | + | + | + | + |
| Dextrose | + | + | + | + | + | + | + |
| Lactose | + | + | + | + | + | + | + |
| Sorbitol | + | + | + | - | - | - | - |
| Mannitol | - | + | + | - | - | - | - |
| Mannose | + | + | + | + | + | + | + |
| Arabinose | + | + | - | + | - | - | - |
| Trehalose | + | + | + | + | + | + | + |
| Galactose | + | + | + | + | + | + | + |
| Melibiose | + | + | + | + | + | + | + |
| Maltose | + | + | + | + | + | - | - |
| **Species identified** | *Vibrio campbelli* | *Photobacterium* sp. | *Vibrio* sp. | *Vibrio parahaemolyticus* | *Vibrio* sp. | *Vibrio* sp. | *Vibrio* sp. |

+ means Positive, - means Negative




NC means negative control PC means positive control

**FIGURE 2:** Image showing the presence of LuxA gene in all 7 isolates at 745bp

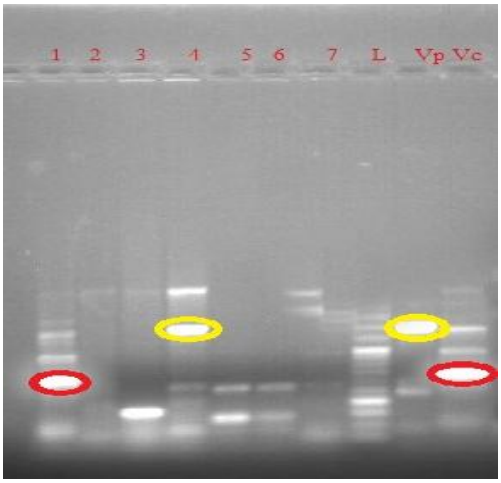



**FIGURE 3:** Multiplex PCR assay specific for *Vibrio campbelli* (328 bp) and *Vibrio parahaemolyticus* (645bp).

The genomic DNA extracted from the seven isolated strains was amplified for the presence of lux gene with a negative control. All strains showed the presence of luxA gene, establishing that all strains are of luminous bacteria (Fig. 2). All seven strains isolates of luminescent bacteria, when subjected to Multiplex PCR assay for identification and confirmation of species, the amplified DNA products of 328 bp, 454 bp and 645 bp correspond to *V. campbelli*, *V. harveyi* and *V. parahaemolyticus*, respectively. The image shows that strain 1 corresponds to *V. campbelli* at 328 bp and strain 4 resembles *V. parahaemolyticus* (Vp) at 645 bp (Fig. 3).

## DISCUSSION

Studies on symbiotic luminescent bacteria especially on *Euprymna scolopes* (Bobtail squid), have been carried out extensively around the world (Nishiguchi, 2002 and Guerrero-Ferreira, 2007). The two most common genus of bioluminescent bacteria are *Vibrio* and *Photobacterium* (Nishiguchi and Nair, 2003). Though, it is evident that *Photobacterium* sp. are most commonly associated with Loligonid squid genera - *Uroteuthis* and *Loliolus*, studies have not been carried out on the association of luminous bacteria with *Uroteuthis duvaucelli*, a member of *Uroteuthis* genera, from Andaman waters. Results from our study corresponds well to the studies of Balan *et al*. (2013) on squids of Eastern coasts of India and Guerreru-Ferreira *et al*. (2013) from Australian waters, where the dominance of *Vibrio* and *Photobacterium* was observed. The isolated strains in our study showed growth in temperature ranging from 16°C to 38°C, which can be attributed to the fact that *Uroteuthis duvaucelli* inhabits at a depth of 3 m to 170 m. This also establishes that the strains isolated were associated microflora as they show their ability to grow in wide range of temperature which varies horizontally in the oceanic waters. This study has elucidated the identification and association of two genera-*Vibrio* and *Photobacterium* of bioluminescent bacteria associated with *Uroteuthis duvaucelli*. It is also evident from the phenotypic characterization that there are minor differences between the strains isolated in the present study from the earlier strains reported in association with Loligonid squids, which can be attributed to the "Horizontal Gene Transfer" phenomenon.

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## REFERENCES

Aylward, F.O., Eppley, J.M., Smith, J.M., Chavez, F.P., Scholin, C.A. and DeLon, E.F. (2015) Microbial community transcriptional networks are conserved in three domains at ocean basin scales. *PNAS*. **112**, 5443-5448.

Baban C. K., Cronin, M., Akin, A. R., O'Brien, A., Gao, X., Tabirca, S., Fransis, K.P. and Tangney, M. (2012) Bioluminescent Bacterial Imaging In Vivo. *J. Vis. Exp*. **69**, 4318.

Balan, S.S., Raffi, S.M. and Jayalakshmi, S. (2013) Probing of potential luminous bacteria in Bay of Bengal and its enzyme characterization. *J. Microbiol. Biotechnol*. **23**, 811-817.

Benson (2001) Microbiological Application Lab Manual, 8th ed. The McGraw-Hill Companies, New York.

Das, S., Lyla, P.S. and Khan, S.A. (2006) Marine microbial diversity and ecology: importance and future perspectives. *Curr. Sci*. **90**, 1325-1335.

Dunlap, P.V. and Urbanczyk, H. (2013) Luminous Bacteria; in *Prokaryotes*. Rosenberg, E. F. DeLong, F. Thompson, S. Lory, and E.Stackebrandt (eds.) pp. 495-528, Springer-Verlag, Berlin.

Flores, O., Belanche, L.A. and Blanch, A.R. (2009) New multiplatform computer program for numerical identification of microorganisms. *J. Clinical. Microbiol*. **47**, 4113-4135.

Guerrero-Ferreira, R., Gorman, C., Alba, A., Willie, S., Nishiguchi, M. K. (2013) Characterization of the bacterial diversity in Indo-West Pacific Loligonid and Sepionid squid light organs. *Microb. Ecol*. **65**, 214-226.

Guerrero-Ferreira, R., Nishiguchi, M.K. (2007) Biodiversity among luminescent symbionts from squid genus *Uroteuthis*, *Loligonus* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics*. **23(5)**, 497-506.

Haddock, S.H.D., Moline, M.A. and Case, J.F. (2010) Bioluminescence in the Sea. *Ann. Rev. Mar. Sci*. **2**: 443-493.

Haldar, S., Neogi, S.B., Kogure, K., Chatterjee, S., Chowdhury, N., Hinenoya, A., Asakura, M. and Yamasaki, S. (2010) Development of haemolysin gene based multiplex PCR for simultaneous detection of *Vibrio campbellii*, *Vibrio harveyi* and *Vibrio parahaemolyticus*. *Lett. Appl. Microbiol*. **50**, 146-152.

Nishiguchi, M.K. and Nair, V.S. (2003) Evolution of symbiosis in the Vibrionaceae: A combined approach using molecules and physiology. *Int. J. Syst. Evol. Microbiol*. **53**, 2019-2026.

Nishiguchi, M.K., Doukakis, P., Egan, M., Kizirian, D., Phillips, A., Prendini, L., Rosenbaum, H.C. (2002) DNA Isolation Procedures; in Methods and Tools in Biosciences and Medicine Techniques in Molecular Systematics and Evolution. R. DeSalle *et al*. (eds.), pp. 249-287, Birkhäuser Verlag, Basel, Switzerland.

Reichelt, J.L. and Baumann, P. (1973) Taxonomy of marine luminous bacteria. *Arch. Microbiol*. **94**, 283-330.

Roura, S., Galvez-Monton, C. and Bayes-Genis, A. (2013) Bioluminescence imaging: a shining future for cardiac regeneration. *J. Cell. Mol. Med*. **17**, 693- 703.

Whitman, K.A. (2004) Finfish and Shell fish bacteriology manual; techniques and procedures. Blackwell Publishers. Iowa.

Widder, E.A. (2010) Bioluminescence in the Ocean. Origins of Biological, chemical and ecological diversity. *Science* **328**, 704-708.

Wimpee, C., Nadeau, T.L. and Nealson, K. (1991) Development of species-specific hybridization probe for marine luminous bacteria by using in vitro DNA amplification. *Appl. Environ. Microbiol*. **57**, 1319-1324.