A Bioluminescent Bacterial Survey from Marine Ecosystems of Puerto Rico for the Determination of their Potential Use as Biosensors

By

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Abstract

Bioluminescence is the phenomenon by which living organisms emit light through an organic oxidation catalyzed by the enzyme luciferase. These luminescent organisms have been discovered in all kinds of ecosystems. Among their diversity, bacteria are the most widespread and abundant bioluminescent organism in nature supporting a unique and necessary ecological role. Research on bioluminescent bacteria had led to the discovery of quorum sensing in which cells can chemically detect their presence and execute various processes crucial for competition such as extracellular transport of virulent factors, plasmid replication, catabolic pathways and light emission. In addition, luciferase coding genes (*luxAB*) have been cloned and express in other biological models with the purpose of biosensing molecules that trigger changes in light emission. The main objective of this study is to evaluate the presence of bioluminescent bacteria in marine ecosystems of Puerto Rico. Among the methods used are Gram stain and morphology confirmation through Scanning Electron Microscopy (SEM). In addition, biochemical assays were performed to test their potential enzymatic capability to metabolize different energy sources such as glycerol, mannitol, starch and sucrose. Phylogeny was done by sequencing both 16S rDNA and *luxAB* amplicons. As a final objective several isolates were exposed to phenanthrene to confirm growth and tolerance for their possible future use as biosensors. *In silico* analysis confirmed the presence of candidates closely related physiologically and molecularly to *Photobacterium leiognathi* and *Vibrio harveyi*. Eleven 16S rDNA sequences did not show close evolutionary relationship with previously reported strains based on neighborhood joining method. Only 14 sequences resulted to group close to the specie *V. harveyi* and only one strain was physiologically and molecularly related to the *Photobacterium* Family. The groups

with the highest frequency in the collection are strains similar to the *Vibrionaceae* Family. This study is the first to report 32 partial sequences coding for luciferase from strains collected along the coast of Puerto Rico. Bioluminescent isolates also grew in presence of more than one antibiotic. In the collection, 66% of the candidates were resistant to ampicillin, 8% to tetracycline and 16% to kanamycin. This result suggests that these strains have mechanisms that contribute to their survival. This provides additional groups of bacteria that can clinically impact society and it will serve to report additional members of *Vibrionaceae* Family that are resistant to ampicillin and kanamycin.

Resumen

La bioluminiscencia es el fenómeno por el cual los organismos vivos emiten luz por medio de una oxidación orgánica catalizada por la enzima luciferasa. Estos organismos bioluminiscentes han sido descubiertos en todo tipo de ecosistemas. Dentro de su diversidad, las bacterias son los organismos bioluminescentes más distribuidos y abundantes en la naturaleza, contribuyendo un papel ecológico único y necesario. Investigaciones en bacterias bioluminiscentes han contribuido al descubrimiento de la autoinducción, en el cual las células pueden detectar su presencia químicamente y ejecutar varios procesos cruciales para la competencia como el transporte extracelular de factores virulentos, replicación de plásmidos, rutas catabólicas y emisión de luz. En adición, los genes que codifican para la luciferasa (*luxAB*) han sido clonados y expresados en otros modelos biológicos con el propósito de bio detectar moléculas ejecuten cambios en la emisión de luz. El propósito principal de este estudio es evaluar la presencia de bacterias bioluminiscentes en ecosistemas marinos de Puerto Rico. Entre los métodos usados fueron la tinción Gram y la morfología fue confirmada usando Microscopía Electrónica de Rastreo (MER). En adición, ensayos bioquímicos fueron realizados para determinar su potencial enzimático para metabolizar distintas fuentes de energía como glicerol, manitol, almidón y sacarosa. La filogenia se realizó secuenciando ambos amplicones de ADNr 16S y *luxAB*. Como objetivo final varios candidatos fueron expuestos a fenantreno para confirmar crecimiento y tolerancia para el futuro uso como biosensores. Análisis *In silico* confirmó la presencia de candidatos fisiológicamente y molecularmente cercanos a *Photobacterium leiognathi* y *Vibrio harveyi*. Once secuencias de ADNr 16S no tuvieron relación evolutiva cercana con cepas reportadas. Solo 14 secuencias resultaron ser cercana a la especie *V. harveyi* y solo una cepa fue fisiológicamente y molecularmente cercana a la de Familia de *Photobacteriaceae*. Los grupos

con la frecuencia más alta en la colección son parecidos a la Familia *Vibrionaceae*. Este es el primer estudio en reportar 32 secuencias parciales codificantes para luciferasa de cepas colectadas a lo largo de la costa de Puerto Rico. Aislados bioluminiscentes también crecieron en presencia de más de un antibiótico. En la colección, 66% de los candidatos fueron resistentes a ampicilina, 8% a tetraciclina y 16% a kanamicina. Este resultado sugiere que los aislados tienen mecanismos que contribuyen a su sobrevivencia. Esto provee grupo adicionales de bacterias que pueden clínicamente impactar la sociedad y servirá para reportar miembros adicionales de la Familia *Vibrionaceae* que son resistentes a ampicilina y kanamicina.

Dedication

Many persons close to my heart helped me build up my faith to continue this journey or rollercoaster throughout these years. First I want to dedicate this work to the one that gives me the example through courage, love and sacrifice; the one that goes further than time and space. To God the only one that teaches me through difficult situations and confirms daily that there is still hope. I wish to also dedicate this work to my family, because they never lost faith in me and supported me always even when I lost hope, thank you Elizabeth Orengo for your endless love and courage, because you have taught me how to fight and never give up. To my dad and best friend, José Domingo Malavé Dieppa, because even when you can't verbally speak or do the things you use to, you always have the strength to love me, to fight and to still be there, because I have it all just because you chose to fight and to love and to accept what God permits. To my brothers José Domingo Malavé and Doely Malavé Orengo, because you carry my blood, my sorrows and the next generation, because you played with me when I was growing up and saw me fall and live. I also I dedicate this work to my brother because he taught me to stand up when one falls and to search and pursuit for what makes your happy. To my sister because she believes in me, thank you for your love and firmness when is necessary. To my grandparents, Diego Orengo and Delfa Aviles because I always think of you when I'm fighting for something. I dedicate this thesis to my mentors: Tío Jimmy and Tío Che, because you taught me that is possible to do great and to do lots of things with few things and with a lot of limitations. Finally but not least to my loving and unique girlfriend Viviana Aimeé Meléndez Muñiz, because not just only you were there for me in the most difficult stage of this process, but also because you decided to support me with love, patience and the one thing I lost hope, because you wanted to be there even though it was tough and frustrating. I love and admire your beautiful heart.

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Chapter One

Introduction and Literature Review

1. 1 Introduction

Bioluminescence is the process by which living organisms emit light, and occurs in many species of bacteria, fungi, insects, dinoflagellates and fishes. Light-emitting bacteria are the most abundant and widespread of luminescent organisms, and occur mainly (though not exclusively) in aquatic ecosystems (Wegrzyn, et al., 2002). The phenomenon of bioluminescence is spread over many ecosystems including terrestrial, but mainly marine ones (Campbell, 1988). Even though, Puerto Rico (PR) is a tropical island, detailed studies based on bioluminescent bacteria have not been done. The only published study was to determine the presence of *Vibrio harveyi* and *Photobacterium phosphoreum* at different depths in two sampling sites located in the north of Puerto Rico (Ruby et al., 1980). Emphasis have been given to study dinoflagellates and their distribution along the island, but the distribution, physiology and possible biotechnological applications of studying bioluminescent bacteria still not been assessed. Important discoveries have been accomplished in many studies conducted on bioluminescent bacteria. For example, early studies on these bacteria lead to the discovery and understanding of the quorum sensing phenomenon that allowed determining how genes are regulated according to cell density basis (Miller et al., 2001, Nealson et al., 1970). This promoted the understanding of similar models in gram-negative such as *P. aeruginosa*, *Agrobacterium tumefaciens* and *Erwinia carotovora* among others (Piper et al., 1993).

In the field of biosensors, genes that encode for bacterial luciferase (*luxA* and *luxB)* has been used extensively to monitor biological processes of many kinds. Their usefulness relies on the fact that light emission mediated by these genes is directly involved with the microbial physiological status, therefore capable of detecting environmental stresses. The use of *luxA* and

luxB transcriptional fusions include detection of hydrocarbons, heavy metals and UV mediated stresses (Burlage et al., 1990).

Characterization of bioluminescent bacteria helps develop more sensitive biosensing tools to detect contamination and to evidence degradation of the most common pollutants in natural environments. The main focus of this thesis is to assess taxonomically unreported bioluminescent candidates from marine environments of Puerto Rico, to characterize these bacteria using traditional morphological and biochemical approaches and to confirm the identity of the isolates molecularly by analyzing sequences of 16S rDNA amplicons and partial sequences coding for the enzyme luciferase.

In order to determine their potential as biosensors, preliminary experiments were done to confirm growth of the bioluminescent candidates in media with phenanthrene as a toxic compound. As a biomedical research component, antibiotic resistance assays were developed in order to evaluate additional candidates capable of growth in the presence of ampicillin, tetracycline, kanamycin and chloramphenicol. This will serve as an opportunity to evaluate mechanisms of luminous bacteria that inactivate antibiotics Finally, the bacterial isolates derived from this work could provide new reporter genes useful for the detection of chemicals impacting human health, may be used as whole cell biosensors and help produce new cell strains transformed with luciferase coding genes useful as biosensors.

1.2 Literature Review

Bioluminescence in nature

Bioluminescence is a form of light produced by a chemical reaction in living organisms and has been witnessed by humans before the scientific method was developed. Reports have been found in religious writings of India and China. The Greeks and Romans were the first ones to manually report the description of luminous organisms and referred to the sea bioluminescence as phosphorescence (about 500 BC) (Harvey 1957). In the sixteenth century documents were found describing this phenomenon in voyages of Christopher Columbus and Sir Frances Drake. The first book dedicated to this "cold light" was published in 1555 by Conrad Gesner (Carter and Kricka 1982; Harvey, 1957). The first known modern scientist that contributed publishing books and articles on bioluminescence was Newton Harvey (1887-1959). He devoted his life searching for molecules and enzymes involved in this emission of light, but the first luciferase activating molecule (luciferin) isolated was by McElroy (Green and McElroy, 1956).

The function of bioluminescence may vary from one organism to the other such as for defense against predators, for predation or for communication with their mates (Hastings et al., 1963). It is exhibited by a diverse group of organisms although their number is very less compared to the total number of known species. It has been estimated that luminous organisms may have come from about 30 different evolutionarily distinct origins (Hastings et al., 1973).

The enzyme luciferase and its substrate luciferin are the agents responsible for bioluminescence. The biochemical and physiological mechanisms responsible for the bioluminescence are different among the luminous organisms and even though components of

the reaction are different in structure they have similar roles. In higher organisms such as fish and squids, evidence suggests that they emit their own light or have an endosymbiosis with luminous organisms to perform a phenomenon known as counter illumination (Berry, 1912; Boettcher et al., 1990). This strategy is performed by the squid *Euprymna scolopes*, which uses luminous bacteria contained in his gut to erase the shadow that is cast by the moon in the oceans bottom that is detected by predator when the squid swims at night (Boettcher et al.,1990) (Nealson, 1978; Nealson, 1979; Nealson and Hastings,1979).

. In the world of insects, the first studies with luciferase were carried out to measure available ATP. The luciferase used was monomeric and was isolated from firefly by the pioneer in bioluminescence William McElroy. He made protein extracts from abdominal tissue of fireflies and noticed that ATP was required for the reaction to take place (McElroy et al., 1985).

In microorganisms such as Dinoflagellates, the mechanism of regulation is unique, because it depends on the circadian cycle, which orientates the organisms to sense what stage of the day is (Bode et al., 1963). When a physical stimuli or stress is perceived by the eukaryotic cell a sudden drop in pH occurs that detaches a protein called luciferase binding protein (LBP), this way the Dinoflagellate protein is released to perform the reaction in charge of sending an alarm for predators in distance that would devour the organisms grazing on the dinoflagellate (Buck et al., 1963). In dinoflagellates the reaction takes place on specialized organelles called scintillons located in the cytoplasm.

The emission of light in Puerto Rico

The smaller of the Greater Antilles has a diverse repertoire of bioluminescent sites; some examples are the luminous bays from the town of Lajas (La Parguera) and Mosquito Bay (Island of Vieques) which both are populated by Dinoflagellates (Bernache, 1995; Seixas, 1983). The most dominant specie in our bays is *Pyrodinium bahamense* (Margalef, 1957). Even though most luminous flagellates are distributed along the marine environments of Puerto Rico certain conditions are met in order to see this bioluminescence on these two bays. Some factors include intensity of oceanic tides and water dynamics (Carpenter, 1968). Also other a biotic factors, such as the lack of wind intensity helps the horizontal and vertical distribution of bioluminescent protists including the movement and transportation of nutrients (Ryther, 1955). In other studies Margalef mentions the presence of different species of bioluminescent protists such as: *Ceratium furca, Dinophysis caudate ventricosa, Pyrophacus horologium steinii, Peridinium divergens*, *Peridinium quinquecorne* and *Cochlodinium polykrikoides*.

The insects contain another traditional example of bioluminescence in PR, locally both are Families belonging to the Order *Coleoptera* and are known as "cucubano" and "luciérnaga". The first one, *Pyrophorus luminosus* is native to PR and is capable of emitting light from its abdomen and from the top of its thorax (Lacordaire, 1857; Lawrence, 1982). The purpose of the first light emission is to attract its partner and the light pattern is caused by two different luminous dots. The second emission is for intimidation purposes of possible predators. These beetles are also known as click beetles and belong to the Family *Elateridae*.

Fireflies ("luciérnagas") on the other hand belong to the Family Lampyridae and have been reported extensively in PR, even though not all the species are luminous (Olivier, 1790; Laporte, 1840). Differences when compared with click beetles lies on only one light emitting organ,

Fireflies generally emit in the yellow to green range, as part of a courtship process; click beetles emit green to orange light. Scientists acknowledged 16 species of Lampyridae of four genera in Puerto Rico, *Erythrolychnia motschulsky, Callopisma motschulsky, Pyractomena melsheimer* and *Photinus laporte* (Olivier, 1790). The bioluminescence substrate (luciferin) between various *Lampyridae* and *Elateridae* species are nearly identical (Seliger and McElroy, 1964). The coding gene for luciferase is the same and has been cloned from over 15 lampyrids and 3 elaterids (Colepicolo-Neto et al., 1986). These studies revealed that luciferases are nearly 48% identical in its aminoacid sequence. It was recently suggested that bettle luciferases have a common origin (Oba et al., 2005).

Taxonomy of bioluminescent bacteria

The *Vibrionaceae* Family is one of the largest taxonomic groups in the field of microbiology. Historically has evolved much to finally incorporate the following genera: *Vibrio, Photobacterium, Aeromonas* and *Plesiomonas*. These are the same genera that were originally proposed by Veron almost 20 years earlier (Veron, 1965). Large series of changes occurred in the classification of *Vibrio* when new methods were generated. These methods helped to examine structure, function and regulation of proteins. Some of the most used techniques are DNA-DNA hybridization, 5S rRNA and 16S rRNA gene comparison and sequencing (Farmer, 1992).

Many vibrios and related organisms are widely distributed in aquatic environments (Campbell, 1957; Baumann, 1981). Many factors govern the distribution but the most important probably are; human and animal presence, plant hosts, inorganic nutrients, carbon sources available, temperature, salinity, dissolved oxygen and depth for the species that are found in the

ocean (Baumann, 1984). Most members of the *Vibrionaceae* grow well when inoculated in ordinary complex media. Isolates are spread onto solid medium with NaCl concentration from .5 to 3%. Incubation temperature is crucial since few species grow only at temperatures less than 25ºC, most at 25ºC but not at 35ºC to 37 ºC (Sakazaki and Balows, 1981).

Most data collected by Campbell and Baumann describes members of this genus as small, straight or slightly curved. Involutions may be seen in old cultures or cultures under adverse conditions. Not a single member produces endospores. All of them are gram-negative and most are facultative anaerobes capable of both fermentative and respiratory metabolism. Molecular oxygen is the universal electron acceptor. Most do not denitrify. All are chemoorganotrophs are able to grow on medium containing glucose as sole carbon source, few may produce gas. Most species grow well on sea water (2.5-3.5% NaCl) and can ferment fructose, maltose and glycerol. A unique property of some Vibrio strains is their ability to convert chemical energy into blue green light in response to certain environmental stimuli. Some bioluminescent species include *V. fischeri, V. harveyi, V. logei, V. splendidus, P. leiognathi* and *P. phosphoreum*. Some species are reported inside the genus of *Photobacterium* or *Vibrio* depending on the taxonomist that isolate and reported the strain. In this thesis *Vibrio sp*. denotes *V. fischeri.*

Light emitting reaction

Bioluminescence is the emission of light by living organisms. It involves the oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde. This even produces a blue-green (490 nm) light that gives bacteria an important ecological role. This mechanism which requires O_2 is catalyzed by an enzyme called luciferase of which different types are present on different organisms. Luciferase is a heterodimer of 80 kDa, coded by two similar

genes it is activated by $FMNH_2$ and reacts with O_2 to produce a compound that will form a highly stable complex with the aldehyde that degrades slowly by emitting luminous energy due to the oxidation of the substrates Miyamoto et al., 1986).

The aldehyde required for the reaction is synthesized by an enzymatic complex coded by three different genes present in all bioluminescent bacteria, *luxCDE*. This substrate is derived from the fatty acid pathway and can be recycled after each reaction. The three proteins that form the enzymatic complex are a reductase, a synthetase and a transferase that were identified through radioactive labeling. Other proteins are involved in the reaction that is only present in certain species of bacteria. For example, lumazine and yellow fluorescent protein (yfp) are two different proteins present in *Photobacterium phosphoreum* and in some strains *Photobacterium fischeri respectively*. Both are called accessory proteins because they are not crucial for the reaction to take place. The first one (lumazine) shifts the color of light to shorter wavelengths, therefore increasing the energy of the emission. The second protein shifts the light produced to such a degree that the color changes completely to yellow ($\lambda_{max} \sim 540$ nm) (Macheroux et al., 1987).

Regulation of the *lux* operon

The characterization of the *lux* operon was determined through the cloning of a 9 kb DNA fragment obtained from *P. fischeri* that was transformed into *E. coli*. Successful expression was witnessed when *E. coli* emitted light similar to *P. fischeri.* Mutational analysis showed five crucial genes required for the reaction (*luxAB* and *luxCDE*). The genetic organization was determined to be arranged as an operon, a diverging with a left and right side containing two different promoters that are transcribed in opposite directions (Engebrecht et al.,

1983). The first gene, *lux I* at the start of the right side of the operon produces a small peptide that works as an autoinductor that triggers the synthesis of the structural genes, *luxCDABE,* in that same order (Figure 1).

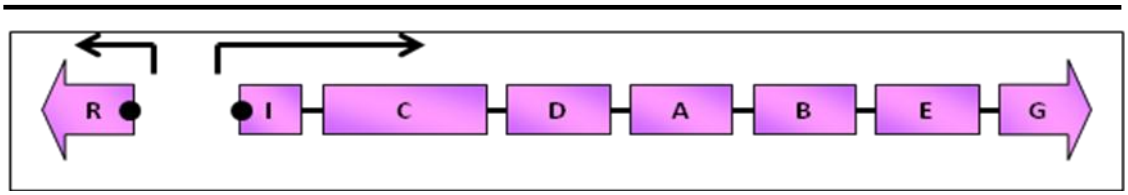


Figure 1. Bioluminescence in bacteria is produced by the expression of *lux* genes. The *Vibrio fischeri* region is organized in two different operons: *luxI* to *luxG* are expressed downstream and opposite to *luxR*. Promoter sites are indicated by dots.

All genes in the right side are closely linked to each other, less than 50bp separates them. Next to *luxE* there is an additional gene that is present only in marine species which is not crucial for the reaction but its function is still unknown. To the left side of the operon, a gene is present that codes for another regulatory protein, a repressor with dual function in all bioluminescent strains. This repressor has one site that interacts with the DNA and a second site that binds to the small peptide (*luxI*). When attached to the autoinducer, it forms a complex that promotes transcriptional activation in the right side through positive regulation. On the other hand when the autoinducer concentration is not high enough, the repressor continues to be produced. Therefore, inhibiting the transcription of structural genes by binding to operator located in the control region. This 218bp intergenic region between the left and right sides of the operon works as the control region (region between *lux R/lux I*). It contains the DNA binding site of the repressor and a site for cAMP receptor protein (CRP) (Dunlap et al., 1988). This CAP-cAMP region located in front of the *luxR* transcription site seems to stimulate the coding of this left

gene that when combined with *luxI* can stimulate the expression of the right side operon. Therefore, this bioluminescent system is directly related to the cells metabolic state.

Biological systems used as biosensors

Biological processes are known to be diverse in nature and also highly specific. Bioelements such as enzymes, toxins and DNA sequences are known to recognize or interact with specific molecules with certain properties or conformations. An application that has grown in use and versatility is known a biosensor (Timmis et al., 1999). This technology is a modified biological model that can detect through a signal production a specific analyte or molecule. It consists mainly of two main elements: a promoter and a bioreporter. The promoter or receptor will interact or bind depending on the situation and will translate that interaction into a detectable signal carried out by the reporter. A DNA sequence that codes for a protein which produces a signal that can be color or light. Some of these biological systems are used to detect concentrations of nitrite, arsenic and mercury at picomolar levels which are not detected by certain equipments (Poole et al., 1996). A specific example is the use of trichloroethylene degrading genes from *Pseudomonas aeruginosa* JI104 to detect and monitor the catabolism of this chlorinated hydrocarbon commonly used as an industrial solvent (Kitayama et al., 1992). Other biosensors are so versatile that can even produce a measurable signal for physical, chemical and genotoxic agents having genes involved such as *recA, uvrA* and *alkA* (Volmer et al., 1997).

Chapter Two

Isolation and characterization of bioluminescent bacteria from marine environments of Puerto Rico

2.1 Introduction

Bioluminescence is the emission of light by living organisms or a chemiluminescent reaction catalyzed by an enzyme. In nature a diversity of different organisms can perform this phenomenon. Some examples include insects, fish, fungi, worms, squids, bivalves among others. Even though aspects of bioluminescence are still not known well, its emission has been studied and applied into science. Being bacteria widely distributed in marine and terrestrial environments new findings are being recorded of new bacterial bioluminescent strains, applications to detect pollutants, evolutionary origins and gene regulation processes related to bioluminescence (Czyż et al., 2000).

For example, the first study of autoinduction or now known quorum sensing was pioneered using bioluminescent bacteria (Chen et al., 2002). This process determines how bacteria detect their cell density and its relation to the emission of light (Fugua et al., 1994). This quorum sensing process has also been discovered in other organism such as *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens* and many others. Also, with the study of bioluminescent bacteria pollutants detection systems have been developed. Rapid effective diagnostics based on bioluminescence are now evolving. Microtox is a system that uses luminous marine bacteria *Vibrio fischeri* to determine water quality and toxicity based on the principle of a healthy metabolism being affected by chemical substances which results in a decrease of light. Several studies confirms that bioluminescence is the most sensitive and quantifiable system today (Dunlap and Kita-Tsukamoto, 2006).

The best studied bioluminescent group is the genus *Vibrio,* which includes the free living specie of *Vibrio harveyi* (*Beneckea harveyi*). Other genus that has some luminous species is *Shewanella*. The distribution of bioluminescent bacteria includes diverse locations (Makemson

et al., 1997). For example, *Photobacterium phosphoreum* can be found in the deep sea, *Vibrio fischeri* (*Photobacterium fischeri*) is found in species specific complexes involving symbiosis with sepiolid squids and monocentrid fishes (Gentile et al., 2008). Other strains, *Photobacterium leiognathi* are found as primary symbionts of leiognathid and morid fishes. Most bioluminescent bacteria are known to live in warmer waters (Urbanczyk et al., 2007). They may be grown from pieces of detritus, tissue or dead fish on the beach (Dunlap et al., 2005).

In Puerto Rico, while having many scientific and touristic attractions due to bioluminescence, hardly any studies involving bioluminescent bacteria have been carried out (http://www.biobay.com/cd/articlew/night.htm; Ruby et al., 1980). Scientific reports or documents on bioluminescence are mainly related to Dinoflagellates, fireflies and click beetle. Even though bioluminescent bacteria are found widespread in the world, hardly any study representative of islands bioluminescent candidates have been conducted (Latz et al., 1995).

2.2 Material and Methods

Processing and sample collection

To accomplish the objectives of this research, samples were collected from marine water sources located around the whole island (Figure 2.1) using sterile autoclaved dilution bottles. Water sampling was performed is the absence of rain to avoid dilution. Sterile bottles (stored in ice) were used to contain enough water volume to use, approximately from 300-500 µL. Luminescent Agar (LA; NaCl 3%, Peptone 1% and Yeast Extract .5%) was chosen as media to perform duplicate spread plates for each sample. After a 25º C incubation period of 24 hours, plates were examined on a dark room to presence bioluminescence. Positive candidates were isolated by performing streak plates using toothpicks to obtain pure colony for morphology examination. All sampling sites were confirmed using a global positioning system unit (Trimble).

Microbiological and physiological characterization

The morphology of the candidates was evaluated after Gram staining and confirmed by Scanning Electron Microscopy (SEM). The isolated bacteria were stored at minus -80ºC in 50% glycerol preservation to avoid cell lysis. To confirm viability of bioluminescent bacteria, inoculation of stored cultures on LA (3% NaCl) media was regularly done. Twelve biochemical assays were used to characterize isolates physiologically including: the indirect oxidase, catalase, urease, gelatine, Simmons citrate, Dnases assay or tests. In addition to tests for catabolic metabolism such as the starch, glucose, glycerol .5%, mannitol, sucrose and lactose assays were used (Georges et al., 2005). Additional NaCl was added to all commercial media for final

concentration of 3%. Anaerobic respiration was confirmed using a GasPak and anaerobic jar with a methylene blue indicator strip. Incubation at 25 °C for a minimum of 24hrs.

Digital photography of bioluminescence

In order to record the light emission, isolates were grown close to early log phase and plated in LA (3%NaCl). Digital pictures were taken no more than 8hrs after second inoculation. Parameters used with a Nikon D90 were: 8s exposition with a 1.8 opening lense, ASA 500, white balance was 10,000K.

Scanning Electron Microscopy (SEM)

The isolates were observed using a JEOL JSM-541 OL SEM microscope. For the analysis, strains were grown in LA (3% NaCl) for 24 hours at 25 ºC while shaking. Then, approximately, 1.5 mL of the culture was transferred to a microtube and centrifuged for 3 min at 1.8 g to obtain a cell pellet. A volume of glutaraldehyde 4% as final concentration was used to fix the cell pellet and was incubated for a minimum of 24 hours at 4ºC. The pellet was rinsed with 1M phosphate buffer three times using for a 10 min incubation period. To dehydrate the pellet, different percentages of alcohol were used (from 10% to 100% in 10% intervals). Every ten minutes the alcohol supernatant was decanted and replaced with fresh alcohol. Critical point drying was used as dehydration method. A sputter coater was used to give the samples a palladium-gold cover in order to protect the sample and improve conductivity. The parameters used above were established by the Scanning Electron Microscopy Center at the University of Puerto Rico, Mayagüez Campus. The photos were taken at 15 kv and a magnification of 7500.

Growth in different percentages of NaCl

This test was done to determine the NaCl inhibiting concentration. Luminescent Broth was used as the base medium. Percentages of salt used were: 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%. Briefly, 5mL of the broth was place in tubes where the bacteria were inoculated. The samples were incubated in an orbital shaker at 25 °C for 72 hours. The bacteria used as controls were *Vibrio fischeri* (ATCC 7744) and *Vibrio harveyi* (ATCC 5881).

Determination of antibiotic resistance and detection of beta-galactosidase

In order to determine the potential of the luminous strains in the Biomedical field, isolates were inoculated in LA broth (NaCl 3%) individually with the following antibiotics: ampicillin (50µg/mL), tetracycline (10µg/mL), kanamycin (25µg/mL) and chloramphenicol (34µg/mL). For the beta-galactosidase detection plates were prepared using X-gal as a substrate (40µg/mL). All of the above concentrations we used as final in the media. Incubation at 25 °C for 12hrs.

Determination of optimal temperature

To determine the optimal temperature for the luminous bacteria, the isolates were streaked on Luminescent Agar (LA, NaCl 3%) plates. Then plates were incubated for four days at different temperatures ranging from 4 ºC to 40 ºC.

Preliminary assays for the possible use as biosensors

To test the biotechnological potential of the isolates and their possible detection of toxic substances through light emission, bioluminescent isolates were grown in the various

concentrations of naphthalene and phenanthrene and compared with the Minimum inhibitory concentration that affected *Escherichia coli* JM109 and *Vibrio fischeri* (ATCC 7744) as certified controls.

Hydrocarbon stocks preparation

A total of 4mg were slowly suspended in 1mL of absolute ethanol, 0.025mL of this stock were dissolve in an additional volume of 25mL of absolute alcohol for a final concentration of 4µg/mL.

Naphthalene and phenanthrene growth assays

Control and experimental strains were grown in optimal temperatures in LA (3% NaCl) and Luria bertoni (LB) respectively. Optical densities were measured (600nm) after 3-4hrs to obtain O.D. of 0.4 to 0.8. After achieving optimum cell densities, 100µL of cell suspension were inoculated in the media with naphthalene or phenanthrene. Incubation at 32 °C for 48 hrs.

Minimum Inhibitory Concentration (MIC)

From 4µg/mL stocks 2mL were dissolved by performing a serial dilution using 5 sterile tubes containing 7.9mL of media each (1/5 dilution). LA for bioluminescent strains and LB for *E.coli* JM109. Final hydrocarbon concentrations for naphthalene and phenanthrene were: 0.8 µg/mL, 0.16 µg/mL, 0.032 µg/mL, 0.0064 µg/mL and 0.00128 µg/mL. After cells were prepared in optimum cell density a final inoculation was made in the second dilution (0.16 µg/mL of hydrocarbon).

2.3 Results

The bacterial bioluminescent collection in this study is composed of 125 strains from different municipalities along the coastline of Puerto Rico. Only sites which bioluminescence was detected when water sample was cultured in the laboratory are reported. A total of 31 purified strains are described due to an observable difference in the physiological or morphological data. Traditional microbiology was done including microscopic analysis to confirm cellular morphology, recording on colony bioluminescence and molecular identification using 16SrDNA and also sequencing of partial luciferase coding genes, *luxA* and *luxB.* Finally, some trains were tested for their resistance to phenanthrene and naphthalene as toxic hydrocarbons. Sampling was carried from 2005 to 2008.

All strains were gram-negative. Physiologically, most of the strains from the north were able to metabolise starch, mannitol, glucose, lactose and sucrose. All bioluminescent strains are oxidase positive and can degrade the DNA in media and show growth was detected in a 0.5% of glycerol.

Strains isolated from the west were all citrate, glucose, mannitol positive and in none the production of urease was detected. In one strain isolated from Cabo Rojo a dark precipitate was clearly observed. The east side all strains were able to grow on NaCl 9%, glycerol 0.5% and on most carbon sources such as: maltose, sucrose, lactose and starch. In strains that degrade starch also liquefaction in gelatine media was observed.

Some of the strains were tested for their growth in media containing phenanthrene and naphthalene (not shown) individually. First a Minimum Inhibitory Concentration (MIC) was determined through serial dilutions in order to confirm the concentration of the hydrocarbons in which the control is not able to grow (*E. coli* JM109). This final concentration was found to be

0.16 µg/mL of phenanthrene in a 1/25 dilution factor. From this concentration on, both controls were able to grow including the control of *V. fischeri* (ATCC 7744). On experimental samples, optical densities were detected higher in the media lacking phenanthrene (Table 2.2). This was a tendency along the strains tested which included isolates from the west, east and southern area of Puerto Rico. From a clinical perspective isolates were able to show antibiotic resistance. Most isolates were able to grow in a final concentration of 50 µg/mL of ampicillin. On the other hand, none were resistant to chloramphenicol. In the west and south are of Puerto Rico a total of five strains were found to have kanamycin resistance. Only in the north 3 candidates were found to be resistant to tetracycline. Finally in the detection of beta-galactosidase 50% of the northern and western isolates were capable to degrade x-gal as a substrate. In the south area only a 25% and in the east no activity was detected.

Figure 2.1 Bioluminescent strains were isolated randomly from a total of 31 different municipalities. Water sources include beaches and estuaries located both on the Atlantic Ocean and Caribbean Sea (red star=open areas such as beaches, blue star=estuary)(http://www.puertorico.com/images/map-new.gif.).

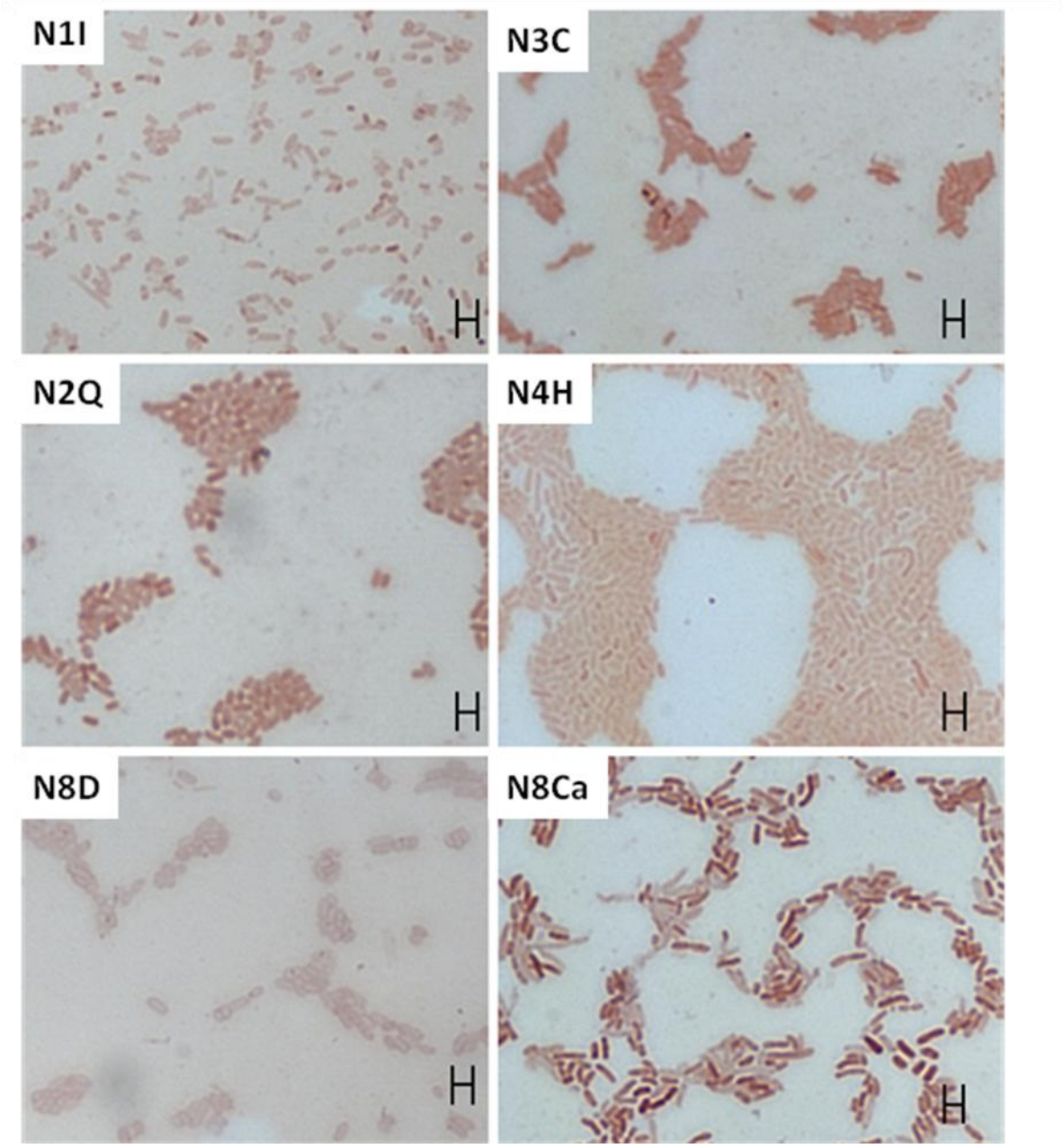


Figure 2.2 Bioluminescent isolates from the north of Puerto Rico. Bacteria were observed using light microscopy after Gram staining at a 1000X magnification. All strains were gram-negative varying in difference lengths of bacilli. Scale length is 2 µm.

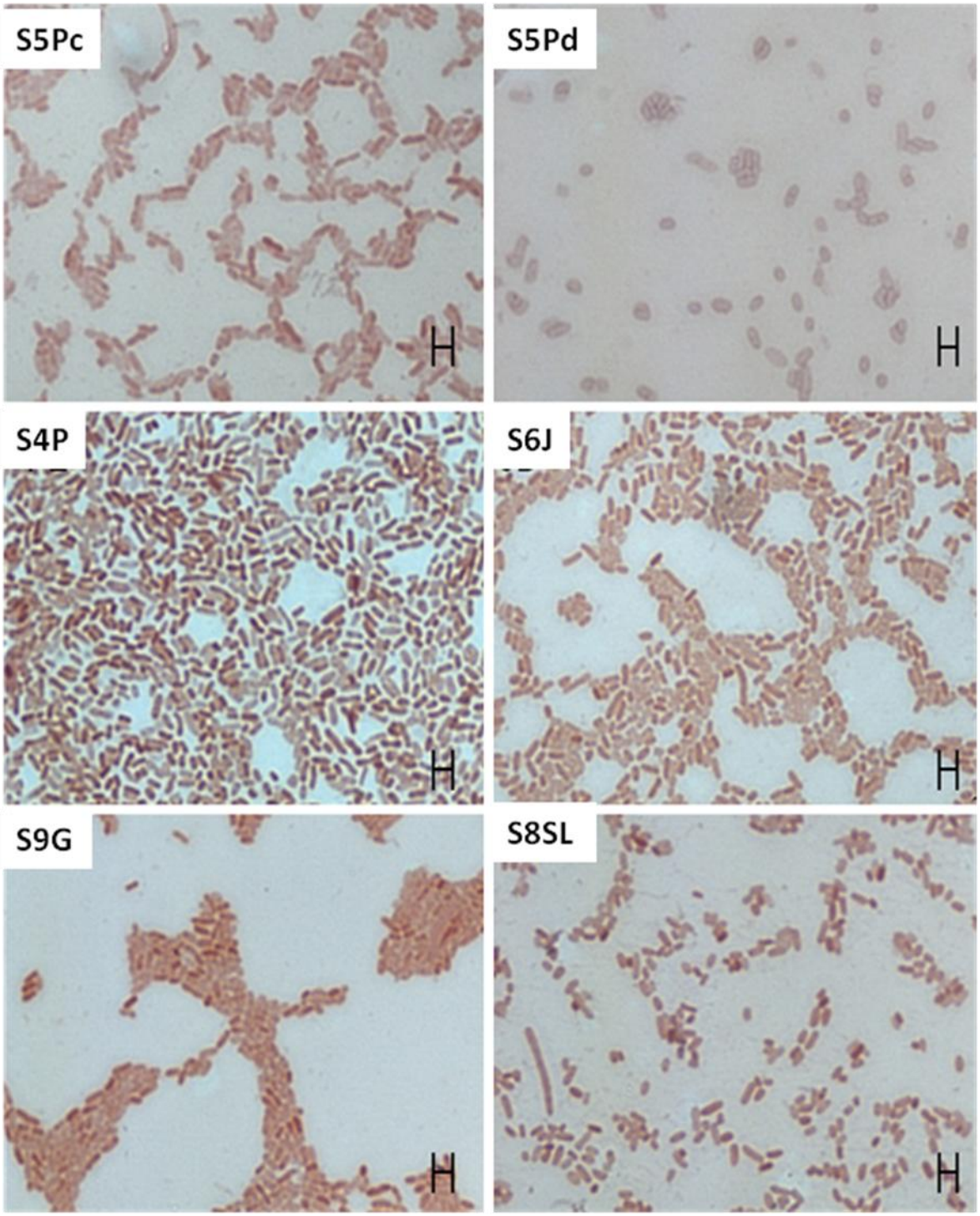


Figure 2.3. Microscopy of bacterial bioluminescent isolates from the South of Puerto Rico. Bacteria were observed through Gram stain in a light microscope 100X. All strains were gram-negative, one short bacilli was observed. Scale length is 2um.

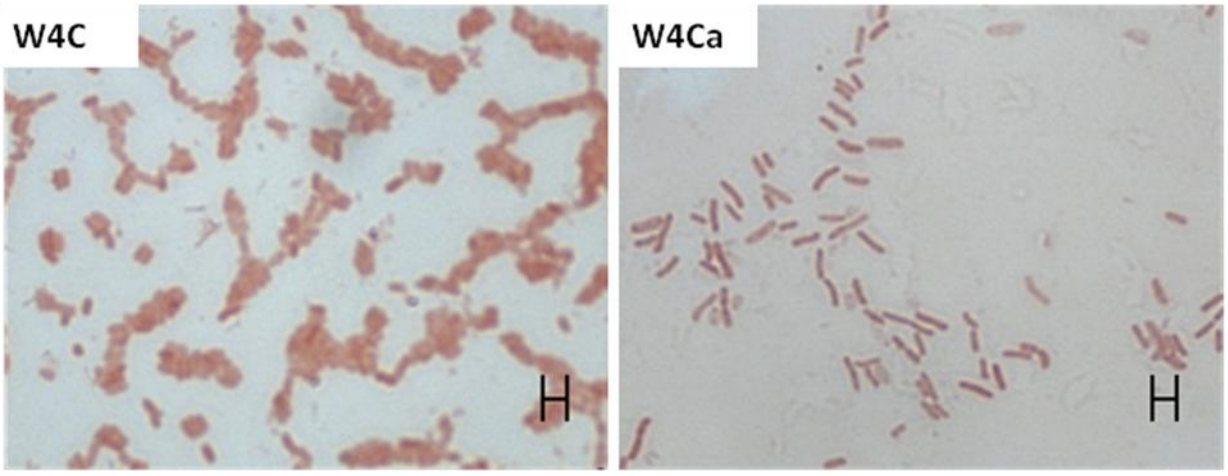


Figure 2.4 Bioluminescent isolates from the west of Puerto Rico. Bacteria were observed using light microscopy after Gram staining at a 1000X magnification. All strains were gram-negative, one long straight rod was observed. Strain W4C capable of hydrogen sulfide production. Scale length is µm.

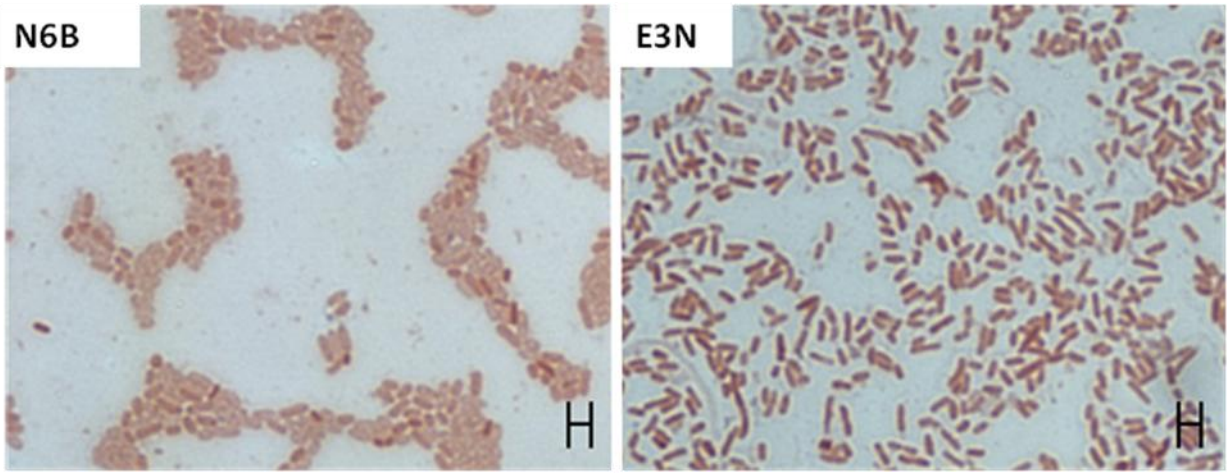


Figure 2.5 Bioluminescent isolates from the East of Puerto Rico. Bacteria were observed using light microscopy after Gram staining at a 1000X magnification. All strains were gram-negative, one long straight rod was observed. Scale length is 2 µm.

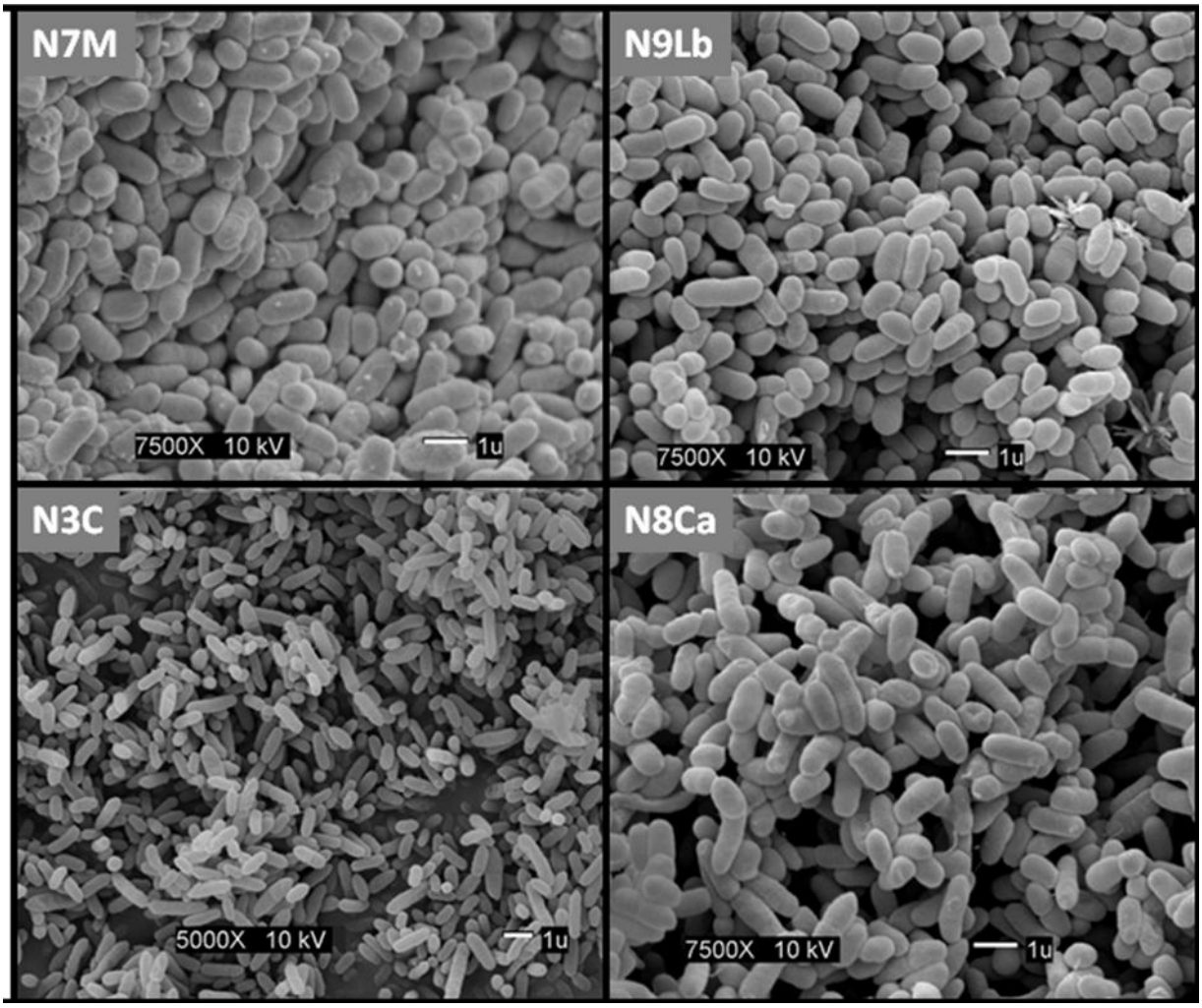


Figure 2.6 Scanning electron microscopy from bioluminescent candidates of the north area (Biology Department Microscopy Center). Sampled Municipalities were: Manati (N7M), Camuy (N3C), Carolina (N8Ca) and Loiza (N9Lb).

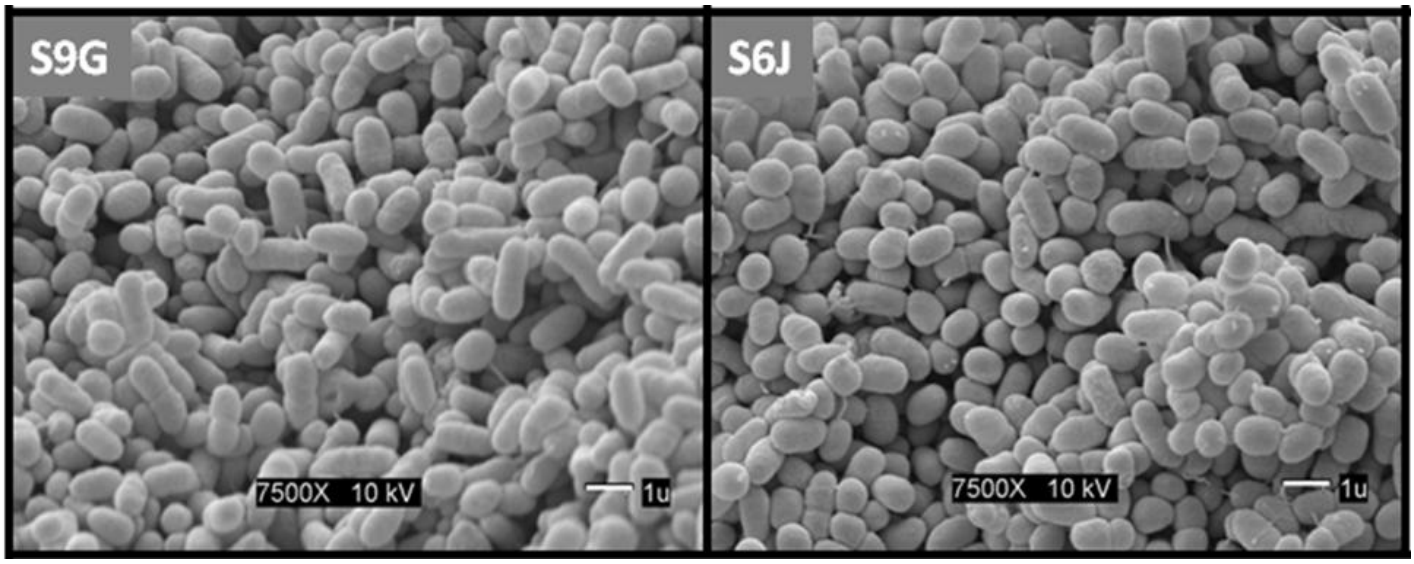
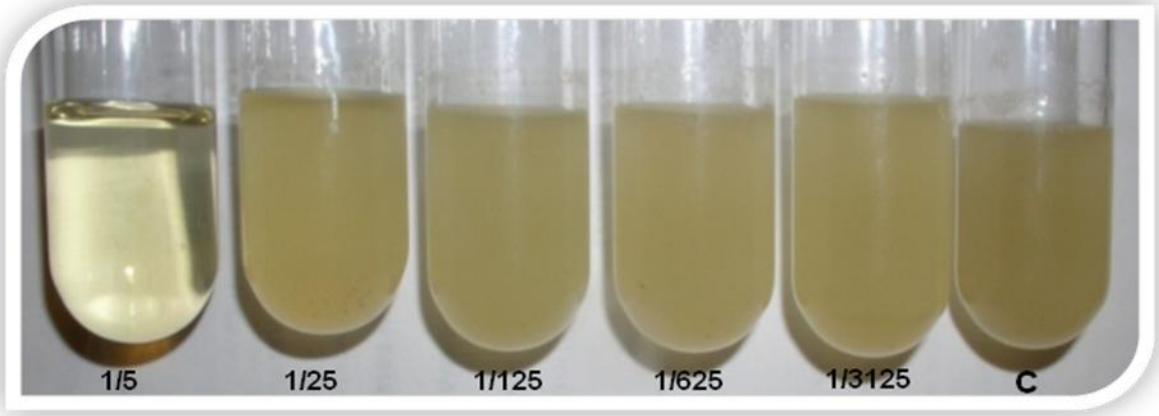


Figure 2.7 Scanning electron microscopy from bioluminescent candidates of the south area (Biology Department Microscopy Center).

A.



B.

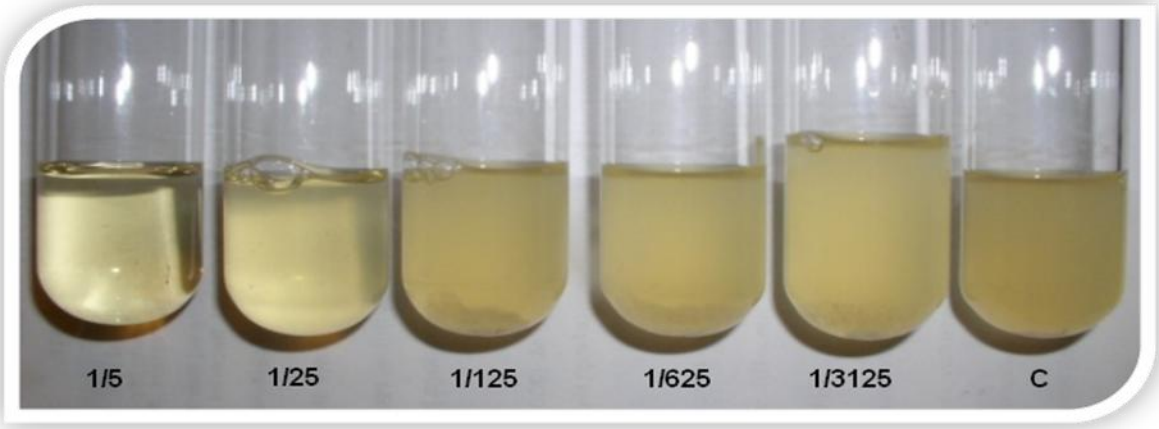


Figure 2.8 Minimum inhibitory concentration for controls. Evidence of hydrocarbon dilution (phenanthrene) that inhibits the growth for both controls (B for *E.coli* JM109, A for *V. fischeri*). C is for the control lacking any phenanthrene. Final phenanthrene concentrations were: 1/5(0.8µg/mL), 1/25(0.16µg/mL), 1/125(0.032µg/mL), 1/625(0.0064µg/mL) and 1/3125 (0.00128µg/mL).

Table 2.1 Location through GPS coordinates of each water sample collected

Sample	Municipality	Beach	Coordinates
N1I	Isabela	Jobos	(18.5135, -67.0775)
N2Q	Quebradillas	Guajataca	(18.4640, -66.9274)
N3C	Camuy	Penon	(18.4844, -66.8449)
N4H	Hatillo	Outlets	(18.4867, -66.8255)
N5A	Arecibo	Sardinera puente	(18.4750, -66.7158)
N6B	Barceloneta	Mar chiquita	(18.4840, -66.5386)
N7M	Manati	Los tubos	(18.4691, -66.4862)
N8D	Dorado	Cerromar	(18.4600, -66.2676)
N8Ca	Carolina	Balneario	(18.4418, -65.9097)
N8Cb	Carolina	Balneario	
N9La	Loiza	Pinones	(18.4460, -65.9085)
N9Lb	Loiza	Pinones	
N10La	Luquillo	Balneario	(18.3854, -65.7301)
N10Lb	Luquillo	Balneario	
W1A	Aguadilla	Ruinas	(18.4382, -67.1609)
W2R	Rincon	Faro	(18.3381, -67.2516)
W3CO	Añasco	Hermanos	(18.2720, -67.1753)
W3M	Mayagüez	Bahía	(18.2039, -67.1450)
W4C	Cabo Rojo	Mangle	(17.9590, -67.1789)
W4Ca	Cabo Rojo	Hotel sucia	(17.9467, -67.1983)
S1G	Juanica	Ballena	(17.9530, -66.8572)
S2Ya	Yauco	Ventana	(17.9508, -66.8417)

Sample	Municipality	Beach	Coordinates
S2Yb	Yauco	Ventana	(17.9508, -66.8417)
S3G	Guayanilla	Triangulo	(17.9805, -66.7949)
S4P	Peñuelas	Puente	(17.9953, -66.7163)
S5Pa	Ponce	Playa Ponce	(17.9689, -66.6114)
S5Pb	Ponce	Hilton	(17.9852, -66.6260)
S5Pc	Ponce	Hilton	(17.9852, -66.6260)
S5Pd	Ponce	Guancha	(17.9826, -66.6145)
S6J	Juana Diaz	Punta Pastillo	(17.9917, -66.4966)
S7S	Santa Isabel	Punta Petrona	(17.9664, -66.3608)
S8SL	Salinas	Bo Coquí	(17.9618, -66.3177)
S9G	Guayama	Punta Barrancas	(17.9409, -66.1907)
S10P	Patillas	Punta Viento	(17.9684, -65.9764)
S11M	Maunabo	Bohío	(17.9937, -65.9060)
E1F	Fajardo	Seven Seas	(18.3440, -65.6357)
E2C	Ceiba	Bahía de Puerca	(18.2241, -65.6019)
E3N	Naguabo	Playa Naguabo	(18.1788, -65.6904)
E4Y	Playa lucia	Pnta Guayanes	(18.0564, -65.7741)

Table 2.2 Optical densities of bioluminescent isolates in phenanthrene. A 100µL of cell suspension were incubated at 32 °C for a minimum of 48hrs. Control= no phenanthrene(*)

Sample	OD (600nm)	Sample	OD (600nm)
VF*	1.21	W4Ca *	3.48
VF	1.492	W4Ca	0.033
W4C *	3.092	N1I *	0.433
W4C	0.073	N1I	0.040
S9G *	3.036	S6J *	2.523
S9G	0.020	S6J	0.003

Table 2.3 Physiological characterization of bioluminescent isolates southern marine environments. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	Catalase	Citrate	DNAse	Urease	Starch	Mannitol	Glucose	Lactose	Sucrose	Maltose (.2%)	Glycerol (.5%)	Gelatin
S1G	+	+	+	-	+	+	+	+	+	+	+	-
S2Ya	+	+	-	-	-	-	+	+	+	-	-	-
S2Yb	+	+	-	-	-	+	+	+	+	-	-	-
S3G	+	-	+	+	-	+	+	+	+	+	+	-
S4P	+	+	-	-	+	+	+	+	+	+	+	+
S5Pa	-	+	+	-	-	+	+	+	+	+	+	-
S5Pc	+	+	+	-	+	+	+	-	-	+	+	+
S5Pd	+	+	+	-	+	+	+	+	+	+	+	+
S6J	+	+	+	-	+	+	+	+	+	+	+	-
S8SL	+	+	+	-	+	+	+	+	+	+	+	-
S11M	-	+	-	-	-	-	+	+	+	-	-	-

Table 2.4 Physiological characterization of bioluminescent isolates northern marine environments. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	Catalase	Citrate	DNAse	Urease	Starch	Mannitol	Glucose	Lactose	Sucrose	Maltose (.2%)	Glycerol (.5%)	Gelatin
N1I	+	+	+	-	+	+	+	+	+	+	+	+
N2Q	+	+	+	-	+	+	+	+	+	+	+	+
N3C	-	+	+	+	+	+	+	+	+	+	+	+
N4H	+	+	+	+	+	+	+	+	+	+	+	+
N5A	+	-	+	-	-	+	+	+	+	+	+	-
N7M	+	+	+	+	+	+	+	+	+	+	+	+
N8D	+	+	+	-	+	+	+	+	+	+	+	+
N8Ca	+	+	+	-	+	+	+	+	+	+	+	+
N8Cb	+	+	+	-	-	+	+	+	+	+	+	-
N9Lb	+	+	+	-	+	+	+	+	+	+	+	+
N10La	+	-	+	-	-	+	+	+	+	+	+	-
N10Lb	+	+	+	-	+	+	+	+	+	+	+	+

Table 2.5 Physiological characterization of bioluminescent isolates eastern marine environments. Positive growth (+), no growth (-). Incubation at 25°C for a minimum of 24hrs.

Sample	Catalase	Citrate	DNAse	Urease	Starch	Mannitol	Glucose	Lactose	Sucrose	Maltose (.2%)	Glycerol (.5%)	Gelatin
E1F	+	+	+	-	+	+	+	+	+	+	+	+
E2C	+	+	+	-	+	+	+	+	+	+	+	+
E3N	+	+	+	-	+	+	+	+	+	+	+	+
E4Y	+	+	+	-	+	+	+	+	+	+	+	+

Table 2.6 Physiological characterization of bioluminescent isolates western marine environments. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	Catalase	Citrate	DNAse	Urease	Starch	Mannitol	Glucose	Lactose	Sucrose	Maltose (.2%)	Glycerol (.5%)	Gelatin
W2R	+	+	+	-	+	+	+	+	+	+	+	+
W3CO	-	+	-	-	-	+	+	+	+	-	-	-
W3M	+	+	+	-	+	+	+	+	+	+	+	+
W4C	+	+	+	-	+	+	+	-	-	+	+	+
W4Ca	+	+	+	-	+	+	+	+	+	+	+	+

Table 2.7 Antibiotic resistance and detection of beta-galactosidase in northern isolates. Growth (+), no growth (-). Incubation at 25 °C for 12 hrs.

Sample	ampicillin (50 µg/mL)	tetracycline (10 µg/mL)	kanamycin (25 µg/mL)	chloramphenicol (34 µg/mL)	beta-galactosidase
N1I	+	-	-	-	-
N2Q	+	-	-	-	+
N3C	+	-	-	-	-
N4H	+	-	-	-	-
N5A	+	-	-	-	-
N6B	+	-	-	-	-
N7M	+	-	-	-	-
N8D	+	-	-	-	+
N8Ca	+	-	-	-	+
N8Cb	+	-	-	-	+
N9La	-	+	-	-	-
N9Lb	+	-	-	-	-
N10La	-	+	-	-	+
N10Lb	-	-	-	-	+

Table 2.8 Antibiotic resistance and detection of beta-galactosidase in western isolates. Growth (+), no growth (-). Incubation at 25 °C for 12 hrs.

Sample	ampicillin (50 μg/mL)	tetracycline (10 μg/mL)	kanamycin (25 μg/mL)	chloramphenicol (34 μg/mL)	beta-galactosidase
W1A	-	-	-	-	-
W2R	+	-	-	-	+
W3CO	-	-	+	-	-
W3M	+	-	-	-	+
W4C	+	-	-	-	-
W4Ca	+	-	-	-	+

Table 2.9 Antibiotic resistance and detection of beta-galactosidase in southern isolates. Growth (+), no growth (-). Incubation at 30 °C for 12 hrs.

Sample	ampicillin (50 µg/mL)	tetracycline (10 µg/mL)	kanamycin (25 µg/mL)	chloramphenicol (34 µg/mL)	beta-galactosidase
S1G	+	-	-	-	+
S2Ya	-	-	+	-	-
S2Yb	-	-	+	-	-
S3G	-	-	+	-	-
S4P	+	-	-	-	-
S5Pa	-	-	+	-	+
S5Pb	+	-	-	-	-
S5Pc	-	-	+	-	-
S5Pd	+	-	-	-	-
S6J	+	-	-	-	-
S7S	-	-	-	-	-
S8SL	+	-	-	-	-

Table 2.10 Antibiotic resistance and detection of beta-galactosidase in eastern isolates. Growth (+), no growth (-). Incubation at 25 °C for 12 hrs.

Sample	ampicillin (50 µg /mL)	tetracycline (10 µg /mL)	kanamycin (25 µg /mL)	chloramphenicol (34 µg /mL)	beta-galactosidase
E1F	+	-	-	-	-
E2C	+	-	-	-	-
E3N	+	-	-	-	+
E4Y	+	-	-	-	+

2.4 Discussion

In order to distinguish among the members of the bacterial bioluminescent collection a physiological profile was developed based on biochemical assays reported in scientific literature (Georges et al., 2005). This data serve as a tool to confirm the diversity of the bioluminescent candidates collected around the island of PR. A minimum of one distinct result in any of the commercial media used for the biochemical assays was accepted as different strain among the collection. Positive results from the starch and nutrient gelatine media were used to confirm the possible presence of *Vibrio harveyi* in the collection. Strains from the north (Table 2.4) of PR were similar regarding the degradation of carbohydrates, their physiological profile were almost identical including common positive results on essays such as mannitol, glucose, lactose and sucrose. A total of 9 candidates were similar to *Vibrio harveyi* due to their capability to degrade both starch and gelatine. Only one sample was catalase negative and one of the isolates were positive in the cleavage of hydrogen peroxide. This result suggests that theses catalase positives strains are close to member of the genus *Photobacterium*.

Southern strains have a more diverse physiological profile was seen due to the observation of gas production in the presence of glucose (Figure 2.3). A total of six strains were similar to *Vibrio harveyi*, this resemblance includes catabolism on glycerol, maltose, citrate and mannitol. Another similarity is seen in sample S5Pc which is not able to metabolise lactose and sucrose. The *in silico* data suggested this strain to be *Vibrio orientalis* (Georges et al., 2005).

Candidates from the west area were unique (Figure 2.6) due to the possible presence of hydrogen sulphide observed. Candidates from this area were exposed to 0.16µg of phenanthrene which had a higher optical density than when inoculated with *E.coli* JM109 as a control. This bioluminescent strain was capable of growing to an absorbance of 3.48 without phenanthrene

and 0.033 in the presence of this hydrocarbon. This final O.D. confirms that most of the strains are not able to grow optimally in the presence of phenanthrene. Therefore, the additional strategies in order to detect phenanthrene should be consider such as transferring luciferase coding genes into strains that can grow better in this toxic organic compound.

An interesting and concerning data from this study was finding strains in the collection resistant to ampicillin, kanamycin and tetracycline. Most studies regarding antibiotic resistance are focused on specific groups of bacteria, most which are gram-positive such as methicillin resistant *Staphylcoccus aureus* (MRSA) that routinely captures the headlines. Gram-negative pathogens attract less attention, even though their emergence is also associated with serious public concerns (Rice et al., 2006). Additional studies are being published that confirms groups of gram-negative bacteria that are resistant to broad spectrum antibiotics such *Pseudomonas aeruginosa*, *Klepsiella pneumoniae* and *Acinetobacter baumannii* (Fish et al., 2006). Bioluminescent strains in this study have also resistance to the same antibiotics of previously described strains. In the north side 71% of the strains showed resistance to ampicillin and and only 21% to tetracycline. In the west side 66% were found to be resistant to ampicillin and only 16% to kanamycin. In the east side all strains were only resistant to ampicillin. This result suggests that isolates have mechanisms of protecting their cell wall through expression of beta-lactamases or closely related enzymes. In the case of tetracycline resistance it is possible that members of this collection can express a membrane protein that actively exports tetracycline out of the cell (Foster et al., 1983; Garrett et al., 2000). These findings can contribute to additional and unstudied groups of bacteria that can possibly affect clinical issues in society and it will serve to upgrade additional members of *Vibrionaceae* Family that are resistant to ampicillin, kanamycin and others. Some species of this family already studied include *V. vulnificus, V. alginolyticus, V. parahaemolyticus*

and *V. logei* (Yie et al., 1998). Most studies of bioluminescent bacteria involve their isolation, characterization and taxonomy through physiological and molecular tools as shown is this study. Most reports have not been carried out the Caribbean. For example, a study performed after the only publication in Puerto Rico (Ruby et al., 1980) was in an estuary located in Mystic River, Connecticut (Feldman et al 1984). The study consisted in the isolation of 111 bioluminescent isolates from nine different stations where 93% of the isolates were closely related to *V.harveyi*. Also this specie was predominant in water containing high nutrient concentrations. A different data in this study were the growth of the strains in NaCl which were able to grow in concentrations that ranged from 6% to 30%, no strains grew below this percentage (Nealson et al., 1983).

Another studied location (Galveston, Texas) was based on *Beneckea harveyi* (now known as *V. harveyi*) distribution and detection over *Photobacterium* groups. A collection of 79 bioluminescent strains were collected from water, marine sediment and shrimp intestines. A 100% was identified as *B. harveyi.* When samples were collected from below 200 to 2,000 members of the *Photobacteriaceae* Family were detected. In this study the physiological profile used was similar. Some carbon sources tested were maltose, mannitol and lactate (O'brien et al., 1979). Detection of enzymes included amylase and gelatinase to confirm the presence of *V. harveyi*.

Physiological knowledge was gained on the bioluminescent bacteria group by studies conducted in the Gulf of Elat and the Mediterranean Gulf. By sampling the waters of these sites the effect of seasonal changes was determined. In the appearance of winter the presence of *V. fischeri* (also known as *Photobacterium fischeri*) was detected while in summer *V. harveyi* was dominant (Shilo and Yetinson, 1979).

Additional studies have been performed with bioluminescent strains isolated from Kuwait, the Gulf of Arabia and in the Indo-Pacific Sea (Makemson et al, 1992; Lapota et al., 1988; Yang et al., 1983). Even though a high amount of information have been discovered about bioluminescent bacteria, there are still some questions to be answered; little is known about their ecology, because some species may exhibit a variety of different behaviors inhabiting several niches including saprophytic, symbiotic and parasitic. Neither the dynamics of bioluminescent bacterial populations have been understood nor most of their distribution and abundance (Nealson et al., 1993; Hastings et al., 1981).

Chapter Three

Phylogenetic analysis and detection of luciferase coding genes present in bioluminescent isolates

3.1 Introduction

In bioluminescence usually there are enzymes termed luciferases, the substrate for this reaction varies among organism such as dinoflagellates, bacteria, fungi, fireflies and worms. Even though in many examples the principle of bioluminescence is similar most luciferases don't have a similar origin from an evolutionary perspective (Engebrecht et al., 1983).

In bacteria, luciferases are coded by two different genes (*luxA* and *luxB*) which form a heterodimeric enzyme. Both units have an aminoacid identity of 30% and unidentical in all bioluminescent strains (Friedland et al., 1957). Luciferase is responsible of catalyzing the oxidation of a reduced flavin mononucleotide with a long fatty aldehyde that results in the emission of visible and quantifiable light close to 490nm (Rozen et al., 1999). Luciferases have been purified from a number of various strains including *Photobacterium leiognathi*, *P.phosphoreum*, *Vibrio harveyi*, *Vibrio fischeri* (also known as *P. fischeri*) and the only terrestrial specie *Photorhabdus luminescens* (Ferri et al., 1991). Structurally, the luciferase enzyme is simple and has no metals, nor prosthetic groups or non-amino acid residues (Cline et al., 1970; Paquatte et al., 1989).

There are some advantages of using *luxAB* genes reporters for biosensor systems. Most luminescent assays require a few seconds. It has been demonstrated that one picogram of luciferase can be detected with scintillation counters and that costs of materials (aldehyde and $FMNH_2$) are not expensive (Kohler et al., 2000). Lux genes can not only been used as reporters but can also be transferred or transformed into other species capable of hydrocarbon degradation or remediation of metals for which light emission serves as a signal for the presence of these processes. In addition, *luxAB* genes are chosen over other reporters due to its sensitivity. The lux cassettes are directly dependant on a stable metabolism which any toxic disturbance would

result in a change of light emission (Ptitsyn et al., 1997). A luciferase biosensor can be used as whole cell models or just using luciferase genes (Polyak et al., 2000). Some analytes detected at picomolar concentrations by these systems include: mercury, naphthalene, estrogen, iron and phosphorus. Different strategies have been used to detect expression of genes related with membrane damage, DNA alkylation and physiological depravation through making transcriptional fusion of an interest gene with luciferase coding DNA (Elsemore, 1998; Vollmer et al., 1997).

After bioluminescent isolates were characterized using physiological and microbiological approaches partial *luxA* sequences were amplified, sequenced and analyzed *in silico* for a the identity of the isolates. Phylogenetic approach provided a superior idea of the diversity among the collection. In addition, the first bank of luciferase sequences will be available from Puerto Rican sites in the Caribbean, which will complement other studies.

3.2 Material and methods

DNA extraction

In order to confirm the identity of the isolates based on the physiological and microbiological data, molecular analysis was started by performing genomic DNA extraction. DNA was extracted using the methodology described by Chen and Kuo (1993). Briefly, the samples were grown in LA (3%NaCl) for 24 hours and then 1.5 µl of the sample were centrifuged to form a bacterial pellet. Cell lysis was performed using lysis buffer (40mM Tris-acetate pH 7.8, 20mM sodium-acetate pH 8.0, 1.0mM EDTA pH 8.0 and 1% SDS) and sodium chloride 5M, then treated with RNAse (20 µg/µl) for 30 minutes at 37 ºC. An organic extraction was made adding one volume of chloroform followed by absolute ethanol precipitation. The isolated genomic DNA was resuspended in 50 µl of deionised sterile water. The final concentration of the DNA was quantified using an Eppendorf Biophotometer (260nm).

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify the 16S unit with universal primers for bacteria (27F, 5-AGAGTTTGATCMTGGCTCAG and R 1492, 5-TACGGYTACCTTGTTACGACT-3). Amplification parameters for bacterial 16S rDNA were as follow: an initial denaturation of 94°C for 3 min., 30 cycles of (94°C 30 sec., 52.7°C 30 sec., and 72°C 1.30 min). DNA sequencing of the PCR product was done in the facilities Macrogene USA (www.macrogenusa.com). *In silico* analysis was done by using available online databases such as Gen Bank in NCBI using the program BLAST. For the amplification of *luxA* primers were used for luciferase (*lux 7* , 5-AAAGGATCCTCAGAACCGTTTGCTTCAAAACC-

3 and *lux8*, 5- ACACAAGCTTCTACTGGATCAAATGTCAAAAGGACG-3)

The following parameters were used: 95°C for 5 min, next 5 cycles were run 94°C for 30 sec, 37°C for 1min and 72°C for 1min. Next, 25 cycles will be the same but with a temperature of 55°C as the annealing.

Phylogenetic Analysis

The 16S rDNA sequences were edited using the Chromas Lite 2.0.0 and then aligned and edited with BioEdit 7.0.0. Phylogenetic analysis was performed using MEGA 5 BETA version 6.1 program. The distance model used was p-distance and the bootstrap test of phylogeny was calculated for 2000 replicates. The construction of the consensus tree was performed by the Neighbor Joining method.

3.3 Results

Products from both 16S rDNA and *luxAB* were successfully amplified and sequenced from a total of 31 bioluminescent strains. An average close to 900 bp was obtained from an expected 1,500bp product (Figure 3.1). For the luciferase coding DNA a size close to 700 bp was obtained from the expected average of 2000 bp for the luciferase coding genes (Figure 3.2). For the phylogenetic analysis both trees had a total of 2000 repetitions. For the first tree out groups were used to confirm the quality of the analysis: *Saccharomyces cerevisiae* and *Escherichia coli*. For both trees known sequences were chosen as control positives were *S. hanedai, P. luminescens, V. cholera, V. fischeri, V. logei, P. phosphoreum, P. mandapamensis, P. leiognathi* and *P. kishitanii*. For the luciferase tree know the out group was *luxA* from *Photinus pyralis* (Figure 3.3).

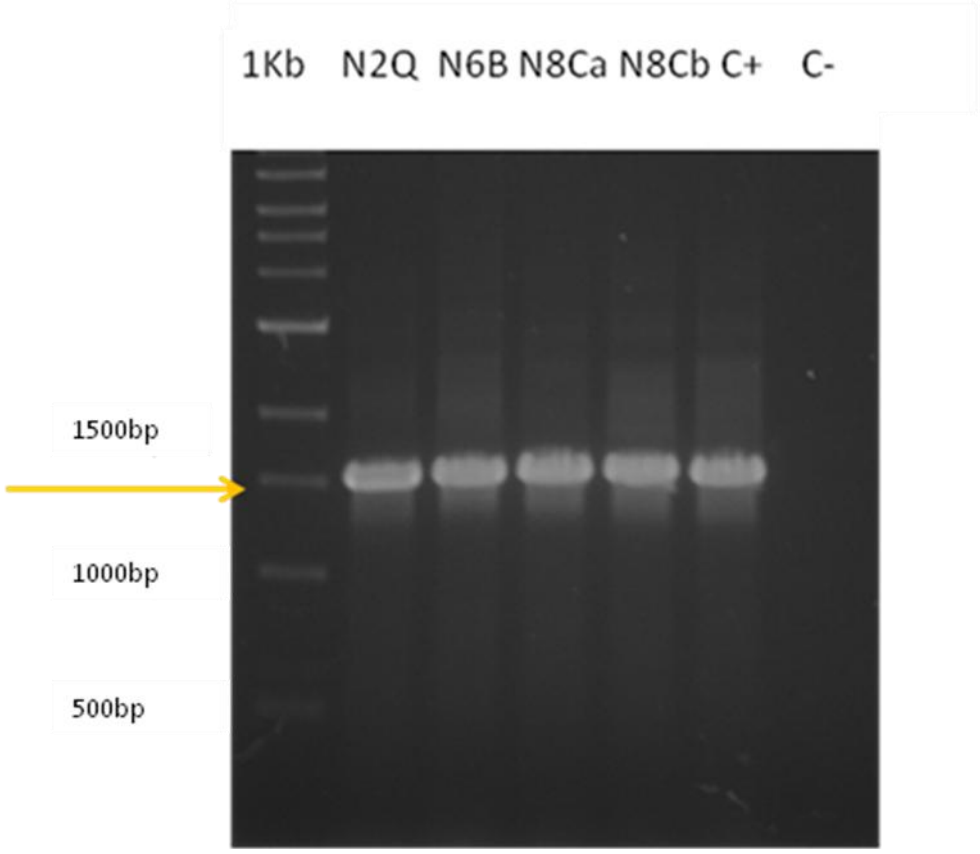


Figure 3.1 The 16S rDNA amplification of bioluminescent isolates .8% agarose. The molecular marker was 1Kb ladder (NEB). The positive control (C+) and the negative control (-) was the PCR mixture without DNA. The amplification of the all strains shows a 1.5Kb band of approximate size.

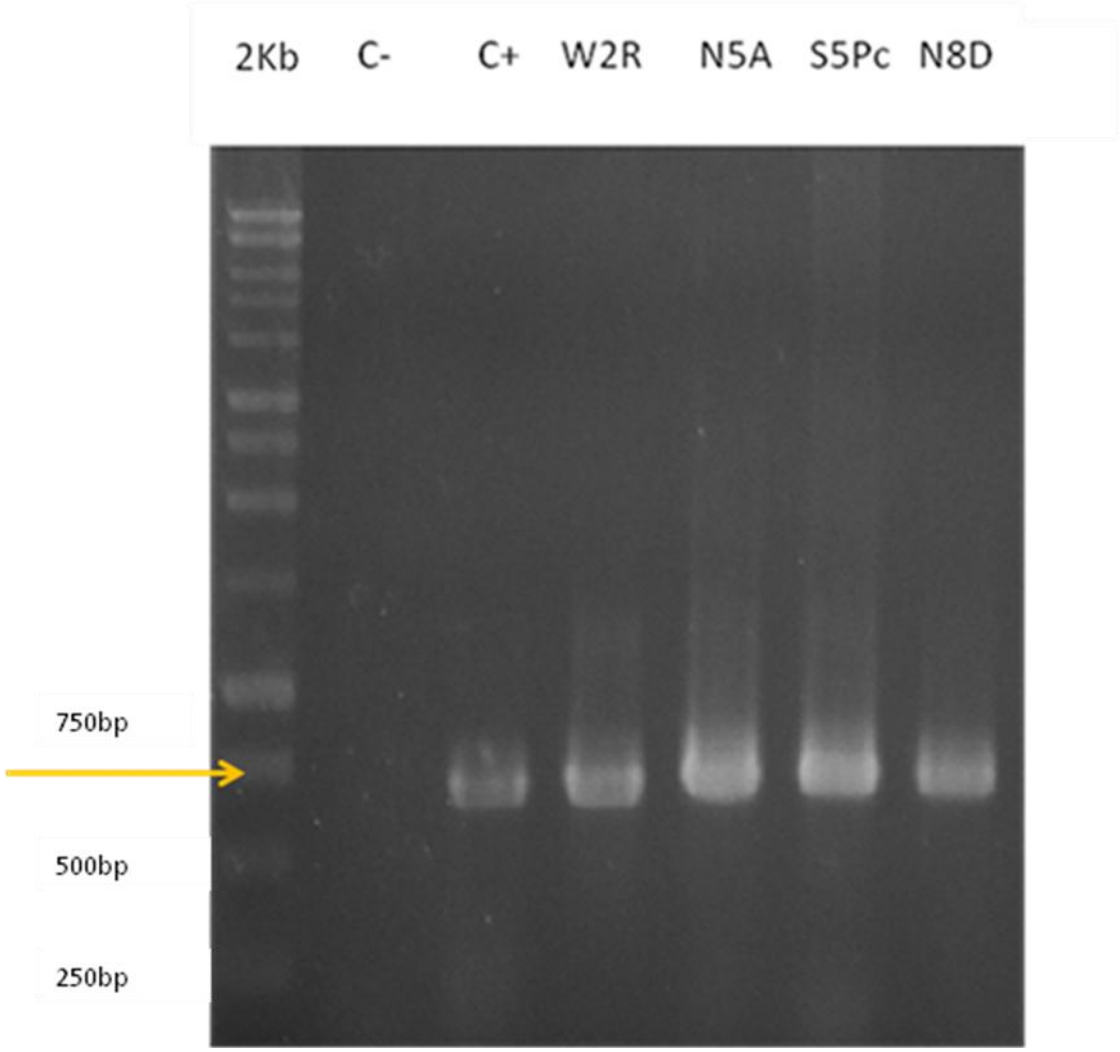
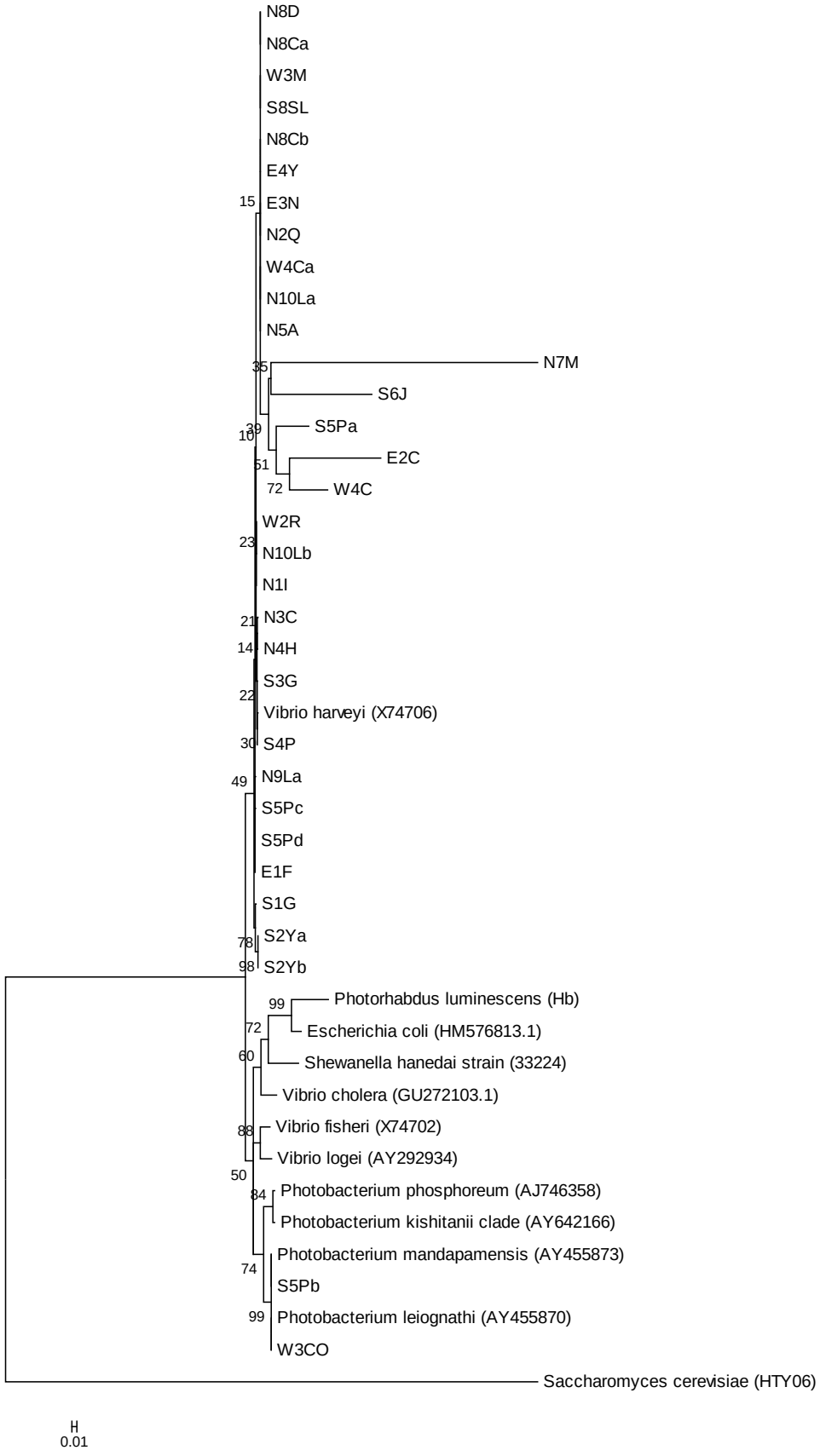
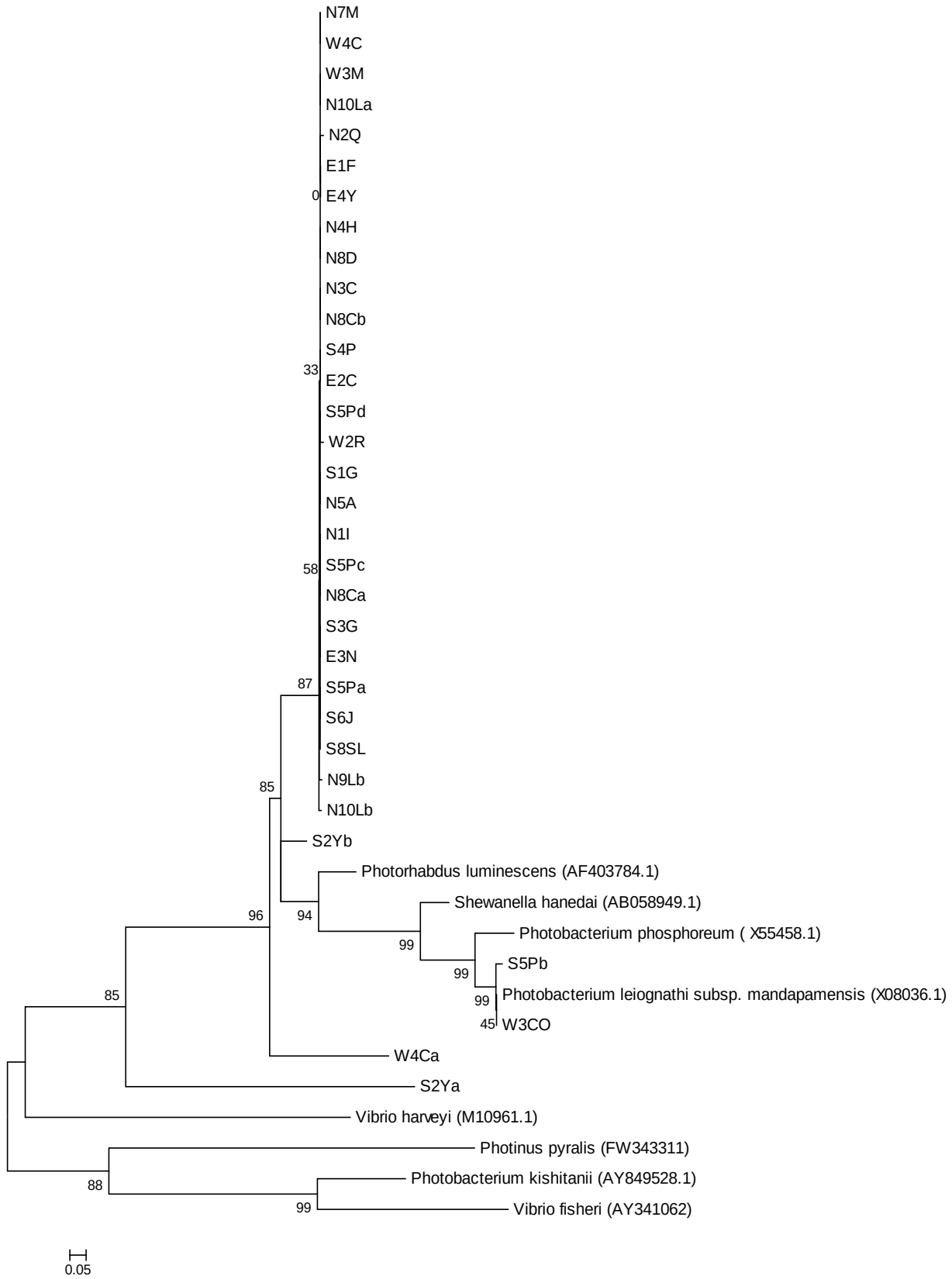


Figure 3.2 The luciferase amplification of bioluminescent isolates .8% agarose. The molecular marker was 2Kb ladder (Promega). The positive control (C+) and the negative control (-) was the PCR mixture without DNA. The amplification of the all strains shows a 750bp band of approximate size.

A.


N8D
N8Ca
W3M
S8SL
N8Cb
E4Y
15
E3N
N2Q
W4Ca
N10La
N5A
35
N7M
S6J
39
10
S5Pa
51
E2C
72
W4C
W2R
23
N10Lb
N1I
21
N3C
14
N4H
S3G
22
Vibrio harveyi (X74706)
30
S4P
49
N9La
S5Pc
S5Pd
E1F
S1G
78
S2Ya
98
S2Yb
99
Photorhabdus luminescens (Hb)
72
Escherichia coli (HM576813.1)
60
Shewanella hanedai strain (33224)
Vibrio cholera (GU272103.1)
88
Vibrio fisheri (X74702)
Vibrio logei (AY292934)
50
84
Photobacterium phosphoreum (AJ746358)
Photobacterium kishitanii clade (AY642166)
Photobacterium mandapamensis (AY455873)
74
S5Pb
99
Photobacterium leiognathi (AY455870)
W3CO
Saccharomyces cerevisiae (HTY06)
H
0.01


N7M
W4C
W3M
N10La
N2Q
E1F
0
E4Y
N4H
N8D
N3C
N8Cb
S4P
33
E2C
S5Pd
W2R
S1G
N5A
N1I
58
S5Pc
N8Ca
S3G
E3N
87
S5Pa
S6J
S8SL
N9Lb
85
N10Lb
S2Yb
Photorhabdus luminescens (AF403784.1)
Shewanella hanedai (AB058949.1)
96
94
Photobacterium phosphoreum (X55458.1)
99
S5Pb
99
85
99
Photobacterium leiognathi subsp. mandapamensis (X08036.1)
45
W3CO
W4Ca
S2Ya
Vibrio harveyi (M10961.1)
Photinus pyralis (FW343311)
88
Photobacterium kishitanii (AY849528.1)
99
Vibrio fisheri (AY341062)
0.05

B.

Figure 3.3 Phylogenetic analyses of the 16S rDNA and *luxAB* sequencing. The development of the consensus tree was made by the Neighbour-Joining method and the final tree was drawn with the use of MEGA 5 Beta version 6.1. Numbers in the nodes are the bootstrap values. For the first figure A (16S rDNA) and B (luciferase) the bar represents 0.01 and 0.05 substitutions per nucleotide position. A total of 2000 repetitions based in a pair wise arrangement were performed to produce both trees. For the first tree (A) *Escherichia coli* (HM576813.1) and *Saccharomyces cerevisiae* (HTY06) were used as out groups and for the second tree (B) *Photinus pyralis* (FW313311) was used.

Table 3.1 *In silico* analysis documentation of sequenced 16SrDNA using basic local alignment search tool (BLAST) as data base

Sample	Accession number	16SrDNA organism	Coverage	E-Value	Identity
S5Pc	FJ161348.1	*V. harveyi* strain D7047	100%	0.0	99%
S5Pd	FJ161348.1	*V. harveyi* strain D7047	100%	0.0	99%
S4P	GQ406738.1	*Vibrio sp.* PaH2.06	100%	0.0	100%
S3G	GQ406738.1	*Vibrio sp.* PaH2.06c 16S	100%	0.0	99%
S2Ya	EF199916.1	*Vibrio sp.* DH108 16S	100%	0.0	99%
S2Yb	EF199916.1	*Vibrio sp.* DH108 16S	100%	0.0	99%
S1G	GQ391933.1	*Vibrio sp.* PaH1.12 16	99%	0.0	100%
W3CO	EU807747.1	*Photobacterium sp.* Raj-7	100%	0.0	100%
W2R	GQ406636.1	*Vibrio sp.* PaD3.04	100%	0.0	99%
N1l	FJ161347.1	*V. harveyi* strain D7046	100%	0.0	99%
N3C	HM590018.1	Uncultured *Vibrio sp*. clone	100%	0.0	99%
N4H	GQ406768.1	*Vibrio sp.* PaH2.22	100%	0.0	100%
N7M*	HQ161745.1	*V. harveyi* strain F75047	90%	2e-47	68%
S5Pa	FJ981864.1	Vibrio sp. BBT42 16S	98%	0.0	90%
S6J	EF584106.1	Vibrionaceae bacterium	97%	0.0	81%
N6B	HM565977.1	*Vibrio sp.*. 08XMYD-1	96%	0.0	82%
W4C	HM565977.1	*Vibrio sp.* 08XMYD-1	96%	0.0	88%
N9Lb	HM008702.1	*V. harveyi* strain NB	100%	0.0	99%
N8D	GQ391965.1	*Vibrionaceae* bacterium PaH	100%	0.0	99%
W3M	GQ406646.1	*Vibrio sp.*. PaD3.13	99%	0.0	100%
N8Ca	GU078673.1	*Vibrio communis* strain R	100%	0.0	100%

(continuation)

Sample	Accession number	16SrDNA organism	Coverage	E-Value	Identity
N8Cb	GQ406800.1	*Vibrio sp.* PaH3.38	99%	0.0	99%
W4Ca	GQ406800.1	*Vibrio sp.* PaH3.38	99%	0.0	99%
N10La	EU276985.1	Vibrionaceae bacterium	99%	0.0	99%
N10Lb	FJ952801.1	*Vibrio sp.* 1tc4	100%	0.0	99%
E1F	FJ457374.1	*Vibrio sp.* S1160	100%	0.0	99%
S8SL	GQ406800.1	*Vibrio sp.* PaH3.38	99%	0.0	99%
E3N	GQ406800.1	*Vibrio sp.* PaH3.38	99%	0.0	100%
E4Y	GQ406800.1	*Vibrio sp.* PaH3.38	99%	0.0	99%
S11M	FJ240422.1	*Photobacterium leiognathi*	100%	0.0	100%
S10P	GQ406646.1	*Vibrio sp*. PaD3.13 16	99%	0.0	100%
N2Q	GQ406646.1	*Vibrio sp.* PaD3.13	99%	0.0	100%

***no similarity found.** Sequence was analyzed using somewhat similar alignment.

3.4 Discussion

In order to confirm the identity of the isolates two biomarkers were amplified in this collection of bioluminescent bacteria. The first biomarker was 16SrDNA which was successfully amplified from all strains and sequenced with an average of 800-900 base pairs (bp). This represents at least half of the total size of the gene (1,500bp), which is a representative and accurate tool of this small ribosomal subunit in order to identify and compare the isolates (Figure 3.1).

From the north region of PR most strains were molecularly similar to *V.harveyi* with more than 99% of homology of the sequenced strand when aligned with the database (BLAST). Among this diversity it can be emphasize that one strain (N7M) was not able to align or found any homology in the *in silico* analysis, which suggests that further investigation is required to confirm that this isolated strain may be a unreported or not previously studied bioluminescent bacterium (table 3.1). Another particular strain verified was closely related to *V. communis* with 100% coverage when compared the isolated strand with the one found homologous in BLAST.

In the south region, other members of the *Vibrionaceae* Family are reported. This strain S5Pb) has 100% identity homologous to *Photobacterium leiognathi*. In this case the physiological data support the similarity in this comparison due to the fact that *P. leiognathi* is one of the few bioluminescent bacteria that produce gas upon the use of glucose in the medium. Most of the other strains sequenced from this region have the tendency to be similar when compare to *V.harveyi* (Figure 3.3). According to the phylogenetic tree (A) a small cluster from the south and north strains are closely related to *V.harveyi* (X74706) type 16S rDNA sequence taken from BLAST.

Samples from the west were not found to be heterogeneous, except for W3CO. This strain was found to behave different when exposed to glucose as a carbon source; it produced a dark

precipitate similar to when H_2S is produced. In addition, when compare with other strains (Figure 3.3) it is placed in a group of phylogenetically distant to most of the strains that are related to *V. harveyi* (X74706). In this neighborhood joining (NJ) tree we have a number of Operational taxonomic units (OTUs) that are relevant to mention. There's an OTU composed of 11 sequences that are similar and were mostly isolated from the north side of the island, a few from the east and south. Based on the 16S rDNA marker this cluster is the most important one from the perspective that there's no direct homology with the known sequences taken from BLAST (*P.phosphoreum*, *V. logei* and *V. harveyi*), which suggests that future studies must be continued to characterize deeply these isolates. A second OTUs stands out which according to the tree is possibly related to *V. harveyi*, this cluster is composed of strains isolated from the south and few from the west side of the island. The only candidate that according to this marker is related to another group besides the *Vibrionaceae* Family is W3CO, which its sequences shares 946bp of homology. None of the others environmental OTUs are related to *P. phosphoreum and V. logei*.

The second marker (*luxAB*) was amplified successfully is an approximate weight of 700bp (Figure 3.2). Primers were used to amplify the *luxA* fragment of this enzyme. The *in silico* analysis confirms that all sequenced samples code for luciferase. Due to the high difficulty of amplifying the complete luciferase coding genes, only a small fragment was possible to analyze and reported in Puerto Rico, when the phylogeny was obtained most of the sequences were similar. It would be possible that the small region amplified is not enough to have a complete comparison with *luxA* amplicons, theres a higher diversity in the first marker, because is more representative and it is traditionally used for phylogeny. Another explanation would be that most proteins tend to maintain conserve regions in order to express a stable function. This is the first

time that *luxA* amplified sequences are reported from a bacterial collection from the whole island of Puerto Rico.

In this *luxA* NJ tree 5 base substitution is expected per each 100bp. The first OTU is composed of 27 that are not closely related to *V.harvey P. kishitanii* or *S. hanedai,* this cluster is composed from samples isolated from each coast of the island. One of the arrangements with the highest rate was between samples S2Ya and W4Ca which belong to different branches in the 16S rDNA tree. The W3CO strain was confirmed in both trees to be closely related to the *Photobacterium* group.

This amplified *luxA* strands will not only serve as an initial report of luciferases in PR, but also can be used to design *lux A* probes to specifically detect other bioluminescent strains in future bacterial surveys. In the Persian Gulf and Kuwait region four different groups of bioluminescent bacteria were detected using probes derive from isolated strain amplification. Members detected included *V.fischeri, V.harveyi*, *P.photobacterium* and *P.leiognathi*. When detected taxonomic, analysis using standard methods confirmed this identification (Nealson et al., 1993).

One of the main contributions of this study is to update the global survey of bioluminescent bacteria in the world. Recent studies have confirm the presence of more than one specie in sites such as: Hawaii, Dominican Republic, Alaska, California, Cancun, Florida,Curacao, Uruguay, France, Israel, Madagascar, Philippines, Taiwan among others (Urbanczy et al., 2008). On the other hand, for more than 30 years no recent study has been published concerning PR.

Chapter 4

Summary, conclusions, recommendations and literature cited

4.1 Summary, conclusions and recommendations

- From this research, the first collection of bioluminescent strains from marine ecosystems of Puerto Rico were reported and characterized. Physiological and molecular analyses were performed to further differentiate the isolates.
- A total of 32 partial 16S rDNA sequences were isolated and reported that will contribute to access the unknown bioluminescent bacterial diversity that we have in Puerto Rico.
- Partial *luxA* sequences are reported from this study with an average of 600 sequenced bp including the start codon.
- To determine the possible *in vivo* coding or expression of newly reported luciferase sequences as gene reporters.
- In the future, start sampling other ecosystems other than marine ones such as soil of fresh water in Puerto Rico.
- In order to further determine the possible potential of the bioluminescent strains as biosensors it is advice to add additional analytes of interest that affects human's quality of life in order to test the bioluminescent strains for their use as biosensor.
- A bank of luciferase coding sequences belongs to several species were confirmed using more than one molecular biomarker.
- Members of this bioluminescent collection will serve as an addition to upgrade the growing antibiotic resistance population of gram-negative bacteria, which will serve in the future as a possible opportunity to study additional mechanisms of resistance.

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Appendix

Figure A. Growth and halo tolerance of bioluminescent strains from the north coast. Percentages represent NaCl concentrations. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	0%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
N1L	-	+	+	+	+	+	+	+	+	-	-
N2Q	-	+	+	+	+	+	+	+	+	-	-
N3C	-	+	+	+	+	+	+	+	+	-	-
N4H	-	+	+	+	+	+	+	+	+	-	-
N5A	-	+	+	+	+	+	+	+	-	-	-
N7M	-	+	+	+	+	+	+	+	+	-	-
N8D	-	+	+	+	+	+	+	+	+	-	-
N8Ca	-	+	+	+	+	+	+	+	+	-	-
N8Cb	-	+	+	+	+	+	+	+	+	-	-
N9Lb	-	+	+	+	+	+	+	+	+	-	-
N10La	-	+	+	+	+	+	+	+	+	-	-
N10Lb	-	+	+	+	+	+	+	+	+	-	-

Figure B. Growth and halo tolerance of bioluminescent strains from the south coast. Percentages represent NaCl concentrations. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	0%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
S1G	-	+	+	+	+	+	+	+	+	-	-
S2Ya	-	+	+	+	+	+	+	+	-	-	-
S2Yb	-	+	+	+	+	+	+	+	-	-	-
S3G	-	+	+	+	+	+	+	+	+	-	-
S4P	-	+	+	+	+	+	+	+	+	-	-
SP5a	-	+	+	+	+	+	+	+	+	-	-
S5Pc	-	+	+	+	+	+	+	+	+	-	-
S5Pd	-	+	+	+	+	+	+	+	+	-	-
S6J	-	+	+	+	+	+	+	+	+	-	-
S8SL	-	+	+	+	+	+	+	+	+	-	-
S11M	-	+	+	+	+	+	+	+	-	-	-

Figure C. Growth and halo tolerance of bioluminescent strains from the west coast

Percentages represent NaCl concentrations. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	0%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
W4C	-	+	+	+	+	+	+	+	+	-	-
W4Ca	-	+	+	+	+	+	+	+	+	-	-
W3M	-	+	+	+	+	+	+	+	+	-	-
W3CO	-	+	+	+	+	+	+	+	-	-	-
W2R	-	+	+	+	+	+	+	+	+	-	-

+ Growth; - No growth

Figure D. Growth and halo tolerance of bioluminescent strains from the east coast. Percentages represent NaCl concentrations. Positive growth (+), no growth (-). Incubation at 25 °C for a minimum of 24hrs.

Sample	0%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
E1F	-	+	+	+	+	+	+	+	+	-	-
E2C	-	+	+	+	+	+	+	+	+	-	-
E3N	-	+	+	+	+	+	+	+	+	-	-
E4Y	-	+	+	+	+	+	+	+	+	-	-

+ Growth; - No growth