

# The Phylogenetic Characterization of a Bioluminescent Bacterium Isolated From Shrimp

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## ABSTRACT

Bioluminescence, the biological production of light, is an unusual characteristic that is observed in certain members of the genera *Vibrio*, *Photobacterium*, *Photorhabdus*, and *Shewanella*. The bioluminescence system is composed of a group of proteins, including luciferase the main enzyme responsible for light production. I have isolated a bioluminescent strain of gram negative cocci from marine shrimp. All known bioluminescent bacteria are gram negative rods, so the different morphology of the isolate indicates that it has not been previously characterized.

The isolate, designated strain BC1, is catalase and oxidase positive and is unable to utilize maltose as a sole carbon and energy source. A fragment of the *luxA* gene, which encodes for the enzyme luciferase, was amplified from the DNA of the unknown isolate using the polymerase chain reaction and primers specific for the *Vibrio fischeri luxA* gene. The 16S rRNA gene was sequenced to determine the phylogeny of the isolate. With sequence data containing approximately 1200 bases, strain BC1 was 83.5% similar to *Photobacterium leiognathi*, its closest relative. Based on this data, I conclude that strain BC1 is a new species in the genus *Photobacterium*.

## INTRODUCTION

Bioluminescence, the biological production of light, is a phenomenon that is carried out by a diverse group of organisms, including bacteria, dinoflagellates, fungi, and insects (3). The enzyme that carries out the light emitting reaction is called luciferase. In bacteria, a blue-green light is released when luciferase oxidizes a reduced riboflavin phosphate (FMNH<sub>2</sub>) and a long chain fatty aldehyde. In the marine bacterium *Vibrio fischeri*, luciferase is encoded by the *luxA* gene, which is grouped together with the genes for the other components of the bioluminescent system. These genes are organized into a single operon known as the *lux* operon. The *V. fischeri lux* genes have been extensively studied, making *V. fischeri* the model bioluminescent system (5).

There are four bacterial genera that have bioluminescent members; *Vibrio*, *Photobacterium*, *Shewanella*, and *Photorhabdus* (6). All known species of bioluminescent bacteria are gram negative and have a bacillus or coccobacillus morphology (3). They are all facultative anaerobes and have the ability to ferment. While a few freshwater species have been isolated, *Vibrio*, *Photobacterium*, and *Shewanella* are found in marine environments while *Photorhabdus* is terrestrial. Bioluminescent bacteria are common in seawater and the intestinal tract, gills, and skin of marine fish (3). Some of the marine bioluminescent species are known to form symbiotic relationships with different species of fish and squid (3, 6).

*P. phosphoreum* is known to inhabit the light organs of five families of fish, while *P. leiognathi* is known to inhabit two families of fish (3).

The *Prokaryotes* recognizes three species in the genus *Photobacterium*; *P. phosphoreum*, *P. leiognathi*, and *P. angustum* (3). The American Type Culture Collection also lists *P. damselae* and *P. histaminum* in their strain collection. All *Photobacterium* are known to be short, gram negative, motile rods or coccobacilli. This paper describes the isolation and phylogenetic characterization of a new strain of bioluminescent cocci, designated BC1. Strain BC1 is most closely related to members of the genus *Photobacterium* according to 16S rRNA phylogenetic analysis. The morphology, growth, and biochemical characteristics of strain BC1, however, clearly distinguish it from known species of *Photobacterium*.

## MATERIALS AND METHODS

**Isolation of BC1.** Fresh shrimp from the Gulf of Mexico were incubated in a Petri dish, partially covered with 3% NaCl at room temperature for 24 hours. The shrimp was then examined in the dark for luminescence. Glowing patches were streaked for isolation on photobacterium plates (8). Once a confirmed bioluminescent colony was isolated, it was Gram stained and a wet mount was examined with phase-contrast microscopy. The isolated strain was designated BC1.

**Bacterial strains, media, and growth conditions.** Strain BC1 was grown in photobacterium broth (shaking at 250 rpm) or on photobacterium agar at room temperature, unless otherwise indicated.

**Growth curves and optimum temperature.** Strain BC1 was grown at different temperatures in photobacterium broth shaking at 250 rpm. Samples of the culture were taken at timed intervals and the optical density measured at 600 nm in a Spectronic 601 spectrophotometer.

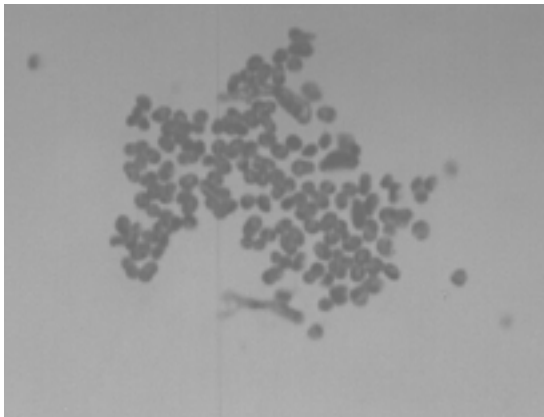
**Utilization of carbon sources.** Strain BC1 was grown in artificial seawater basal salts broth (1) with a sole carbon and energy source. Glucose, cellobiose, galactose, maltose, and sucrose were tested at a concentration of 0.05M. Overnight cultures were grown up in photobacterium broth, cells were harvested and washed in the basal salts broth. The washed cells were used to inoculate the test media to a starting OD<sub>600</sub> of 0.01. Samples were taken after 1, 2, and 4 days and the OD<sub>600</sub> was measured to check for growth.

**Polymerase chain reaction (PCR).** A cell extract was prepared by boiling a 1-2 mm colony in 15 µl of sterile water for 12 minutes. The resulting extract was diluted 1/100 for use as the template in the PCR reaction. PCR was performed using *Taq* polymerase (Promega, Madison, WI) and primers for the bacterial 16S rRNA gene or the *luxA* gene (2,8). The same primers were used to set up a positive control with *Escherichia coli* DNA and a negative control with sterile water. The reactions were cycled in a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT) with the following program: hold at 94°C for 5 minutes; five cycles of 94°C for 30 seconds (denature), 37°C for one minute (primer anneal), and 72°C for one minute (primer extension); and 25 cycles of 94°C for 30 seconds (denature), 55°C for one minute (primer anneal), and 72°C for one minute (primer extension). The PCR amplification products were electrophoresed on a 1% agarose gel with DNA standards. The PCR product was sized to confirm that the product was the expected size.

**DNA sequencing of 16S rRNA gene.** The PCR fragment obtained with primers specific for the 16S rRNA gene was used as the template for the sequencing. Unincorporated nucleotides were removed from the product by washing the reaction three times with sterile distilled water in a Ultrafree microfuge filter, MWCO 30,000, (Millipore, Bedford, Ma). The product was brought up in an appropriate volume of sterile distilled water and used as a template in the sequencing reactions using [ $\alpha$ - $^{32}$ P]-ATP and the SequiTherm kit (Epicenter, Madison, WI). The reactions were run on a 6% polyacrylamide gel and visualized by autoradiography. Sequence data of the 16S rRNA gene was analyzed with a sequence match program at the Ribosomal Database Project website (4).

## RESULTS AND DISCUSSION

Microscopic examination revealed that strain BC1 is a motile, gram negative coccus (Figure 1). Since all known bioluminescent bacteria are rod-shaped, the coccoid cell morphology suggested that strain BC1 has not been previously characterized. The biochemical properties of strain BC1 were investigated and compared to literature values of the *Photobacterium* reference strains (Table 1) (7). Strain BC1 was both catalase and oxidase positive. This differentiated strain BC1 from *P. angustum* and *P. leiognathi*, which are both catalase negative. Strain BC1 was able to grow with glucose and galactose as a sole carbon and energy source, but was unable to grow on cellobiose, sucrose, and maltose. The inability of strain BC1 to utilize maltose as a carbon and energy source further differentiates strain BC1 from the reference *Photobacterium* strains, which are all able to use maltose. The optimum growth temperature of strain BC1 is approximately 30°C, differentiating it from *P. phosphoreum*, which has an optimum growth temperature of 18°C.



**Figure 1: Gram stain of strain BC1 at 1000X magnification.**

**Table 1: Characteristics of strain BC1 compared to *Photobacterium* reference strains (7).**

Characteristics	<i>P. ang</i> <sup>1</sup>	<i>P. dams</i> <sup>1</sup>	<i>P. hist</i> <sup>1</sup>	<i>P. lei</i> <sup>1</sup>	<i>P. phos</i> <sup>1</sup>	str. BC1
Oxidase	-	+	+	+	+	+
Catalase	-	+	+	-	+	+
Optimum growth temp. (°C)	25	26	26	26	18	25-30
Carbon sources for growth:						
Glucose	+	+	+	+	+	+
Cellobiose	-	+	+	-	-	-
D-Galactose	+	+	+	+	-	+
Maltose	+	+	+	+	+	-
Sucrose	+	-	-	-	-	-

<sup>1</sup> *P. ang* - *P. angustum* American Type Culture Collection (ATCC) 25915, *P. dams* - *P. damsela* ATCC 33539, *P. hist* - *P. histaminum* Japan Collection of Microorganisms 8968, *P. lei* - *P. leiognathi* ATCC 2552, *P. phos* - *P. phosphoreum* ATCC 11040

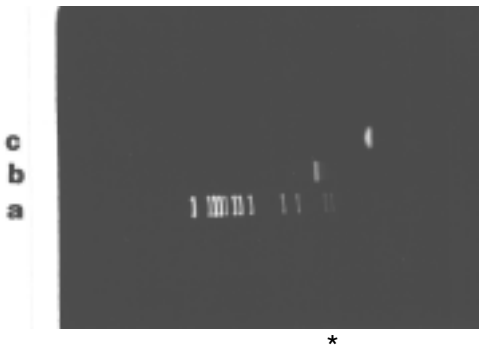
The 16S rRNA gene sequence was used to determine the phylogeny of strain BC1. Similarity comparisons were made using an almost complete sequence of the 16S rRNA gene (1196 bp). The sequence was aligned manually to a conserved secondary structure in order to ascertain homologous sequence positions. From the similarity search, strain BC1 was most closely related to *P. leiognathi* (83.5% similarity), followed by *P. angustum* (78.8%), and *P. phosphoreum* (78.2%) (Table 2). This is a considerable difference, indicating that strain BC1 is a separate species.

**Table 2: Comparison of the 16S rRNA sequence of strain BC1 to that of other prokaryotes found in the Ribosomal Database.**

Organism	Percent Similarity <sup>1</sup>
<i>P. leiognathi</i> str. L1 ATCC 25521	83.5
<i>P. angustum</i> ATCC 25915	78.8
<i>P. phosphoreum</i> ATCC 11040	78.2
<i>V. iliopiscarius</i>	77.8
<i>Vibrio</i> sp. Str. DSJ4	75.6
<i>P. damsela</i> subsp. piscicida	73.0

<sup>1</sup>Similarity comparisons were made using data from seven different primers which gave four separate fragments of 16S rRNA sequence totaling 1196 bases.

A 700 base pair fragment was amplified using PCR with primers specific for the *luxA* gene of *V. fischeri* (Figure 2). The amplified fragment was approximately 700 bp long, the expected size of the *luxA* PCR product. These data suggest that there is some degree of similarity between the *luxA* gene of *V. fischeri* and that of strain BC1.



**Figure 2: Polymerase chain reaction.** Lane a:  $\lambda$ /BstEI DNA size standards. Lane b: PCR product of 16S rRNA gene, ca. 1500 base pairs. Lane c: PCR product of *luxA* gene, ca. 700 base pairs. The asterisk denotes the dublet bands in lane a with sizes of 1371 and 1264 base pairs.

In summary, strain BC1, a gram negative coccus, was isolated from fresh marine shrimp. Strain BC1 is motile, catalase and oxidase positive, and cannot utilize maltose as a carbon and energy source. Its optimum growth temperature is higher than that of *P. phosphoreum*. 16S rRNA sequence analysis has determined that strain BC1 is related, but not identical to the known *Photobacterium* species. Strain BC1 has a *luxA* gene similar to that of *V. fischeri*. Taken all together, the data clearly indicates that strain BC1 is related to the members of the genus *Photobacterium*, but is not currently a recognized species.

## ACKNOWLEDGMENTS

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