The Purple Non-Sulfur Anoxyphototrophic Bacteria from Subtropical Hypersaline Microbial Mats in the Cabo Rojo Salterns

By

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Abstract

Purple non-sulfur bacteria (PNSB) are a diverse group of microbes with versatile physiological traits. As part of the microbial community present in subtropical hypersaline microbial mats, they are exposed to seasonal changes, such as pluvial precipitation, as well as variable oxygen and sulfur concentrations. The main objective of this study was to isolate and characterize cultivable PNSB present in benthic and ephemeral microbial mats from two different locations in the Cabo Rojo salterns, during the rainy and dry seasons. Microbial mats were dissected in layers, and the pink layer was cultivated in solid and liquid media. After being incubated anoxyphototrophically (anaerobic in photosynthetic conditions), colonies were isolated by streaking the bloomed samples on selective marine media. The PNSB isolates were characterized using light and scanning electron microscopy. The presence of a photosynthetic apparatus was molecularly confirmed by amplification and sequencing of the *pufM* gene. Molecular analysis was completed with *in silico* and phylogenetic analyses using *16S rDNA* and *pufM* gene sequences. During the two seasons 23 PNSB were isolated; 16 and 7 from benthic and ephemeral mats, respectively. All the isolates were gram-negative; and the morphology ranged from straight rods, vibrios, and spirals of variable sizes. Spectral analysis suggested the presence of bacteriochlorophyll *a* and carotenoids pigments peaks. *In silico* analysis confirmed the presence of *pufM* gene in all the amplicons. The cultivable microbial members were *Rhodospirillum,Rhodobacter, Rhodothalassium*and *Rhodovulum*. In addition, a *pufM* clones library was used to describe the cultivable and uncultivable purple non-sulfur bacteria present in the mats. Based on the rarefaction curve, the PNSB in both microbial mat samples were below

the diversity of PNSB, but most of the cultivable *pufM* sequences were represented in the clone library from both microbial mats.

Resumen

Las bacterias púrpuras no sulfurosas (BPNS)son un grupo de microorganismos diverso con una gran versatilidad fisiológica. Estas forman parte de las comunidades microbianas presentes en los tapetes microbianos subtropicales e hipersalinos, los cuales están expuestos a cambios estacionales, como por ejemplo precipitación fluvial y variaciones en oxígeno y azufre. El objetivo principal de este estudio fue aislar y caracterizar bacterias púrpuras no sulfurosas cultivables presentes en tapetes microbianos bénticos y efímeros en dos localidades distintas en las salinas de Cabo Rojo durante época seca y época lluviosa. Las muestras de tapetes fueron disectadas en capas y la capa rosa fue cultivada en medio sólido y líquido. Las muestras fueron incubadas en condiciones anoxifototróficamente (condiciones anaeróbicas en presencia de luz), y posteriormente las colonias que presentaron un color rojizo fueron aisladas en medio selectivo marino. Las BPNS aisladas fueron caracterizadas microscópicamente usando microscopia de luz y microscopia electrónica de rastreo. La presencia del aparato fotosintético fue confirmada molecularmente llevando a cabo amplificación y secuenciación del gen *pufM*. El análisis molecular fue completado realizando un análisis *in silico* y filogenético, utilizando las secuencias de los genes 16S rDNA y *pufM*. Durante las épocas estudiadas se aislaron 23 candidatos de BPNS; 16 y 7 de los tapetes béntico y efímero, respectivamente. Todos los aislados fueron Gram -, y exhibieron una morfología de bacilos rectos, vibrios y espirilos de tamaños variables. El análisis espectrofotométrico sugirió la presencia de bacterioclorofila *a* y pigmentos carotenoides de la serie espiriloxantin y esferoidina. Por otro lado, el análisis *in silico* confirma la presencia del gen *pufM* en los amplicones obtenidos y entre las BPNS cultivadas encontramos miembros de los generos*Rhodospirillum, Rhodobacter, Rhodothalassium y Rhodovulum.* En adición, el

desarrollo exitoso de una biblioteca de clones del gen *pufM* fue usada para descubrir presencia de las BPNS cultivables y no cultivables en los tapetes. Basado en el análisis filogenético y de diversidad las BPNS en ambos tapetes microbianos se encuentran por debajo de la diversidad total de BPNS. Mientras que a su vez las secuencias de *pufM* cultivables se encontraron representadas en la biblioteca de ambos tapetes.

Dedication

Very special people closed to my heart helped and inspired me to make this dream come true. God you always gave me the strength and taught me the value of sacrifice (with God all and without him nothing). During this journey, he blessed me with a little angel that makes every morning bright, and in the bad days she gives me a sweet smile and bring me happiness. I dedicate this work to you, Valentina S. Muniz Soto, because you showed me the real love and brought me the motivation to end this journey.

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Chapter One

Introduction and Literature Review

1.1 Introduction

Microbial mats are laminated organo-sedimentary structuresdivided by a gradient of oxygen and sulfide (Gemerden, 1993). The multilayer communities of prokaryotic microorganisms likely represent the first kind of ecosystem that evolved on Earth about 3,500 million years ago(Urmeneta, 2002). Microbial mats played a key role in the evolution of the Earth, especially during the Precambrian when stromatolites mats were abundant in coastal environments. These microenvironments are remarkably cosmopolitan in their occurrence, because they tolerate extremes both in temperature and energy availability, nutrients and water. Their distribution today is limited largely by predation and competition for space with other organisms (Des Marais, 1990). Habitats where mats are still common include hostile environments such as hypersaline bodies of water (Casillas-Martínez *et al*., 2005), hot springs (Hiraishi *et al*., 1999), dry temperate deserts (Guerrero and Wit, 1992) and cold dry environments (Clocksin *etal.*, 2007).

In their fully developed state, microbial mats found in many salt marshes are composed of three consecutive layers. These layers are denominated from top to bottom as: green, pink and black. At the green layer, aerobic organisms like cyanobacteria and diatoms can be found. The pink layer is characterized by the presence of anoxygenic phototrophic bacteria, specially green and purple sulfur and non-sulfur bacteria. The black layer is composed by sulfate-reducing bacteria, which produce sulfide, a compound that acts as an electron donor for both sulfur bacteria and some cyanobacteria (Des Marais, 1990).

Purple non-sulfur anoxyphototrophic bacteria (PNSB)have different strategies to acquire energy. One of the strategies that these bacteria use is the photosynthesis process (Oesterhelt, 1975; Yurkov, 1998). Depending on the electron donor and carbon source used by the

organismcan be described as photoheterotrophs or photoautotrophs, which use organic compounds and CO_2 as carbon sources, respectively (Imhoff, 1989). The energy production by photosynthesis can be classified as oxygenic or anoxygenic. The oxygenic photosynthesis is described as the use of light energy to synthesize ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate) by noncyclic photophosphorylation with the production of O_2 from water. Instead, the anoxygenic photosynthesis is the use of light energy to synthesize ATP by cyclic photophosphorylation without O_2 production (Madigan *etal.,* 2010). Cyanobacteria, plants and some algae (Jones, 2001) are examples of oxygenic photosynthetic organisms. In contrast, microbes such as members of the green and purple sulfur bacteria, green and purple non-sulfur bacteria and *Heliobacter* (Sasikala, 1998) are considered anoxygenic phototrophs.

Photosynthetic Purple Non-Sulfur Bacteria are a physiologically and biochemically diverse group of microorganisms due to their ability to grow as photo-heterotrophs, photoautotrophs or chemoheterotrophs (Imhoff, 1989). They are known to be prevalent in water bodies below the layer of oxygenic photosynthetic organisms such as algae, aquatic plants, and cyanobacteria (Cogdell, 2001). They can also be found in habits such as: wastewater ponds, sediments, moistened soils, seawater pools, hypersaline environments and lagoons (Okubo, 2006). In these habitats, the PNSB participate in the carbon, nitrogen, and sulfur cycles (Imhoff, 1989; Wang, 1993; Madigan, 2002). Because of the diverse electron and carbon sources that this group of photosynthetic bacteria can utilize, they have been used as a biotechnological tool in areas such as bioremediation (Huang *et al.,* 2001; Rajasekhar, 1998; Sasaki *et* al., 2002; McGrath, 1997).

The microbial mats studied in this research arelocated in two hypersaline lagoons in the Cabo Rojo Salterns,southwestern coast of Puerto Rico (Casillas-Martinez *etal.,* 2005).TheCabo Rojo Salterns are part of the Cabo Rojo National Wildlife Refuge,administrated by the U.S. Fish and Wildlife Service (USFWS). Therefuge is composed by several ecosystems, such as mangrove forests,hypersaline and marine lagoons, grassland of marine grass, and coral reefs. The hypersaline lagoons in this area are Candelaria and Fraternidad, they are characterized by low annual precipitation, strong winds and high temperatures (Figure 1). Candelaria lagoon is located innorthwest and Fraternidad in northeast of the salt ponds in an area that floods regularly during high tides. Both areconsidered as artificial, thalassohaline lagoons because of the entry of seawaterwhich is controlled by the USFWS by means of a dam. Candelaria lagoon receives seawater from Bahía Salinas, while Fraternidad receives it from BahíaSucia. The control of the seawater entrance has two main purposes. The first isto manipulate the salinity atthe site. By closing the dam, the concentration ofsalts increases from evaporation of the seawater. When the water reaches aspecific salinity, it is transported to the pools, where the evaporation and therainfall of salts are enhanced. Then, the salts can be extracted and used forcommercial purposes. The second purpose to control the seawater entrance is toprovide asuitable environment for endemic and migratory birds that visit theplace (Casillas-Martinez, 2005 and Colon, 2008).

Figure 1.Location of the Subtropical Hypersaline Microbial Mats in the Cabo Rojo Salterns. The arrows showed the two sampling locations, Candelaria and Fraternidad Lagoons. Figure provided by the U.S. Fish and WildlifeService.

Two different seasons are present in the Cabo Rojo salterns; dry and rainy seasons. The dry season occurs during the months of January to April and the rainy season from August to December. The period from May to July is considered the intermediate period, because it is characterized by occasional precipitation. During most of the year the mats are submerged, except towards the end of the dry season, when they are temporarily exposed to the surface (Casillas-Martinez *et al*., 2005).

Previous studies compared the communities structure using light and transmission microscopy, andreported a geological characterization and mineral composition of the Candelaria microbial mat in dry and rainy season. Still much remains to be study and describe from the hypersaline microbial mats from Cabo Rojo Salterns, for this reason this work provides the first microbiology, molecular and diversity study based on the anoxyphototrophic purple non-sulfur bacteria. The aim of this studywas to isolate and characterize microbial species that belong to the PNSB group from two subtropical hypersaline microbial mats in the Cabo Rojo Salterns of Puerto Rico. PNSBwere identified by general microbiology and molecular genetics tools, such as 16S rDNA and *pufM* genes amplification and sequencing. In addition, culture independent techniques such asgenetic libraries and cloning were used to compare the cultivable and uncultivable PNSB based on *pufM* gene. To comparethe taxarichness and diversity of PNSBa rarefaction curve were applied.

1.2Literature Review

Microbial Mats

Microbial mats have descended from the oldest and most widespread biological communities on Earth. Matsare home to microbes that are crucial for studies of bacterial phylogeny and physiology (Des Marais, 1990). Mats built by photosynthetic organisms created the most studied stromatolites in fossil record. Typically, several discrete layers can be recognized, arranged vertically; where the organisms distribute themselves according to physiological requirements, such as amounts of light, oxygen, nutrients and temperature. The upper layer is denominated green layer, which is dominated by cyanobacteria and organoheterotrophic bacteria (Van Gemerden, 1993; Garcia-Pinchel *et al*., 1998). Below this layer, the pink layer is found which contains anoxyphototropic and chemolitotrophic sulfur bacteria (Visscher *etal*., 1991; Teske *etal*., 1998). The bottom layer of the microbial mats is often black due toiron sulfide(FeS) precipitation by dissimilatory sulfate and sulfur reducing bacteria (Krumbein *et al*., 1979; Van Gemerden, 1993).

Mats are ubiquitous in nature, commonly foundover the sediment surfaces or as floating masses inmarine waters (Bebout *et al*., 1987; Otte *et al*., 1999;Steppe *et al*., 1996), hypersaline bodies of waters (Hoehler *et al*.,2001; Nuebel *et al*., 2001),estuaries (Olendzenski, 1999), fresh waters (Brunberg*et et al*., 2002), hot springs (Hiraishi *et al*., 1999; Nakagawaand, 2002; Skirnisdottir *et al*., 2000), dry deserts (Guerrero and Wit, 1992), soils(Steppe *et al*., 1996; Watanabe *et al*., 2000), deep oceanhydrothermal vents (Lutz *et al*., 2001; Taylor *et al*.,1999) and Antarctic ponds and sea ice (Madigan *et al*.,2000; Clocksin *et al*., 2007).

Examples of previously studied hypersaline microbial mats include those in Salins-de-Girauds (Camargue, France) (Wieland *et al*., 2004), Eilat (Israel) (Krumbein *etal*., 1979; Sorensen *et al*., 2005), Guerrero Negro (Baja California, USA) (Des Marais, 1995; Ley *et al.*, 2006), San Salvador Island (Bahamas) (Bebout, 1993, Yannarell *etal.*, 2006) Salt Pound Eleuthera Island (Bahamas), Salar de Llamara (Chile) (Demergasso *et al.*, 2003) and Cabo Rojo Salterns (Puerto Rico) (Casillas *etal.*, 2005).

A. Microbial Mats Applications

The 3,500 million-years survival of mats provided evidencetotheir capacity in adapting to and altering hostile environmentsthrough cellular and community-mediatedactivities (Des Marais, 1990; Hoehler *et al*., 2001). Innature, mats generally bondtightly to soil or submergedsediments, and then rise to the surface due to buoyancy exertedby the formation of gases in the mat's matrix. Subsequently,another mat begins to form over the vacatedsediment surface. Ecological success ofmicrobial mats and their broad array of microbialactivities suggest that these microbial ecosystems might be useful to reduce coastal erosion (Sarkar *etal.*, 2005),biogeneration of useful products and bioremediation of environmental pollutants(Abed *et al.*, 2002; Martínez-Alonso and Gaju, 2005; Abed *et al.*, 2007).Studies performed by Cohen in 2002 showedthat microbial mats can develop in oil contaminated flat coastal salt crust. Those microbial mats showed crude oil degradation in light by the action of cyanobacteria, like *Phormidium spp.* and *Oscillatoria spp*., and in darkness by sulfate reduction bacteria.

Micobial mats propertiesprovide promising uses in agriculture and energy production. Some studies demonstrated the functional use of mats in aquaculture; because they produce proteins via nitrogen fixation and are capable of supplying nutrition to *Oreochromis niloticus* (tilapia fish) (Bender *etal.*, 1989; Phillips *etal.*, 1994). Also, based on the capability of the

microbial mats to remove ammonia from wastewater (Goodroad *et al.,* 1995), a simple filter for a recycled water aquaculture system was constructed with microbial mats (Craing *et al.,* 1996; Adey and Loveland, 1998).

Microbial mats also developed mechanismsto remove and sequester heavy metals metalloids, radionuclides and oxyanions and degrade organic and some inorganic contaminants (Bender and Phillips, 1995). The accumulationand production of nutrients generates self-sufficiencyamong microbial members as materials are passed alongwide-ranging redox gradients within the mats. Metabolizingorganic materials can be generatedas byproducts likehydrogen, methane (Hoehler *et al*., 2001) and hydrogen sulfide (Taylor *et al*., 1999). These mechanisms take place at the cellular level of the constituent microorganisms and at the community level of the entire micro-ecosystem (Bender and Phillips, 2004).

B. Function and importance of the Microbial Mats

The structure, function and dynamics of microbial mats have been studied before to analyze the development of the microbes that live in this ecosystem. This ecosystem is considered an ideal model to study microbial interactions, element cycling and microbe-mineral interactions (Decker *et al.,* 2004). The availability of oxygen, sulfide, redox potential, pH and light have been measured and revealed steep and fluctuating gradients, particularly of oxygen and sulfide, in which organisms in the mats interact (Jorgensen *et al.,* 1983; Van Gemerden, 1988; De Wit *et al.*, 1989; Lassen *et al.*, 1992). In 1991, Canfield and Des Marais, measured sulfate reduction and dissolved oxygen in hypersaline bacterial mats from Baja California.That studyrevealed that sulfate reduction occurred consistently in the oxygenated photosynthetic zone of the mats. These

evidences challenged the conventional view of sulfate reduction and bring the idea that is not a strictly anaerobic process.

Anoxyphototroph Purple Non-Sulfur Bacteria

Many groups have recognized the ecological and biogeochemical importance of the Purple Non-Sulfur Bacteria (PNSB). These organisms supply carbon by fixation and also are excellent nitrogen fixers (Madigan, 1995), contributing to the productivity of the ecosystems (Pfennig, 1989; Madigan 1998). This bacteria group has an important role in the elemental cycles of carbon and sulfur (Pfennig, 1989).

A. Physiology

The PNSB is a diverse and widely distributed group of gram-negative anoxyphototroph microorganisms. The taxonomic distribution of these bacteria has changed in recent years due to the discovery of new species and the redistribution of the genera. Molecular tools have allowed scientiststo make geneticdistinctionsbetween PNSB genera and new genera of PNSB have emerged. At the time of this research 60species of PNSBhave been reported. These are distributed in five orders: *Rhodospirillales*, *Rhodobacterales*, *Rhizobiales* in the Alpha-proteobacteria group, and *Burkholderiales* and*Rhodocyclales* in the Beta- proteobacteria group(Woese *et al.*, 1984). Most of the bacteria in this group are found in the orders *Rhodospirillales* (family *Rhodospirillaceae*) and *Rhodobacterales* (family *Rhodobacteraceae*) (Woese *etal.*, 1984; Imhoff and Trüper, 2005). This group is composed of genera like: *Phaeospirillum* (stagnant and anoxic freshwater), *Rhodocista* (warm water, wastewaterand hot springs), *Rhodopila* (warm sulfur springs), *Rhodobacter* (freshwater, sewage ponds and eutrophic lakes), *Rhodopseudomonas* (freshwater),*Roseospirillum* (marine sediments and

microbial mats),*Rhodobaca* (alkaline soda lakes), *Rhodomicrobium* (mud and water of ponds and lakes), *Rhodocyclus* (freshwater ponds, sewage ditches and swine waste lagoon), *Rhodovulum* (marine and hypersaline environments), *Rhodoferax* (ditchwater, activated sludge and Antarctic microbial mats), *Rhodoplanes* (freshwater and wastewater environments), *Rubrivivax* (freshwater, activated sludge and sewage ditches), *Rhodobium* (seawater), *Roseospira* (warm sulfur springs), *Rhodothalassium* (anoxic zones of hypersaline environments), *Rhodovibrio* (anoxic zones of hypersaline environments) and *Rhodospirillum* (freshwater) (Imhoff *etal.*,1984, 1998;Kawasaki *et al.*, 1992; Glaeser and Overmann, 1999; Milford *etal.*, 2000; Plenning *etal.*, 1978; Hiraishi and Ueda, 1994; Willems *etal.*, 1991; Hiraishi *etal.*,1991and 1995). Members of the PNSB show morphological and physiologicaldifferences that can be used as taxonomic tools (Oda, 2002).

Some families in this group of bacteria are:

Family *Rhodospirillaceae*

The purple non-sulfur bacteria group is composed of 8 families and 21 genera, being*Rhodospirillaceae* the main family of these bacteria. This family includes the genera:*Rhodospirillum, Phaeoospirillum, Rhodocista*, *Rhosospira, Rhodovibrio* and *Roseospira*. Until 2008, the genus *Rhodospirillum* consisted of ten species, the last speciesdescribed was *Rhodospirillum sulfurexigens* isolated from fresh water (Kumar *etal.*, 2008). Members of this family are characterizedby cell morphology like rods and spirilla. Habitats that home thesespecies are fresh water ponds, wastewater, marine water, salterns and microbial mats. The species*Rhodospira trueperi* was isolated in 1997 from a microbial mat and is the only species in this family that has bacteriochlorophyll b. Also, some genera have the capability to grow in high sodium chloride (NaCl) concentration, such as *Roseospira, Roseospirillum, Rhodovibrio* and

Rhodospira; while genera such as *Rhodospirillum* and *Phaeospirillum*are common in fresh water (mesophilic environment) (Imhoff and Trumper, 2005).

Family *Rhodobacteraceae*

Family with most species of this group of bacteria, with 16 species reported until 2010. *Rhodobacteraceae* family included the genera *Rhodobacter, Rhodothalassium*, *Rhodobaca* and *Rhodovulum*. These bacteria have been isolated from fresh water bodies and marine water, but they were also found in the nasal cavities of pneumonia patients, in the Himalaya mountains, alkaline-salt lagoons, agriculture ponds, coastal sediments and hypersaline environments. The last species described was *Rhodobacter johrii* (2010) in a semi arid subtropical soil from India (Girija *etal.,* 2010).The genus *Rhodothalassium* is the only in this family with vibrio (curvedbacilli) morphology. The optimal temperature of growth is 40ºC and 6-8% NaCl. Most of the species show short bacillus morphology (Imhoff and Trumper, 2005).

Family *Bradyrhizobiaceae*

Bradyrhizobiaceae is characterized by its physiological versatility and include the genera *Rhodoblastus* and *Rhodopseudomonas*. *Rhodopseudomonas palustri* is the most studiedspeciesin this family, because ithas many scientific applications. Many species in this family can be grown in four metabolic ways: (1)photoautotrophicusing light as its sole source of energy and CO_2 as its sole carbon source, (2) photoheterotrophic using light as a source of energy and organic compounds as a carbon source, (3) chemoheterotrophic usingenergy from the oxidation of organic compounds,and (4) chemoautotroph using energy from oxidation of inorganic compounds (Madigan *etal.,* 2010). Their habitats include fresh water bodies, wastewater, marine environments, microbial mats and sediments (Imhoff and Trumper, 2005).

Purple non-sulfur bacteria have the ability of growing under anaerobic conditions in the presence of light. They do this as photoheterotrophs or as photoautotrophs, with electron donors such as molecular hydrogen and low levels of sulfide, thiosulfate or elemental sulfur (Imhoff, 1989). They can also grow under microaerobic conditions in the dark, as chemoheterotrophs or chemoautotrophs by respiration or fermentation (Van Niel, 1941). They tolerate low concentrations of selenate and selenite (Van Fleet, 2000), and have the ability to use aliphatic and aromatic compounds as carbon sources (McGrath, 1997).

Selective media used for the isolation of PNSB include: (1) low concentration of sulfate and a carbon source (not used by sulfate-reducing bacteria), (2) a trace salt solution, and (3) a ferroussolution (Sistrom, 1960). They use different carbon sources and electron donors such as malate, succinate, pyruvate and acetate. If isolated from hypersaline environment, the PNSB grow in NaCl concentrations between 0.5%-10% (optimum 2%-5%). Their optimum pH is in the range of 5.0-8.0. The PNSB are mesophilic with optimum temperature between 30°C-35°C (Mouné *et al.,* 2002; Okubo *et al.,* 2006; Hisada *et* al., 2007).

The anoxyphototroph bacteria can be found in different environments like wastewater, ponds, sediments, costal lagoons, lakes and microbial mats. The purple non-sulfur bacteria are particularly found in regions where water and sediments receive enough light to perform photosynthesis. (Imhoff, 2001; Massé, 2002). Some PNSB can be found in soils and marine environments as halotolerant (Dang, 2002).

PNSB are involved in the CO_2 fixation and are extremely important to photosynthetic bacteria; CO_2 becomes a major electron donor when the bacteria are grown under photosynthetic conditions in the presence of organic carbon (Wang, 1993). This group of bacteria can assimilate

acetate, pyruvate and dicarboxylic acids, some assimilate fatty acids, methanol, ethanol, sugars, sugar alcohols and organic nitrogen sources (yeast extract) (Clayton, 1978). These bacteria depend on the activity of chemoorganotrophic bacteria that degrade organic macromolecules such as starch, cellulose, pectin, proteins and neutral lipids that they cannot break down. The PNSB tolerate low concentrations of hydrogen sulfur (H_2S) and produce CO_2 and elemental sulfur (S^0). Also, they reduce nitrate to nitrite, nitrous oxide, and gaseous molecular nitrogen (Clayton, 1978). This ability to denitrify is very important because the bacteria remove fixed nitrogen from the environment and produce nitrite that is used by other organisms (Madigan, 2002).

B.Bacteriochlorophyll and Carotenoid Pigments

Purple non-sulfur bacteria are phototrophic bacteria that have the capability to use light energy to synthesize ATP by cyclic photophosphorylation without O_2 production. These phototrophic organisms contain bacteriochlorophyll. This pigment is related to porphyrins, which are tetrapyrroles that are parent structure of the cytochromes. But unlike cytochromes, bacteriochlorophyll contains magnesium instead of iron at the center of the ring. Bacteriochlorophyll also contains specific substituents on the rings as well as a hydrophobic alcohol that help to anchor the bacteriochlorophyll into photosynthetic membranes. There are a number of different bacteriochlorophylls, and each is distinguished by its unique absorption spectrum. Bacteriochlorophyll *a* is present in most purple bacteria, absorbs maximally between 800nm-925nm, depending on the species. Different species produce slightly different pigments-binding proteins, and the absorption maxima of the bacteriochlorophyll *a* in any given organism

depends to some degree on the nature of these proteins and how they are arranged to form photocomplexes in the photosynthetic membrane (Madigan *etal.,* 2010).

Although bacteriochlorophyll is required for photosynthesis, phototrophic organisms contain an assortment of accessory pigmentsthat includes carotenoids pigments. Carotenoids are hydrophobic light-sensitive pigments that are firmly embedded in the photosynthetic membrane. They tend to mask the color of bacteriochlorophyll, therefore carotenoids are responsible for the brilliant red, purple, pink, green, yellow and brown colors that are observed in different species of anoxygenic bacteria (Madigan *etal.,* 2010). In purple non-sulfur bacteria, these are present in the series of carotenoid pigments named spheroidenone and spirilloxanthin.

Bacteriochlorophyll molecules within a photosynthetic membrane are attached to proteins to form photocomplexes consisting of 50 to 300 molecules. Only a small number of these pigment molecules participate directly in reactions where light energy is converted into ATP and structure the reaction center. Reaction centers are surrounded by more numerous light harvesting bacteriochlorophylls. These antenna pigments (light harvesting pigments) function to absorb light and funnel its energy to the reaction center (Pullerits and Sundstrom, 1996). Reaction centers of purple bacteria consist of three polypeptides, designated L, M, and H. These proteins, along with a molecule of cytochrome *c*, are firmly embedded in the photosynthetic membrane. The L, M, and H polypeptides bind pigments in the reaction center. The photocomplex consists of two molecules of bacteriochlorophyll *a*, called the special pair, two additional bacteriochlorophyll *a* molecules that function in photosynthetic electron flow, two molecules of bacteriopheophytin (bacteriochlorophyll *a* minus its magnesium atom), two molecules of quinine, and one carotenoid pigment (Madigan *etal.,* 2010).

For each species of PNSB the light-harvesting complexes absorb at different wavelengths, but in many PNSB the LHI and LHII have absorption peaks at 850nm-875nm and 800nm-850nm, respectively.Bacteriochlorophyll *a* absorbs specifically between 800nm-895nm. Carotenoid pigments from the series spheroidenone and spirilloxanthin absorb between 400nm-600nm wavelengths (Cogdell, 1980; Pullerits, 1996; Zeilstra, 1998, Madigan, 2010).

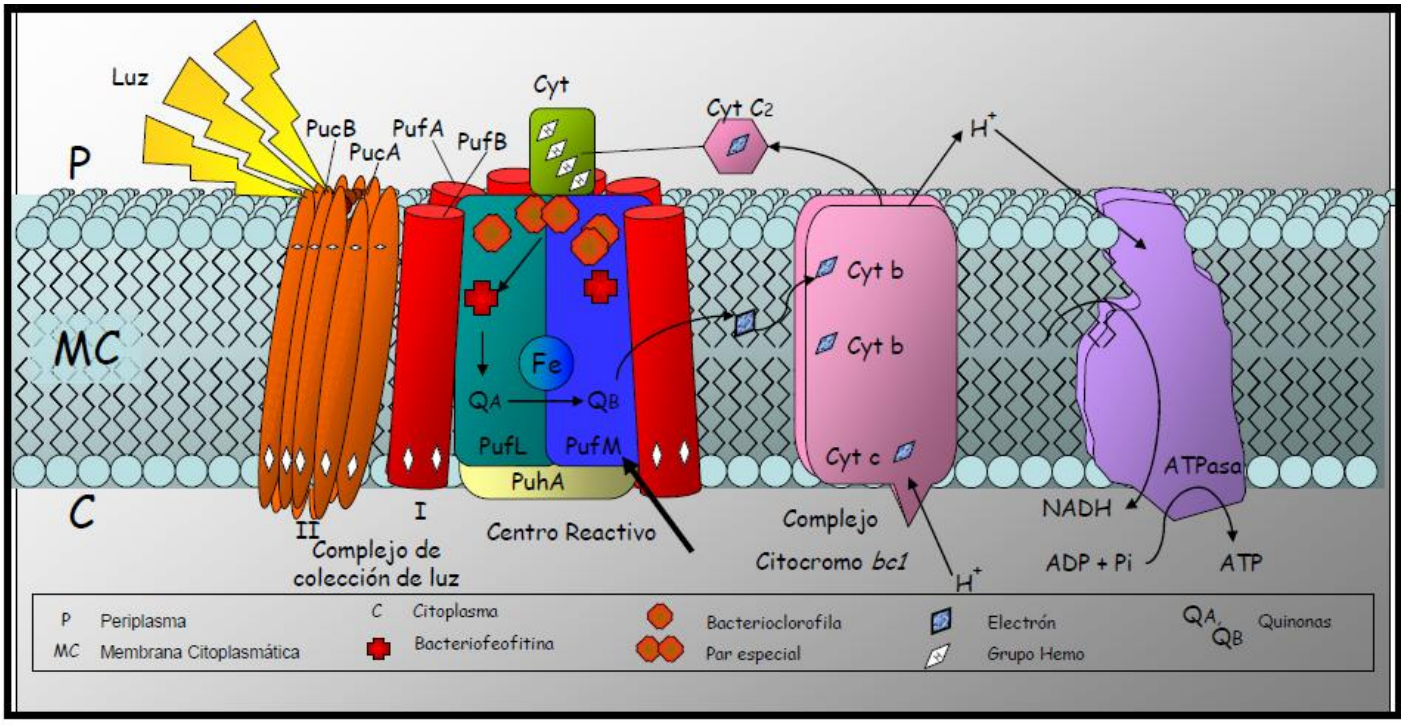


Figure 1.2 **Photosynthetic apparatus of purple non sulfur bacteria in the cytoplasmic membrane.** The process starts when light is directed to the reaction center by light harvesting complexes and then is absorbed by two molecules of bacteriochlorophyll, located in the cytoplasmic membrane closed to the periplasm. This pair of chlorophylls is known as the special pair. Once the photon is absorbed, an electron is released and transferred, by another bacteriochlorophyll molecule, to the bacteriopheophytin (located in the L-subunit of the reaction center). The electron is transferred to quinone A and subsequently to quinone B. To fully reduce

quinone B to QH_2, two high-energy electrons are necessary. The reduced quinone is diffused through the membrane to get to another protein complex, called cytochrome bc_1, where it is oxidized. During the process, the reduction power of QH_2 is used to pump protons to the periplasmic space (through the membrane). Electrons from the cytochrome bc_1 complex are transferred to cytochrome c_2 in the periplasm (functions as an intermediate and transports these electrons again to the cytochrome in the reaction center – cyclic system). The produced proton is targeted to electrons transport chain, to produce energy in the form of ATP. The arrow indicates the protein product of the *pufM* gene. (Picture courtesy of Juan Vega Sepulveda)

C. The Photosynthetic Machinery of the PNSB

Photosynthesis is a process regulated by genes that are organized in clusters and have been identified and characterized before (Cogdell *et al.,* 1980; Kiley *et al.,* 1987, 1988; Braun, 1990, 1996; Zeilstra *et al.,* 1998.). These gene clusters occupy around 46kb of the bacterial chromosome and are composed mainly of genes that encode for (1) bacteriochlorophyll (*bch*), (2) carotenoids (*crt*), (3) light-harvesting complexes (LH), and (4) the reaction center (*puh, puf*). Light is captured by the light-harvesting complexes (LHC) and the electrons are transferred to the bacteriochlorophyll pigments located in the reaction center (Zeilstra, 1998). Each one of the light-harvesting complexes has a characteristic absorption spectrum (Braun, 1990).The microbial ecology and diversity of anoxygenic phototrophic bacteria have been investigated using a variety of techniques like pigments and quinone analysis, electron microscopy, physiological tests, 16S rRNA gene analysis and *pufM* gene analysis (Achenbach *etal.,* 2001; Guyoneaud *etal.,* 2002; Okubo *et al.,*2006; Hisada *etal.,* 2007).

A techniquefor the identification of the PNSB was developed by Achenbach *et al*., (2001)using *pufM* gene. This gene encodes for a pigment binding protein in the photosynthetic reaction center of all purple phototrophic bacteria (Karr *et al.,* 2003). This technique was based on design and application of PCR primer sets to assess the biodiversity, metabolic activity, and ecology of these phototrophs. The primers can be used with isolated anoxyphototropic bacteria and for the analysis of environmental samples (Achenbach *et al.,* 2001). A metabolic gene, *pufM*(total size of 229 base pairs), was chosen because it is unique to purple phototropic bacteria and universally distributed across the group. Because the *pufM* primers are based on a gene unique for organisms that have a purple bacteria light-harvesting reaction center, they can be used to detect any anoxyphototropic bacteria member of the *Proteobacteria* (Corso, 1999; Watanabi, 1995; Madigan *et al.*,2010).

Chapter Two

Isolation and Characterization of Purple Non Sulfur Bacteria from Subtropical Hypersaline Microbial Mats from the Cabo Rojo Salterns

2.1 Introduction

Purple non-sulfur anoxyphototrophic bacteria (PNSB) are ubiquitous in fresh and marine water, wastewater ponds, sediments, moistened soils, seawater pools, activated sludge and hypersaline environments (Okubo *etal.*, 2006).Metabolically, they are the most versatile among all prokaryotes because they are able to grow under anaerobically photoautotrophic and photoheterotrophic in the light and aerobically chemoheterotrophic in the dark. Thus, they can use a broad range of organic compounds as carbon and energy sources (Imhoff, 2005). PNSB are also active participants in the carbon, nitrogen and sulfur cycles (Wang *et al.*, 1993). For example, they have been used for treatment of many types of wastes: concentrated latex wastewater (Choorit *etal.*, 2002), aquarium wastewater (Nagadomi *etal.*, 1999), and agricultural wastes (Hiraishi *etal.*, 1989).

PNSB are divided in eight families distributed in alpha and beta Proteobacteria, with 21 genera and approximately 60 described species (Imhoff and Trümper, 2005). All of these genera are capable of growing under anoxygenic conditions in the presence of light. They produce a characteristic reddish pigment that allows their isolation in culture. The colony texture, size, form and pigmentation are useful macroscopic characteristics to distinguish between genera. Under anaerobic conditions in presence of light, the colonies show a reddish pigmentation in the range of pink to light brown and under aerobic conditions in absence of light the colonies generally do not show any pigmentation (Imhoff, 2005). The microscopic characteristics are considered the most important to determine differences between genera. The cell size, morphology, Gram stain and the presence of external structures were some features used in this research to determine the identity of the isolates. This group of bacteria has a diverse cell

morphology includingrods, spirilla and vibrios with different sizes and arrangements (Madigan, 2002).

This study aimed to isolate purple non-sulfur anoxyphototrophic bacteria from subtropical hypersaline microbial mats and characterize them microscopically, biochemically and molecularly. The present study shows the first report of PNSB in the subtropical hypersaline microbial mats at Cabo Rojo Salterns.

2.2 Materials and Methods

Sampling Areas and Samples Collection

To achieve the objective of this research, microbial mats and water samples (from the microbial mat area) were collected from two hypersaline lagoons located in the Cabo Rojo salterns at the southwestern coast of Puerto Rico (Figure 1.1). Samples were taken during two different seasons: dry and rainy; and two different hypersaline lagoons: Candelaria and Fraternidad. For the dry season 3 samples were collected between the months of Januarythrough May in 2008and 2009, and the locations coordinates were 17.95ºN, 67.19ºO (Candelaria) and 17.98ºN, 67.21ºN (Fraternidad). For the rainy season 3sampleswere collected in the months ofSeptemberthrough Decemberin 2008and 2009, at the same sites used for dry season. A microbial mat piece of approximated 12cm^2 and one liter of water were collected from Candelaria and Fraternidad hypersaline lagoons.

Sample Processing

Microbial mats samples were dissected in the three colored layers and a piece of mat samples of approximately 4cm^2 of the pink layer was selected. The pink layer was processed by diluting the microbial mat piece into a 10mL tube with 5mL of physiological saline solution (0.85% NaCl) at pH 7.1. Serial dilutions from 10^{-1} to 10^{-5} were performed to the pink layer and water (collected from the sampling site). The diluted pink layer and water samples were filtered through a nitrocellulose membrane with a pore size of 0.2µmattached to a Nalgene® Analytical Funnel. The nitrocellulose membranes were placed on Petri dishes with marine agar media (Difco™) and incubated in an anaerobic jar of polycarbonate BBL®, attached to a Gas-Pack®

(following the manufacturer specifications), in presence of light at 30°C-32°C for 48-72 hours and verified the presence of reddish colonies. The reddish colonies were isolated and purified. All the isolates were growth under anaerobic conditions in a anaerobic jar and under photosynthetic conditions irradiated with incandescence light of 60V.

Macroscopic and Microscopic Analysis

The isolates were analyzed at macroscopic level by general morphological description of the colony such as elevation, form, and margin, using the criteria described by Harley and Prescott (2007). The colonies pigmentation was also measured and described using Munsell Color Chart® (Tella *etal.,* 1998).

The bacteria were measured using an Olympus Optical Co., LTD CHT light microscope. To determine the dimension of the bacteria, 100 of them were measured randomly to calculate the average size. Gram stain was performed using heat-fixed smears. To observe the shape and size of the bacteria, simple stain was performed (Harley and Prescott, 2007)

Scanning Electron Microscopy

For the Scanning Electron Microscopy (SEM), the bacteria were observed using the JEOL JSM-541 OL SEM microscope. For the analysis, the isolates were grown in Luria Bertani broth for 24 hours at 32 ºC in an orbital shaker. Then, approximately 1.5 mL of the culture were transferred to a microtube and centrifuged for 1 min at 0.8 g (3,000 RCF) to obtain a cell pellet. Glutaraldehyde at 4% was added to fix the cell pellet and then left resting for 24 hours at 4ºC. The cell pellet was then rinsed with Phosphate Buffer Saline 1M three times. To dehydrate the pellet, different percentages of alcohol, ranging from 10%-100% in 10% intervals, were used.

Every ten minutes the pellet was transferred from one alcohol solution to another. To completely dry the sample, a critical point drier (Critical Point Drying Apparatus Polaron E3000) was used. A sputter coater was used to give the samples a palladium-gold cover in order to protect the sample and to improve the conductivity of the specimen. The parameters used above were established by the Scanning Electron Microscopy Center at the University of Puerto Rico, Mayaguez Campus. Electron micrographs were taken at an accelerating voltage of 15 kV.

Photosynthetic Pigment Analysis of Purple Non-Sulfur Bacteria

During this study, we aimed to determine the presence of photosynthetic pigments, like bacteriochlorophyll *a* and *b,* and carotenoids pigments. After growing the samples in marine broth under anaerobic and photosynthetic conditions, the bacteria cultures were diluted in a solution of 30% bovine serum albumin (BSA). Then, the whole cell spectra were recorded using UV-Vis spectroscopy within a range of 380nm-1100nm. The spectra were recorded in a Genesis2 Thermospectronic®spectrophotometer.

Growth in different percentages of NaCl

This test was done to determine the optimal NaCl percent of growth of the PNSB. Percentages of salt used were: 3%, 5%, 7%, 9%, 10%, 12% and 15%. Briefly, 20mL of broth were place in tubes of 20mL where bacteria were inoculated. The samples were incubated in photosynthetic and anaerobic conditions for 48-72 hours. The bacteria *Escherichia coli*(ATCC-8739) were used as a negative controland *Rhodobacter sphaeroides* as a positive control.

Optimal temperature determination

To determine the optimal temperature of growth, the isolates were inoculated in 15mL of marine broth and incubate for 72 hours in different temperatures ranging from 4 ºC to 42 ºC. For 4ºCthe lowest area of the refrigerator was used and for 32ºC to 42ºC laboratory incubators were used.

Optimal pH determination

To determine the optimal pH for the bacteria, tubes with marine broth at different pHs were prepared. The pH range used was from 4.0-10.0 at 0.5 unit's intervals. HCl and NaOH were used to adjust the corresponding pH. The medium was inoculated with the isolated bacteria. The samples were incubated at 32ºC in anaerobic and photosynthetic conditions for 72 hours.

Biochemical Tests

In order to determine the physiology of the anoxyphototrophic bacteria, a series of biochemical tests were performed. As controls, *Rhodobacter sphaeroides* 241(ATCC 1540), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9721)were used. To determine a physiological and enzymes profile for the isolates bacteria, different tests were done. Urea broth was used to see if the bacteria could degrade urea. Sulfide indole motility (SIM) medium was used to see if the isolates could degrade tryptophan to indole (presence of tryptophanase), it was also used to determine the production of H_2S and to see if the bacteria were motile. Starch Agar Medium was used to see if the bacteria produced the enzyme amylase. The Nitrate Broth was used to see if the bacteria had the ability to reduce nitrate to nitrite. The Nutrient Gelatin Agar was used to see if the isolates produced the enzyme gelatinase. To

determine the carbohydrate fermentation profile, an assay was performed with sucrose, dextrose, galactose, lactose, xylose, fructose, glucose, arabinose, maltose and mannitol broths with phenol red as indicator. The ability to use glucose, lactose, and sucrose was measured using Triple Sugar Iron Agar (TSIA) slant; the production of H_2S and gas was also determined. The utilization of citrate as sole carbon source was measured by using Simmons Citrate Agar slants. Methyl Red (MR) test was used to determinethe capability of the bacteria to produce and maintain acid substance and to see the fermentation of glucose and its transformation to pyruvic acid, we used Voges Proskauer (VP) test. All samples were incubated at 32ºC for 72 hours under anaerobic and photosynthetic conditions. Then, a post incubation treatment was added to those tests that required it. To the SIM medium, 2-3 drops of the Kovac reagent was added. To the MR medium, 7 drops of Methyl Red reagent were added. To the VP medium, 10 drops of α–naphthol and 5 drops of KOH 40% were added, and then results were observed after 30 minutes. To the nutrient gelatin medium, the post incubation treatment was 30 minutes in the refrigerator. To the starch agar medium, 2-3 drops of iodine were added. To the nitrate medium, 1 drop of sulfanilic acid and 1 drop of α-naphtilamine were added; if the result was negative, a small portion of zinc powder was added. All these tests were previously described by Harley and Prescott (2007).

DNA extraction

The genomic DNA of the isolated PNSB was extracted using the method described byChen and Kuo (1993)**.** The samples were inoculated into marine broth for 48 hours under anaerobic and photosynthetic conditions. The cultures were transfer to 1.5mL microtubes and centrifuged at 13,000 rpm (15.7 x g) to obtain a cell 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 NaCl 5M, then treated with RNAse (20 µg/µL) for 30 minutes at 37ºC. The organic extractions were performed using one volume of chloroform and centrifuged at 13,000 rpm (15.7 x g); this step was repeated twice. The samples were precipitated with one volume of absolute ethanol at -20ºC. The isolated genomic DNA was resuspended in 50µL of 1X TE buffer (10mM Tris-Cl pH 8.0 and 1.0mM EDTA pH 8.0). The final concentration of the DNA was measured using an Eppendorf Biophotometer®.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed with the genomic DNA of the isolate as template in order to amplify part of the 16S rDNA gene. The amplification reactions were done using the GoTaq® Green Master Mix of Promega (GoTaq DNA Polymerase 2X, Green GoTaq Reaction Buffer, 400µM, dATP, 400µM, dGTP, 400µM, dCTP, 400µM, dTTP and 3mM $MgCl_2$) and universal primers oligos 14-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTTAGGACTT-3') (Willmotte and Wachter 1993). The PCRs were performed in a GeneAmp® PCR System 2700 (Applied Biosystem Company) using the following parameters: initial denaturalization at 95ºC for 3 minutes; followed by 30 cycles which included denaturalization at 95ºC for 1 second, annealing at 52ºC for 30 seconds, extension at 72ºC for 1 minute and a final extension at 72ºC for 10 minutes.

Also, PNSB were analyzed by PCR amplification of *pufM* gene using specific primer sets: 557F [5'-CGCACCTGGACTGGAC-3'] and 750R [5'-CCCATGGTCCAGCGCCAGAA-3']; and the GoTaq® Green Master Mix of Promega (Achenbach*etal.,* 2001). The PCR parameters were the following: initial denaturalization at 94ºC for 3 minutes; followed by 30 cycles which included denaturalization at 94ºC for 1 minute, annealing at 55ºC for 1 minute,

extension at 72ºC for 1 minute and a final extension at 72ºC for 10 minutes. All the amplifications were confirmed by performing an agarose gel electrophoresis (0.8% agarose prepared with Tris-EDTA 1X) at 80V in Tris-EDTA 1X buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0). The amplicons were purified using a QIAGEN PCR purification kit. The PCR products were sequenced at Macrogene facilities (Maryland, USA). *In silico* analysis was performed by using online databases such as GenBank in NCBI and the program BLAST (http://www.ncbi.nlm.nih.gov).

Phylogenetic Analysis

The isolated PNSB 16S rDNA and *pufM* sequences were edited using bioinformatics tools such as Chromas Lite 2.0.1 and then aligned and edited with BioEdit 7.0.9. The phylogenetic analysis was performed using the MEGA 4.1 software. The distance model used was p-distance and the bootstrap test of phylogeny was calculated for 2000 replicates. The generation of the consensus tree was performed by the Neighbor Joining method and the final tree was drawn using MEGA 4 Tree Explorer. For the first tree the outgroups used were *Aspergillus niger* (HQ600982.1) and *Bryophagus gloeocapsa* (AY300880.1) and for the second tree *Arabidopsis thaliana* (NC003076.8)and *Oscillatoria margaritifera* (GU724208.1) were used.

2.3 Results

Twosubtropical hypersaline microbial mats samples were studied; one from Candelaria Lagoon and other from Fraternidad Lagoon in the Cabo Rojo Salterns (Figure 2.1). The physicochemical parameters of each microbial mat sample are shown in Table 2.1. The pH values ranged from 7.09 to 7.21. The temperatures varied between 34ºC to 37ºC, and the salinity ranged from 6% to 25% over the two seasons. A total of 23 anoxyphototrophic bacteria strains were isolated; 13 from dry season (7 from Candelaria and 4 from Fraternidad) and 10 from rainy season (9 from Candelaria and 3 from Fraternidad), respectively.

The 23 isolated colonies showed circular shape, convex elevation, entire margin and 3 different pigmentation tones between light red to dark red (Figure 2.2) (Munsell® Color Chart). The isolated PNSB were gram-negative rods, vibrios and spirillum, of sizes that ranged from 0.5µm to 2.7µm approximately. Endospores or capsules were not observed in the PNSB isolates.

The optimal pH of growth of PNSB was between 6.0 and 7.5 and the temperature between 30ºC and 32ºC and 3% of salinity. Some of the candidates were able to grow at pH 8.5 and a highest salinity growthwas found at 10% (Table 2.2and Table 2.3).The biochemical tests performed to the isolated PNSB show that all the candidates have the enzymes oxidase and catalase, and were able to ferment ribose, arabinose, galactose and mannitol. None of isolated had the enzyme gelatinase, but had the capability to use citrate as carbon source and reduce nitrate to nitrite (Table 2.4).

The photosynthetic pigments were analyzedusing whole cell spectra. UV-Vis spectroscopy showed whole cell absorption peaks from 300nm to 1100nm. Characteristic bacteriochlorophyll signals were found at 805nm and 865nm. Carotenoids pigments of the

spirilloxanthin and spheroidenone series were found due to absorbance peaks at 400,492, 515, 530, 560 and 600nm (characteristic of purple non-sulfur bacteria)(Figures2.4-2.5) (Table 2.5).

Amplification of the 16S rDNA was done by PCR with positive amplification of a DNA fragment of approximately 1500bp (Figure 2.6).In the amplification of the *pufM* gene by PCR from genomic DNA, all the isolatesshowed positive amplification with bands at approximately229 bp (Figure 2.7). The *in silico* analysis performed to the 16S rDNA suggested the presence of purple non-sulfur bacteria from the genus *Rhodospirillum, Rhodobacter, Rhodothalassium* and *Rhodovulum*(Table 2.6). The phylogenetic tree constructed with the 16S rDNAsequences (715 bp), showed the evolutionary relationship between the isolated PNSB from microbial mats, previously described PNSB and other cultivable bacteria such as *Rhodobacter sphaeroides, Rhodovulum marinum, Rhodovibrio salinarum* and *Rhodothalassium salexigens* (Figures 2.8-2.9).

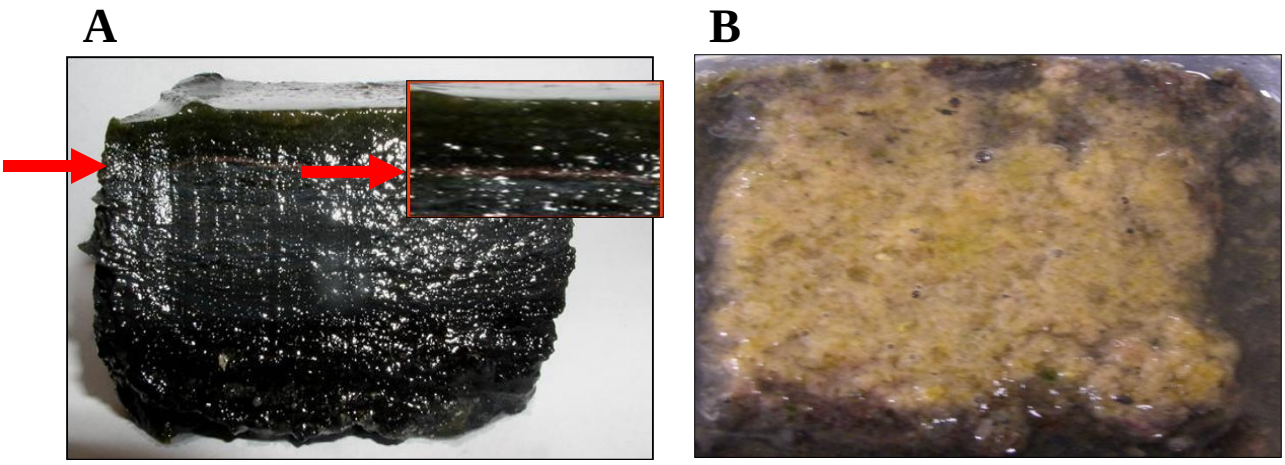


Figure 2.1. Ephemeral (Candelaria-A) and benthic (Fraternidad- B)subtropical hypersaline microbial mats from the Cabo Rojo Salterns. Candelaria mat showed a define pink layer, the pink layer but in Fraternidad mat was disperse across the entire sample.

Table 2.1 Physico-chemical parameters of microbial mats samples

Sampling site	Season	Temperature	pH	Salinity	Coordinates
Candelaria	Dry	32ºC-34ºC	7.03-7.09	24%-25%	17.95ºN, 67.19ºO
	Rainy	31ºC-34ºC	7.00-7.02	5%-6%	
Fraternidad	Dry	32ºC-36ºC	7.15-7.21	24%-26%	17.98 ºN, 67.21ºO
	Rainy	32ºC-35ºC	7.13-7.16	5.0%-5.5%	

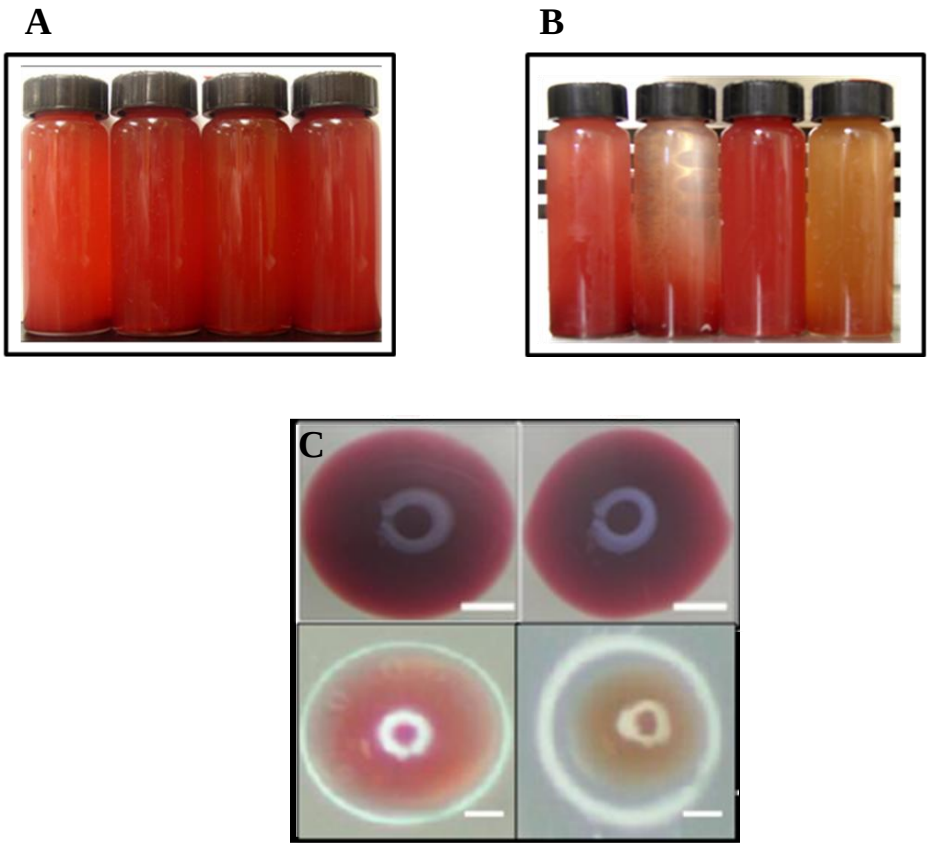


Figure 2.2 Isolated PNSB candidates from subtropical hypersaline microbial mats. Isolated candidates from the two microbial mats samples (Candelaria-A, Fraternidad-B) showing the characteristic reddish bloom after incubated in anaerobic and photosynthetic conditions. The bloomed samples were streaked, and the colonies were analyzed macroscopically and microscopically. The colonies sizes varied from 2-6mm (C). Scale bars represent 1mm.

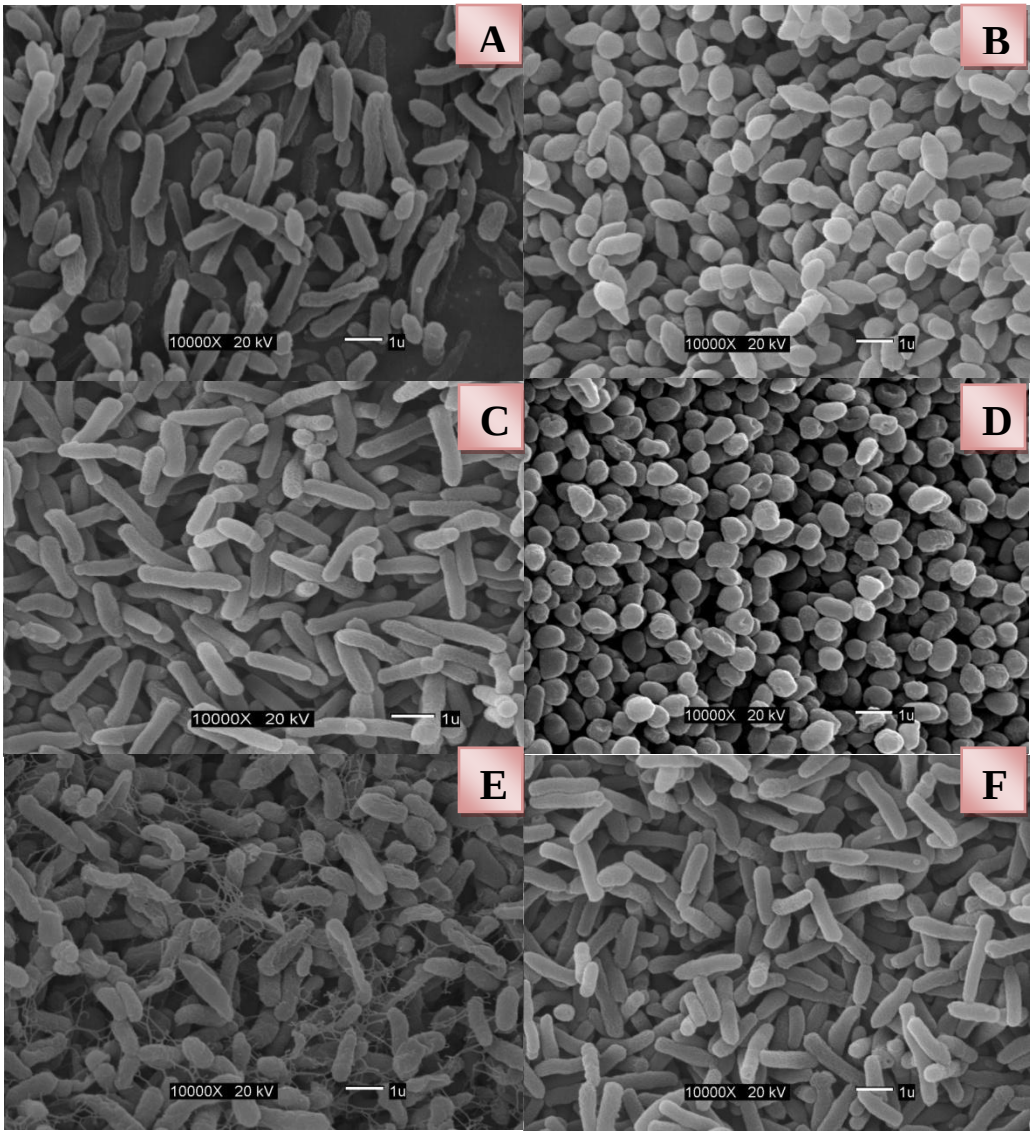


Figure 2.3Isolated PNSB showed morphologies as rods, vibrios and spirilla of different sizes. By using Scanning Electron Microscopy (SEM) three main bacterial shapes were found. The cells sizes ranged from 0.5µm to 2.7µm. The SEM image (E) presented the filamentous structures around the cells and the (F) image showed the same cells after growth in LB broth (2% NaCl). The pictures were taken at the UPRM- Biology Department Microscopy Center.

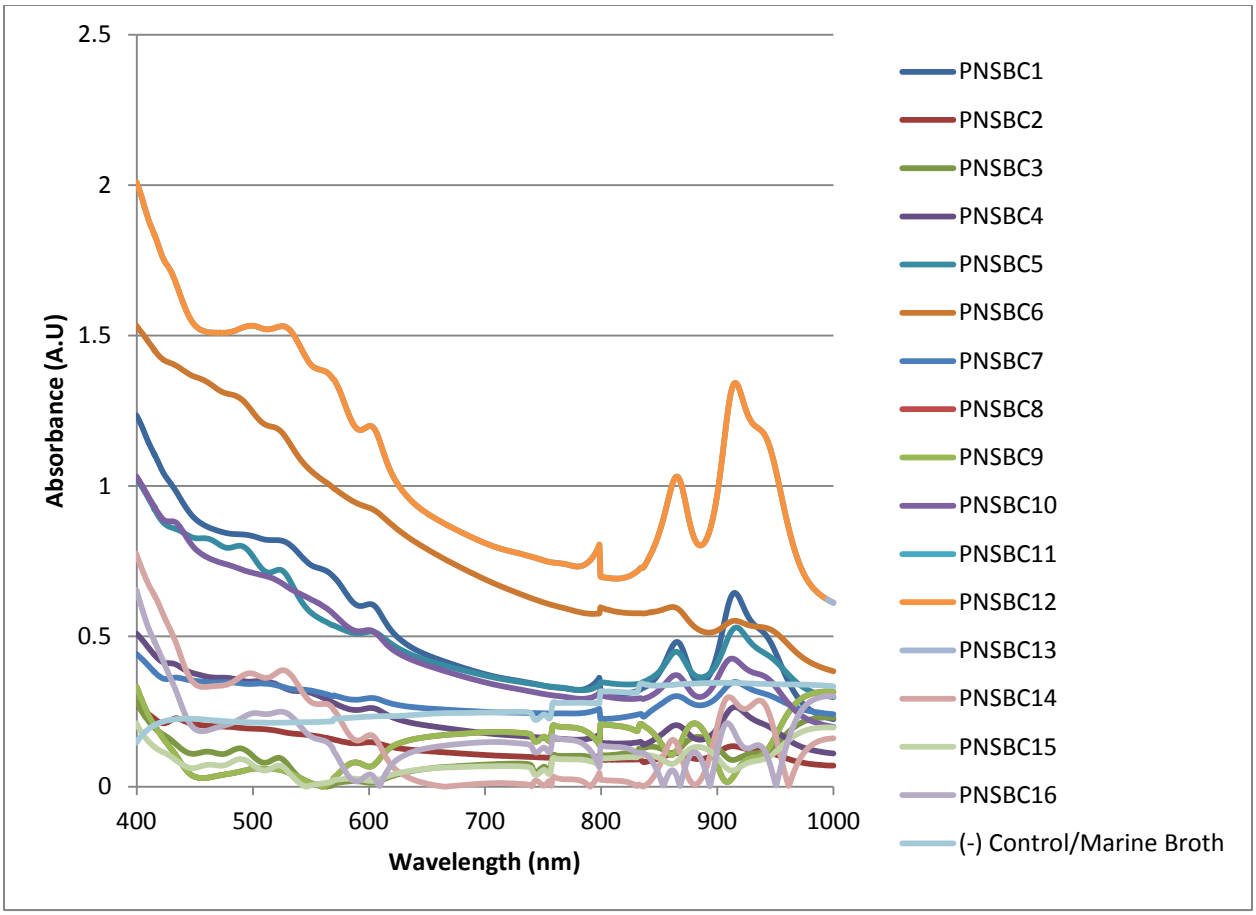


Figure 2. 4 Whole absorption spectra of isolated purple non-sulfur anoxyphototrophic bacteria isolates from Candelaria mat. The whole cell absorption spectra were done from 400nm to 1100nm. Marine broth was used as negative control.

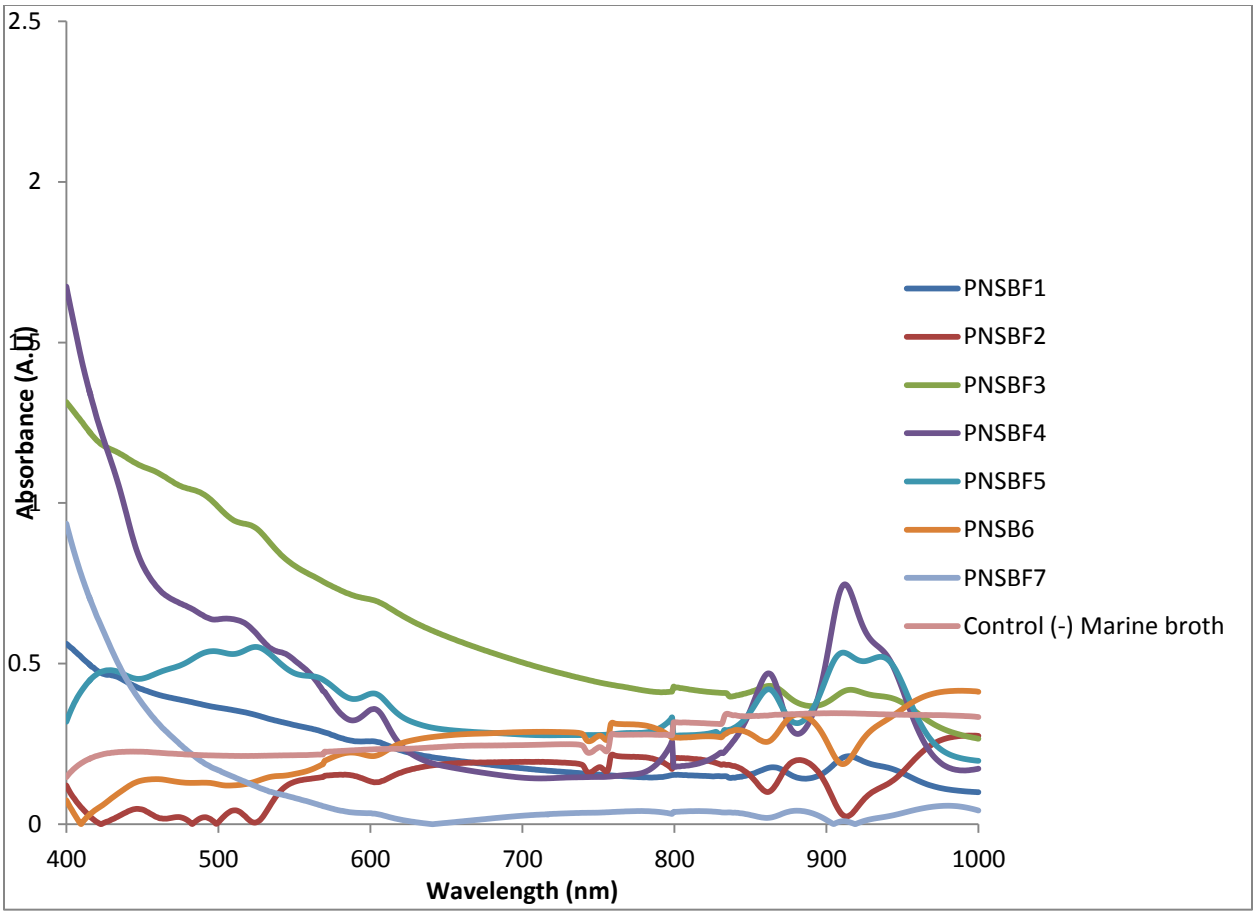


Figure 2. 5 Whole absorption spectra of isolated purple non-sulfur bacteria isolates from Fraternidad mat. The whole cell absorption spectra were done from 400nm to 1100nm. Marine broth was used as negative control.

Table 2.2 Growth of Purple Non Sulfur Bacteria at different salinity percentage

Isolates ID	3%	5%	8%	10%	12%	15%
***R. sphaeroides* 241**	+	+	-	-	-	-
PNSBC1	+	+	+	-	-	-
PNSBC2	+	+	+	+	-	-
PNSBC3	+	-	-	-	-	-
PNSBC4	+	+	+	+	-	-
PNSBC5	+	+	+	+	-	-
PNSBC6	+	+	+	-	-	-
PNSBC7	+	+	+	-	-	-
PNSBC8	+	+	+	+	-	-
PNSBC9	+	+	+	+	-	-
PNSBC10	+	+	+	+	-	-
PNSBC11	+	+	+	+	-	-
PNSBC12	+	+	+	+	-	-
PNSBC13	+	+	-	-	-	-
PNSBC14	+	+	+	+	-	-
PNSBC15	+	+	+	+	-	-
PNSBC16	+	+	+	+	-	-
PNSBF1	+	+	+	+	-	-
PNSBF2	+	+	+	-	-	-
PNSBF3	+	+	+	-	-	-
PNSBF4	+	+	+	+	-	-
PNSBF5	+	+	+	+	-	-
PNSBF6	+	+	+	+	-	-
PNSBF7	+	+	+	+	-	-

PNSBC- Purple non sulfur bacteria from Candelaria, PNSBF- Puple non sulfur bacteria from Fraternidad

Table 2.3 Rangeof optimal pH for isolates purple non sulfur bacteria

Group	Isolates	pH range
A	PNSBC1, PNSBC2, PNSBC3, PNSBC4, PNSBC6, PNSBC7, PNSBC11, PNSBC13, PNSBC15, PNSBF3, PNSBF7	5.0-8.5
B	PNSBC5, PNSBF1	7.0
C	PNSBC12, PNSBC14, PNSBC9, PNSBC10, PNSBC8, PNSBC16, PNSBF2, PNSBF5, PNSBF6, PNSBF4	6.5-8.0

PNSBC- Purple non sulfur bacteria from Candelaria, PNSBF- Puple non sulfur bacteria from Fraternidad

Table 2.5 Biochemical tests

Isolates ID	Oxidase	Catalase	Urease	Gelatin	Citrate	Nitrate	Starch	Mannitol	SIM	TSIA
R. sphaeroides	+	+	+	-	+	+	+	+	-/-	+/-
P. aeruginosa	+	+	-	-	+	+	+	-	-/+	+/-
PNSBC1	+	+	+	-	+	+	+	+	-/-	+/+
PNSBC2	+	+	+	-	+	+	+	+	-/-	-/+
PNSBC3	+	+	+	-	+	+	+	+	-/-	+/-
PNSBC4	+	+	+	-	-	+	+	+	-/-	+/-
PNSBC5	+	+	+	-	+	+	+	+	-/-	+/+
PNSBC6	+	+	+	-	+	+	+	+	-/+	+/+
PNSBC7	+	+	+	-	+	+	+	+	-/-	+/+
PNSBC8	+	+	+	-	-	+	+	+	-/-	+/-
PNSBC9	+	+	+	-	+	-	+	+	-/-	-/-
PNSBC10	+	+	+	-	-	+	+	+	-/-	-/+
PNSBC11	+	+	+	-	-	+	+	+	-/+	-/+
PNSBC12	+	+	+	-	-	+	+	+	-/-	+/+
PNSBC13	+	+	+	-	-	+	+	+	-/-	-/-
PNSBC14	+	+	+	-	-	+	+	+	-/+	+/-
PNSBC15	+	+	+	-	+	+	+	+	-/-	-/+
PNSBC16	+	+	+	-	-	+	+	+	-/-	-/-
PNSBF1	+	+	+	-	+	+	+	+	-/-	-/+
PNSBF2	+	+	+	-	-	+	+	+	-/-	-/+
PNSBF3	+	+	+	-	-	+	+	+	-/-	+/-
PNSBF4	+	+	+	-	-	+	+	+	-/+	+/+
PNSBF5	+	+	+	-	-	+	+	+	-/-	-/+
PNSBF6	+	+	+	-	-	+	+	+	-/-	-/-
PNSBF7	+	+	-	-	-	+	-	+	-/-	-/-

PNSBC- Purple non sulfur bacteria from Candelaria, PNSBF- Puple non sulfur bacteria from Fraternidad

Table 2.5 Purple non- sulfur bacteria maximum absortion peaks of bacteriochlorophyll and carotenoid pigments

Groups	Isolates	Bacterichlorophyll *a* (nm)	Carotenoid pigments (nm)
1	**PNSBC1**	940, 920, 860	560, 500, 530, 430
2	**PNSBC2, PNSBC4, PNSBC5, PNSBC7, PNSBC10**	940, 900, 860	600, 560, 400
3	**PNSBC3, PNSBC8, PNSBC9, PNSBC15**	930, 880	560, 520, 500, 400
4	**PNSBC6**	940, 920, 860, 800	600, 560, 530
5	**PNSBC11, PNSBC12, PNSBC13**	940, 920, 860, 800	600, 560, 530
6	**PNSBC14, PNSBC16**	930, 910, 880, 860	600, 530, 500
7	**PNSBF1, PNSBF3, PNSBF7**	950, 900,860, 850	600, 560, 480, 450
8	**PNSBF2, PNSBF6**	885, 830	550, 500, 420
9	**PNSBF4**	930, 915, 860	610, 530
10	**PNSBF5**	940, 915	606, 550, 530, 520

PNSBC- Purple non sulfur bacteria from Candelaria, PNSBF- Puple non sulfur bacteria from Fraternidad

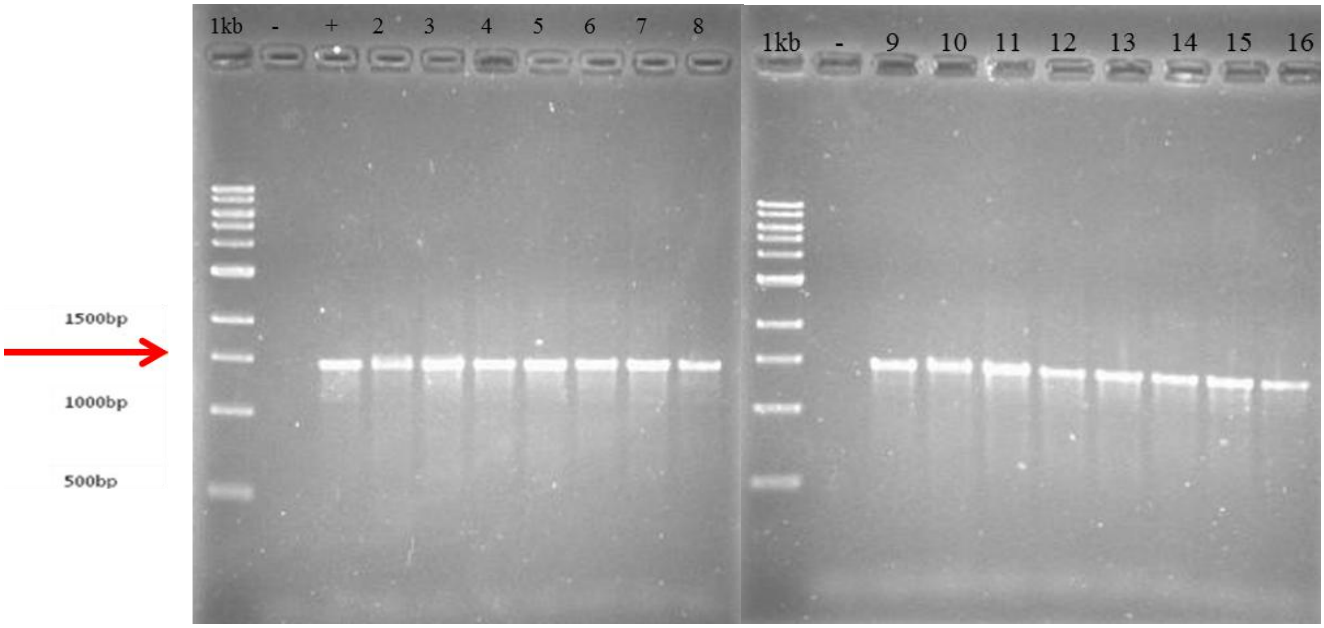


Figure 2.6 16S rDNA gene amplification of Purple Non Sulfur Bacteria isolates in 1% agarose. The molecular marker used was 1Kb ladder (NEB). The positive control (+) was a PCR using *Escherichia coli* genomic DNA. The arrows indicate the position of the 16S rDNA amplicons (~1.5kb).

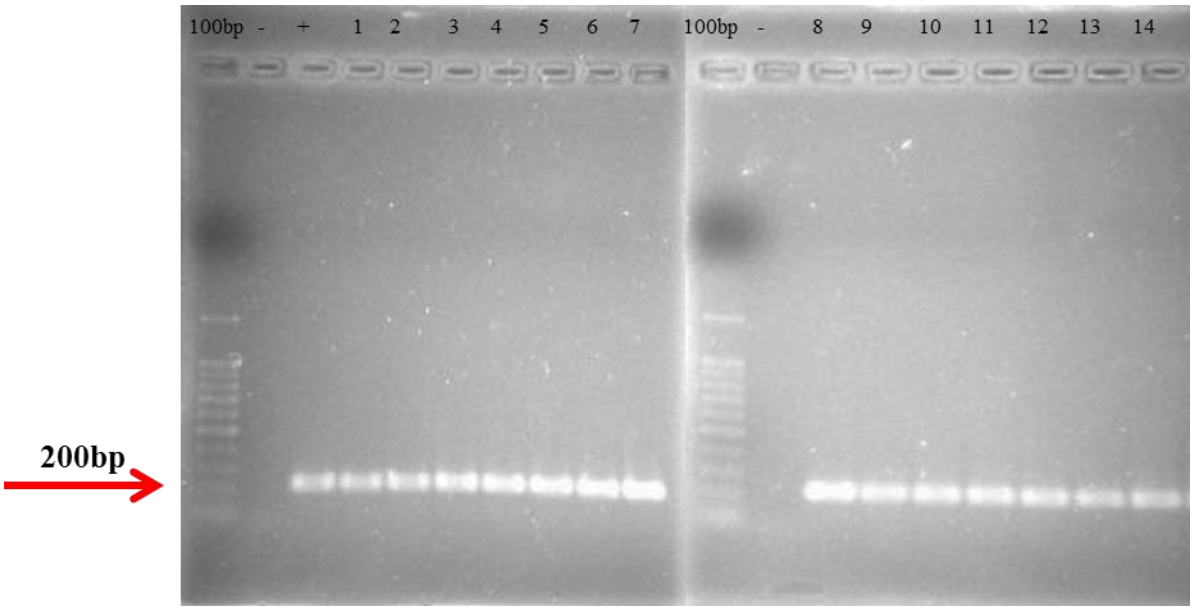


Figure 2.7 Amplification of the *pufM* gene in the isolates. The molecular marker was 100bp ladder. The arrows indicate the position of the *pufM* gene amplicons (~229bp).

Table 2.6 *In silico* analysis documentation of sequenced 16S rDNA using basic local alignment search tool (BLAST)

Isolates ID	Suggested Candidate (BLAST)	Coverage %	E- value	Max. Identity	Accession Num.
PNSBC1	*R. sphaeroides* JA460	99%	0.00	99%	Pending
PNSBC2	*R. sphaeroides* JA491	99%	0.00	100%	Pending
PNSBC3	*R. sphaeroides* S2-1	98%	1e-63	99%	Pending
PNSBC4	*Rhodospirillaceae bacterium* LA45	100%	0.00	99%	Pending
PNSBC5	*R. sphaeroides* JA460	99%	0.00	99%	Pending
PNSBC6	*Vibrio spp.*HS1	98%	0.00	99%	Pending
PNSBC7	*R. sphaeroides* P221	94%	5e-25	95%	Pending
PNSBC8	*Rhodospirillaceae bacterium* LA45	93%	4e-15	95%	Pending
PNSBC9	*Rhodothalassium spp.*JA389T	99%	0.00	99%	Pending
PNSBC10	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBC11	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBC12	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBC13	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBC14	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBC15	*R. sphaeroides* JA460	100%	0.00	99%	Pending
PNSBC16	*Rhodospirillaceae bacterium* LA45	100%	0.00	99%	Pending
PNSBF1	*R. sphaeroides* JA460	99%	0.00	99%	Pending
PNSBF2	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBF3	*Rhodospirillaceae bacterium* LA45	100%	0.00	99%	Pending
PNSBF4	*Rhodospirillaceae bacterium* LA45	100%	0.00	99%	Pending
PNSBF5	*Rhodospirillaceae bacterium* LA45	100%	0.00	99%	Pending
PNSBF6	*Rhodospirillaceae bacterium* LA45	99%	0.00	99%	Pending
PNSBF7	*Rhodovulum marinum*	99%	0.00	99%	Pending

PNSBC- Purple non sulfur bacteria from Candelaria, PNSBF- Puple non sulfur bacteria from Fraternidad

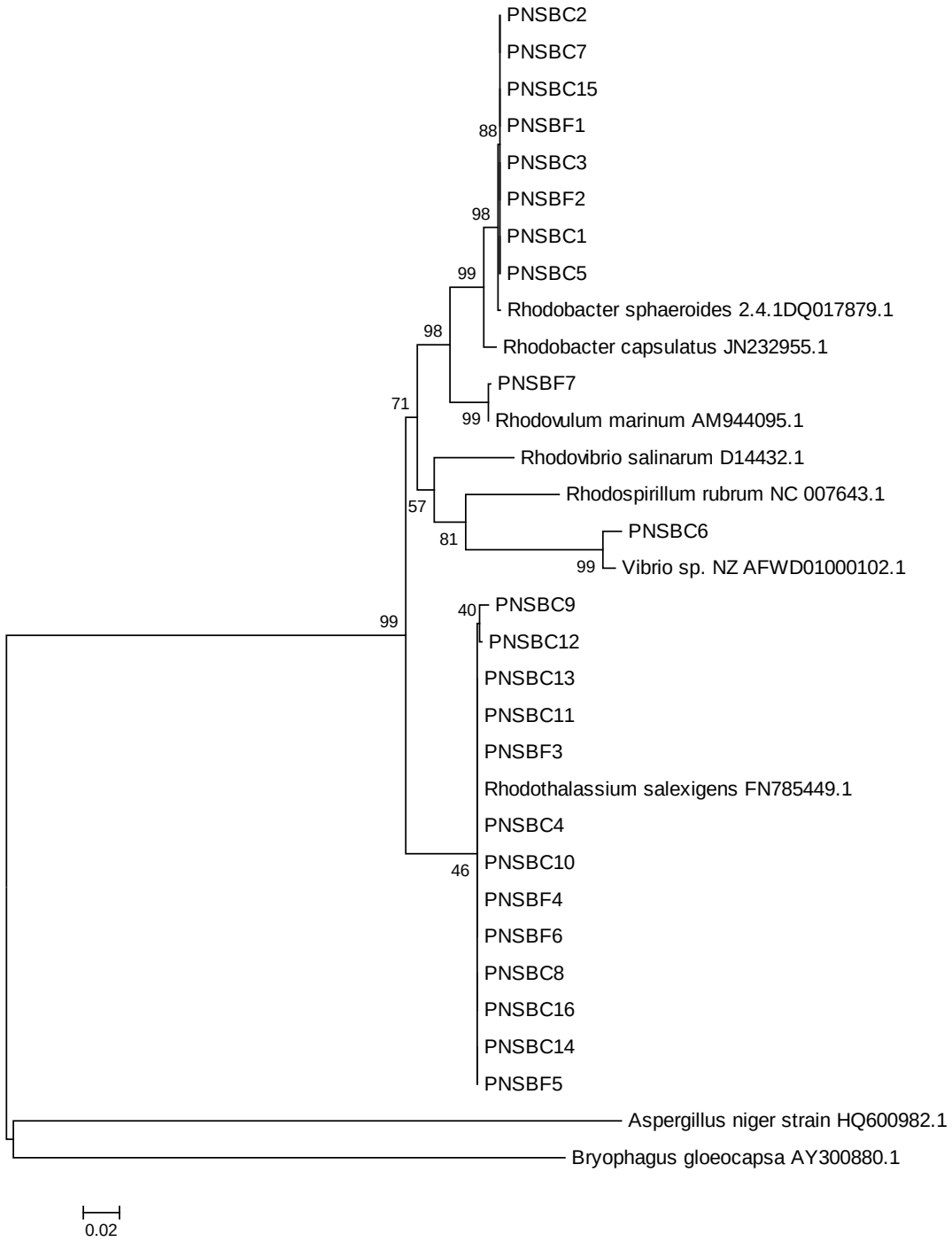


Figure 2.8 Phylogenetic analysis of the 16S rDNA gene sequencing. The generation of the consensus tree was made by Neighbor Joining method and the final tree was drawn with the use of Mega 5 beta version. Numbers in the nodes are the bootstraps values. The bars represent 0.02 substitutions per nucleotide position. A total of 2000 repetitions based in a pair wise arrangement were performed. *Aspergillus niger* (HQ600982.1) and *Bryophagus gloeocapsa* (AY300880.1) were used as outgroups.

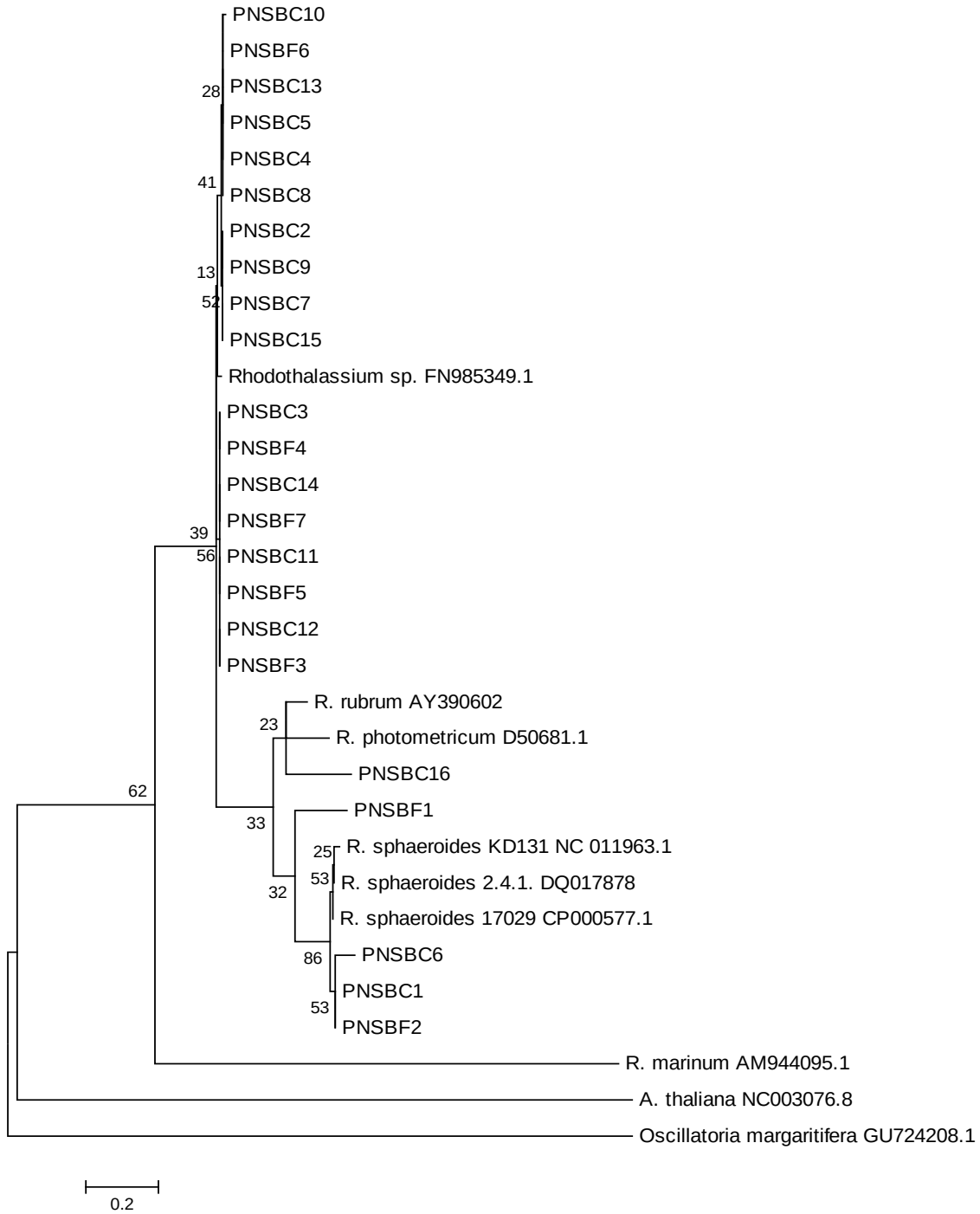


Figure 2.9 Phylogenetic analysis of the *pufM* gene sequencing. The generation of the consensus tree was made by Neighbor Joining method and the final tree was drawn with the use of Mega 5 beta version. Numbers in the nodes are the bootstraps values. For *pufM* the bars represent 0.2 substitutions per nucleotide position. A total of 2000 repetitions based in a pair wise arrangement were performed. *Arabidopsis thaliana* (NC003076.8) and *Oscillatoria margaritifera* (GU724208.1) were used as outgroups.

2.4 Discussion

The main purpose of this research was to isolate and characterize Purple Non SulfurBacteria (PNSB) from subtropical hypersaline microbial mats from the Cabo Rojo salterns. The results showedthe presence of this group of microorganisms in the two different microbial mats studied (Figure 2.1). The highest number of isolates were found in the Candelaria mat sample in both seasons, being the rainy season the one with highest number of isolates overall.In studies performed by Fernandez *et al.,* (2003) found that during dry season, microbial communities colonization in Rio Pinto Microbial Mat (Spain) was not favored due to an increase in pH, high erosion rates, and high saturated salt levels. In contrast, they found that the highest rate for microbial communities colonization and growth occured during rainy season periods, because this is the period with low saturation waters and less ferric concentration that can interfere with photosynthesis. Another research performed by Casillas *et al*., (2005) studied the changes in community composition during the dry and rainy season in Cabo Rojo salterns mats, and suggested that the low humidity affect the microbial communities composition and the microbial activities. Base on the studies described above, together with our findings, hydro-periods plays an important role in the quantity (amount) and diversity (different genera) of microbial communities found in the mats.

One important aspect of our research is the inclusion of two different types of microbial mats. The Candelaria mat is considered a benthic microbial mat; meaning that it is located at the lowest level of a body water such as an ocean. Instead, Fraternidad mat is an ephemeral microbial mat; meaning that it only exists for a short periods following precipitation or floods. Our findings support the concept that the diversity of microorganism is higher in benthic mats, because they maintain a more permanent structure and integrity compared with an ephemeral

environment (Madigan*et al.,* 2010). Rok Tkavc *et al.*,(2010) studied two aspects in the microbial mat that also apply to the Cabo Rojo mats; that included (1) the presence of three different layers and (2) the sea water level fluctuations. The hypersaline microbial mat described was benthic and located in Secovlje salterns in Slovenia. In their study the bacterial communities such as Acidobacteria, Actinobacteria, Cyanobacteria, α- Proteobacteria and β- Proteobacteria were found and described across the hypersaline microbial mat at different seasons. The research revealed an increase in diversity during rainy season when compared with the dry season. This study suggests the concept that microbial communities can be established better in a benthic microbial mat because it keeps the interactions between microorganism through the different layers, maintaining the necessary gradient flux of nutrients.

Previous enrichment and nutrient studies (data not showed) of PNSB were performed in the laboratory. The results showed that the better optical growth conditions of the PNSB were obtained using marine media (agar and broth); probably it provided the necessary salts, nutrients and minerals that this special group of microbes needs (Figure 2.2).

After enriching the samples with the environmental conditions required for anoxyphototrophic growth, a total of 23 purple non sulfur bacteria were isolated based on monitoring the presence of reddish color in the colony. The pigmentation observed ranged from light red to dark red. This color is influenced byfactors such as the amount and type of pigment produced, and mutations in the genes that encode for the pigmentation (Melendez-Martinez*et al.,* 2007). The intensity and color of the pigmentation indicate the presence of photosynthetic pigments like carotenoids and bacteriochlorophyll *a* or *b* (Glaeser and Overmann, 1999). Melendez-Martinez *et al.,* (2007) have described spirilloxanthin and spheroidene as the most common carotenoid pigments in this group of bacteria. Only the genera *Rhodocyclus* and

Rhodopila have rhodopinal (purple-violet) and keto-carotenoids (purple-red) respectively (Imhoff and Trüper, 2005). The production of spheroidenone was studied by Yelissev *et al*,. (1996) and they determined the involvement of this carotenoid pigment in the adaptation to light intensity changes in*Rhodobacter sphaeroides*. Also, they described using spectrophotometric analysis the maximum absorption peaks for carotenoids pigments, specifically spirilloxanthin (490,510 and 530nm) and spheroidene (400, 460 and 500nm). In our study and as described by Melendez-Martinez, all the isolates showed the characteristic absorption peaks for spirilloxanthin and spheroidene.

As described before, the characteristic reddish pigmentation of the carotenoids can be detected spectrophotometrically, specifically due to its distinctive chemical structures such as methoxy and methyl groups. The characteristic peaks of these pigments can be detected using a whole cell spectrum in an interval of 400nm to 1100nm. Table 2.5 showed the maximum peaks recorded in whole cell spectra for the PNSB isolatedin this study and which of them corresponded to bacteriochlorophyll *a* and carotenoid pigments. In this research we only detected bacteriochlorophyll *a* because the presence of absorbance peaks was detected only until 1000nm, and the peaks that correspond to bacteriochlorophyll *b* are present at 1020-1040nm.The negative control used for the study was marine broth medium. The spectra for the negative control showed two peaks instead of a flat line because there is a change in detectors (lasers). The first laser reads at 550nm, and the second laser reads at 800nm.Based on the carotenoids and bacteriochlorophyll found, we can relate that with certain PNSB genera. For example, *Rhodobacter*. has a characteristic peaks at 474-510, 580-590 and 800-880nm, in contrast peaks at 450-590 and 800-860nm and 400-600, 800-930 are present in *Rhodovulum*. and *Rhodospirillum*. respectively (Imhoff and Truper, 2005). Based on the peaks described above,

spectrophotometrically our study suggests only one PNSB isolate (PNSBC1) which is related to *Rhodobacter*, nineteen (PNSBC2-C16, PNSBF2, PNSBF4-F6) to *Rhodospirillum*, and only three (PNSBF1, PNSBF3, PNSBF7) to *Rhodovulum*.

According to Oda *et al.*, (2002), the genera of purple non sulfur bacteria may show morphological differences that can be used as a taxonomic tool. For example, members of the genus *Rhodobacter* are ovoid or rod shape, and can be motile by polar flagella or not motile. Also, *Rhodospirillum* which cells show vibrio to spiral shape and are motile by bipolar flagella. The microscopic analysis performed in our study describes the sizes and morphology of PNSB as rods, vibrios and spirillum. The rods and vibriovaried in sizes and ranged from 0.5-2.7µm and spirillum were rangedfrom 1.0-2.5µm (Figure 2.3). This result agrees with the studies performed by Okubo *et al.*, (2006) where they described the anoxyphototrophic bacteria present in a colored microbial mat from a wastewater ditch, as rods with a size that ranged from 0.5-2.0µm. Using the SEM we were able to detect the presence of filamentous structures through the samples. These structures were eliminated after we modified the SEM protocol; instead we growing the PNSB in marine broth, we grew the isolates in Luria Bertani broth (LB) with 2% NaCl (Figure 2.3).

Purple non-sulfur bacteria are some of the metabolically most versatile organisms known. *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* and *Rhodopseudomonas palustris*are capable of using a variety of organic and inorganic compounds as electron donors and acceptors (respiratory and fermentative growth). This remarkable metabolic versatility of purple non-sulfur bacteria extends to the utilization of a large spectrum of carbon sources from CO_2 (autotrophic growth) to fermentative products generated by other organisms in the same habitats (Alber *etal.*, 2006). These important characteristics allow a physiological characterization of this group of

bacteria. The isolates in this study were grown in different media to determine the fermentation and catabolism of some carbohydrates (Table 2.4), the use of different carbon sources, like citrate and nitrate and the detection of enzymes like gelatinase, catalase, amylase and urease. The carbohydrates fermentation test divided the isolated purple non-sulfur bacteria intothree groups. In the first group,eight of the isolates were similar to *Rhodobacter,* because they showed the same carbohydrate fermentation profile. Only one of the isolates (PNSBF7) did not have the capability to ferment maltose, sucrose and lactose. All the isolates have the capability to reduce nitrate to nitriteand generated the enzymes amylase, catalase and oxidase. Fifteen of the isolated PNSB generated the gelatinase enzyme; and only one of the isolates was urease positive. At the same time, these results suggest that ten of the isolates had similar biochemical profile of *Rhodobacter,* fourteen were related with *Rhodospirillum* and one with *Rhodovulum*.

Physiologically, the optimal temperature of growth to the PNSB isolated in this study was 30ºC-32ºC. Based on this data the isolates can be classified mesophilic microorganisms. The pigment production was not affected by the temperature changes, but it was affected by aerobic conditions of grow.The optimal conditions to produce the pigment was anoxyphototrophically under photosynthetic conditions (anaerobicallyin presence of light).

The capability of growing at different concentrations of NaCl was also measured in the isolated PNSB. The highest concentration that most of the isolates were able to growth was 10%, with the exception of one of the isolates (PNSBC3), which grew up to 3% of NaCl. These results demonstrated agood adaptation of the strains to their environment where the highest salinity is 20-25% during the dry season. The majority of PNSB require 1-3% NaCl for optimalgrowth except for members of the genera *Rhodospirillum,Phaeospirillum*, *Rhodocista* and *Thiospirillum,* which have not salt requirements. The salt- requiringbacteria have been isolated from coastal

marineor hypersaline environments (Drews, 1982; Imhoff *et al.*, 1998). In a study performed by Hirschler *et al.,* (2003) a series of PNSB were isolated from hypersaline environments and described as halophilic bacteria belonging to the genera *Rhodovibrio,Rhodothalassium, Roseospira*and *Rhodobacter*. Onlya few strains have been described as extreme halophilesand have been included in a single genus*Halorhodospira*. In our study, there were no extreme halophiles isolated, in contrast all the isolates can be considered as halophiles, because marine microorganisms usually have a specific requirement of NaCl and typically organisms from hypersaline environments grow best with 3%-12% NaCl (Madigan *etal.*, 2010). Also, all the isolates were growth in media such as Luria Bertani broth (1% NaCl) and none of the isolates have the capability to growth.

The pH analysis classified the 23 isolates in three different groups (Table 2.3). The first group wascomposed by twelve isolates (PNSBC1-C7, PNSBC11, PNSBC13, PNSBC15, PNSBF3 and PNSBF7). These isolates showed results very similar to *Rhodobacter sphaeroides*which hasan optimal growth at pH that range from 5.0-8.5. The optimal pH for PNSB from the family of *Rhodobacteraceae* (*Rhodobacter spp.*and *Rhodovulumspp.*) was pH 5.0-8.0. Just two isolates were able to growth only in pH 7.0 such as the genus *Rhodovibrio spp.*The membersfrom the third group only grew at pH 6.5-8.0.They compared to bacteria from the genus *Rhodospirillum spp.*(Imhoff and Trumper, 2005). In this study, the isolated PNSB can be considered neutrophiles, which are organism that grow optimally at a pH value in the range of 5.0-8.5.

In order to confirm the molecular identity of the isolates, two biomarkers were amplified in the isolated purple non-sulfur bacteria. The first biomarker was 16S rDNA, which was successfully amplified from all strains and sequences with an average size of 700-900 base pairs

(bp). This represents at least half of the total size of the gene (1500bp), which is a representative and accurate tool to identify and compare the isolates (Figure 2.6). According to Neighbor-Joining phylogenetic tree (Figure 2.8) there are four principal clusters. The first cluster was dominated by isolates PNSBC4, PNSBC8-C14, PNSBC16, PNSBF3, PNSBF4, PNSBF5 and PNSBF6, which are closely related to*Rhodothalassium salexigens*. *In silico* analysis suggested that all the isolates in the first cluster have 99% homology with purple non-sulfur bacteria from the family *Rhodospirillaceae*. Based on the 16S rDNA marker, this cluster is the most important one from the perspective that there is no direct homology with known sequences in BLAST database (Basic Local Alignment Search Tool) (*Rhodobacter sphaeroides, Rhodospirillum rubrum, Rhodovulum marinum*), which suggests that future studies must be continued to characterize in more details these isolates. The second cluster presents a specific relationship between isolate PNSBF7 and *Rhodovulum marinum*. The third cluster is composed by isolates related to purple non-sulfur bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, isolates in this cluster were PNSBC1-C3 PNSBC5, PNSBC7, PNSBC15, PNSBF1 and PNSBF2. The last cluster was composed by the isolate PNSBC6, which is closely related with Gamma Proteobacteria *Vibrio* spp.

The second marker, *pufM*, was amplified and sequenced successfully from all isolates (average of 185 bp-210bp). This represents more than a half of the gene size that is approximately 229bp (Figure 2.7). The *in silico* analysis confirmed that all sequenced samples coded for PufM protein. According to Neighbor-Joining phylogenetic tree (Figure 2.9) there are three principal clusters. The first cluster related eighteen of the isolates (PNSBC2,PNSBC4-C5, PNSBC7-C16,PNSBF3, PNSBF5 and PNSBF7) with *Rhodothalassium salexigens*. The second cluster is only constituted by the isolate PNSBC16, which is related with known sequences of

pufM from *Rhodospirillum rubrum*. Finally, the last cluster related four of the isolates (PNSBF1-F2, PNSBC6 and PNSBC1) with *Rhodobacter sphaeroides.*The isolates PNSBC1, PNSBF1 and PNSBF2 are related with *Rhodobacter sphaeroides* in the phylogenetic tree of the 16S rDNA also, and in the case of *Rhodothalassiumsalexigens* eleven of the isolates (PNSBC10-C14, PNSBC4, PNSBC8, PNSBC9, PNSBC16, PNSBF5 and PNSBF3) have both trees related (16S rDNA and *pufM)* too.

Recapitulating and based on the microscopic, biochemical and molecular analysis only eight of the isolated purple non sulfur bacteria were different. In Candelaria lagoonmicrobial mat the amount of microorganisms belonging to purple non-sulfur bacteria groupwere higher than in Fraternidad lagoon mat, but only five of them were different from the total of sixteen isolated. In Fraternidad only three were different from a total of seven isolated. Three different morphologies were showed in the isolates as rods, vibrios and spirilla; of variable sizes. All the isolates from both mats exhibited the reddish pigmentation and the presence of bacteriochlorophyll *a* and carotenoid pigments. The isolates represent halophiles microbes community, because they have the capability of growing in NaCl concentrations between 3%-12%. According to *in silico* and phylogenetic analyses, at least five of the isolates may be considered from the genus *Rhodobacter*, and one of them may be considered as the already described PNSB *Rhodovulum marinum*. The last seventeen isolates belong to the PNSB family of *Rhodospirillaceae*, but we could not identify a specific genus or species for these organisms with the performed analysis.

Chapter Three

Culture Independent Approaches to Identify Purple Non Sulfur Bacteria from Subtropical Hypersaline Microbial Mats

3.1 Introduction

Photosynthetic ability is widely distributed among microorganism. Anoxygenic phototrophic bacteria are prokaryotes capable of utilizing light as an energy source, by unlike cyanobacteria, do not evolve molecular oxygen. The photosynthesis genes are organized in clusters occupy around 46kb of chromosome and are mainly composed of genes encode for bacteriochlorophyll, carotenoids, light harvesting complex and reaction center. The reaction center is form by PufM, PufL and PuhA proteins (Zeilstra, 1998). Achenbach and collaborators designed a PCR primer sets based on *pufM* genes which encoded for a pigment binding protein in the photosynthetic reaction center of all purple non sulfur bacteria. The diversity andphylogenetic composition of PNSBcan be study using culture independent approaches such as biomarker profile andPCR cloning and sequencing of the *pufM* gene (Okubo *etal.,* 2006). Theclone library analysis of the *pufM* gene has been reported as apromising approach to the phylogenetic characterization ofphototrophic bacterial communities in aquatic and hot springenvironments (Achenbach *etal.,* 2001, Yutin *et al.,* 2005).

In this study we isolated cultivable purple non sulfur bacteria, buy also the uncultivable PNSB are important for the diversity of the microbial mats. The pink layer of the microbial mat samples were used to extracted metagenomic DNA. The DNA were used to amplify the *pufM* genes and cloned in a vector. The *pufM* libraries were screened for insert of *pufM* gene and sequences, to compare the sequence of cultivable and uncultivable PNSB. The diversity index and the richness were estimate with a rarefaction curve.

3.2 Materials and Methods

DNA Extraction

Total DNA was extracted from the pink layer of the microbial mat samples from Candelaria and Fraternidad (dry and rainy season) using a Power Soil DNA Isolation Kit (MoBio) following the manufacture specifications. Briefly, samples are added to a bead beating tube for rapid and thorough homogenization. Cell lysis occurred by mechanical and chemical methods. Total genomic DNA was captured on a silica membrane in a spin column format. DNA was washed and eluted from the membrane and then ready for PCR analysis.

PCR and cloning

A portion of the *pufM* gene was amplified from the DNA extracted by PCR using *pufM* specific primer set 557F [5'-CGCACCTGGACTGGAC-3'] and 750R [5'-CCCATGGTCCAGCGCCAGAA-3']and the GoTaq® Green Master Mix of Promega (Achenbach, *etal.,* 2001).The PCR parameters were the following: initial denaturalization at 94ºC for 3 minutes; followed by 30 cycles which included denaturalization at 94ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºC for 1 minute and a final extension at 72ºC for 10 minutes. The amplifications were confirmed performing an agarose gel electrophoresis (0.8%) at 80 volts in Tris-EDTA 1X buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0).

The PCR product amplicons were cloned into the pCR® 4 TOPO® vector (Invitrogen) following the manufacture specifications. The *pufM* microbial mats libraries were grown in 5mL Luria Bertani Broth with 5ug/mL of kanamycin and incubate in shaker at 37ºC for 24 hours. The clones were verified by plasmid extraction using QIAprep® Spin Miniprep Kit

(Qiagen®)following the manufacture recommendations. All clones libraries were screened for inserts by PCR amplification using pufM gene primers. Amplification was carried out with 30 cycles at 94ºC for 60 s, 55ºC for 60 s, and 72ºC for 60 s. After, they were checked by agarose gel electrophoresis (1.5%) at 70 volts in Tris-EDTA 1X buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0).

DNA sequencing and *In silico* analysis

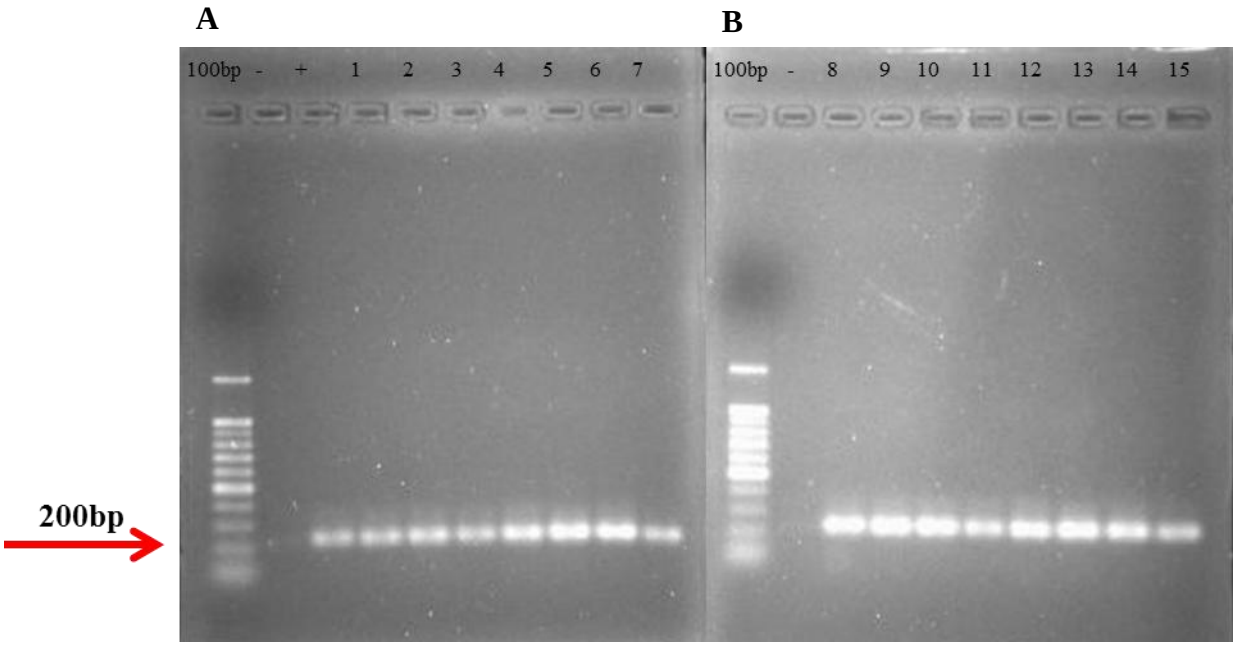
The clones were sequences using ABI3730XL DNA Analyzer in Macrogene facilities (Maryland, USA). All the *pufM* sequences obtain from the clones were aligned along with the closest BLAST matches from GenBank. The sequences wereedited using bioinformatics tools such as Chromas Lite 2.0.1 and then aligned and edited with BioEdit 7.0.9. The phylogenetic analysis was performed using the MEGA 5.1 software. The distance model used was p-distance and the bootstrap test of phylogeny was calculated for 2000 replicates. The creation of the consensus tree was performed by the Neighbor Joining method and the final tree was drawn with the use of MEGA 5 Tree Explorer.

Rarefaction curve

To compare the cultivable and uncultivable PNSB isolated were used a rarefaction curve (Simberloff, 1972). The rarefaction curves were developed using RDP Pyrosequencing Pipeline Rarefaction Tool (http://pyro.cme.msu.edu/spring/rarefaction.spr); that was designed to calculate various diversity indices and richness estimator. Diversity indices and richness estimators are useful to compare the relative complexity of two or more communitiesand to estimate the completeness of sampling of a community (Schloss and Handelsman, 2004).

3.3 Results

Two clone libraries were achieved using TOPO TA Cloning Kit (Invitrogen). A total of 872 clones from Candelaria and 1031 clones from Fraternidad were obtained. The screening of the clone libraries resulted in an amplification of the *pufM* gene; with an amplification product of 229bp (Figure 3.1). Products were successfully sequenced from a total of 80 clones (40 from Candelaria and 40 from Fraternidad). An average close to 190bp was obtained from the expected 229bp product (Figure 3.1). For the phylogenetic analysis, both trees had a total of 2000 repetitions. For both trees the out groups (*Ostreococcus lucimarinus* CP000600 and *Arabidopsis thaliana* NC003076.8) were used to confirm the quality of the analysis. For both trees, known sequences that were chosen as control positives were from *R. sphaeroides, R. capsulatus, R. rubrum, R. photometricum, R. marinum* and *Rhodothalassium spp.*(Figure 3.2). A rarefaction curve was performed to estimate the species richness from microbial mats samples (Figure 3.3).



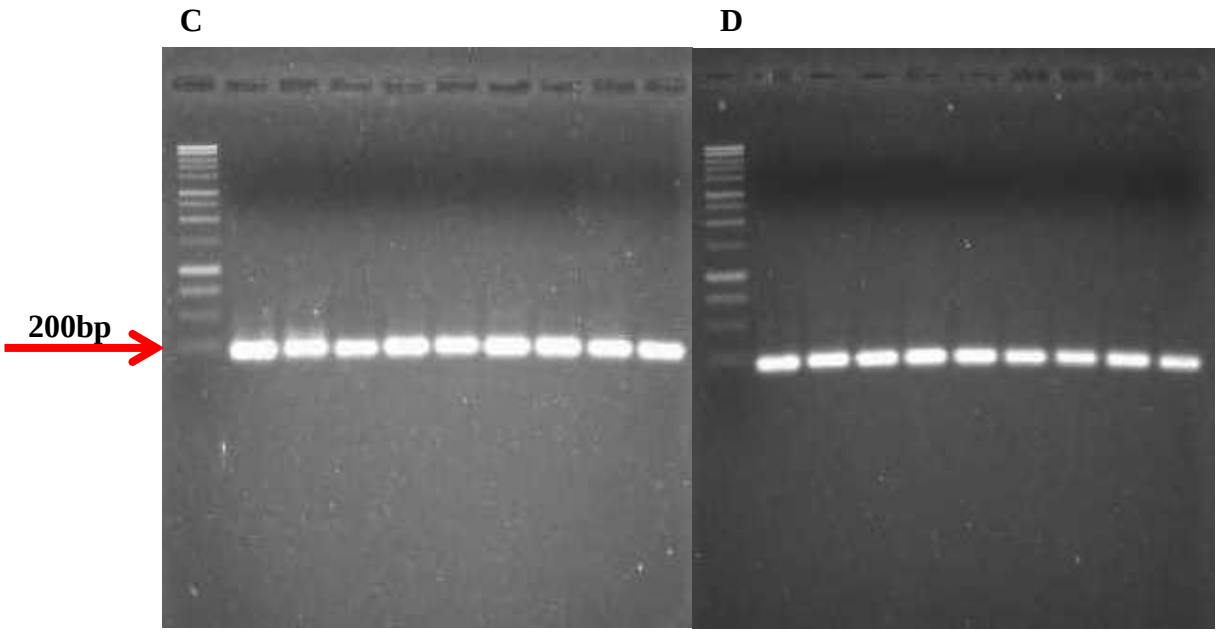
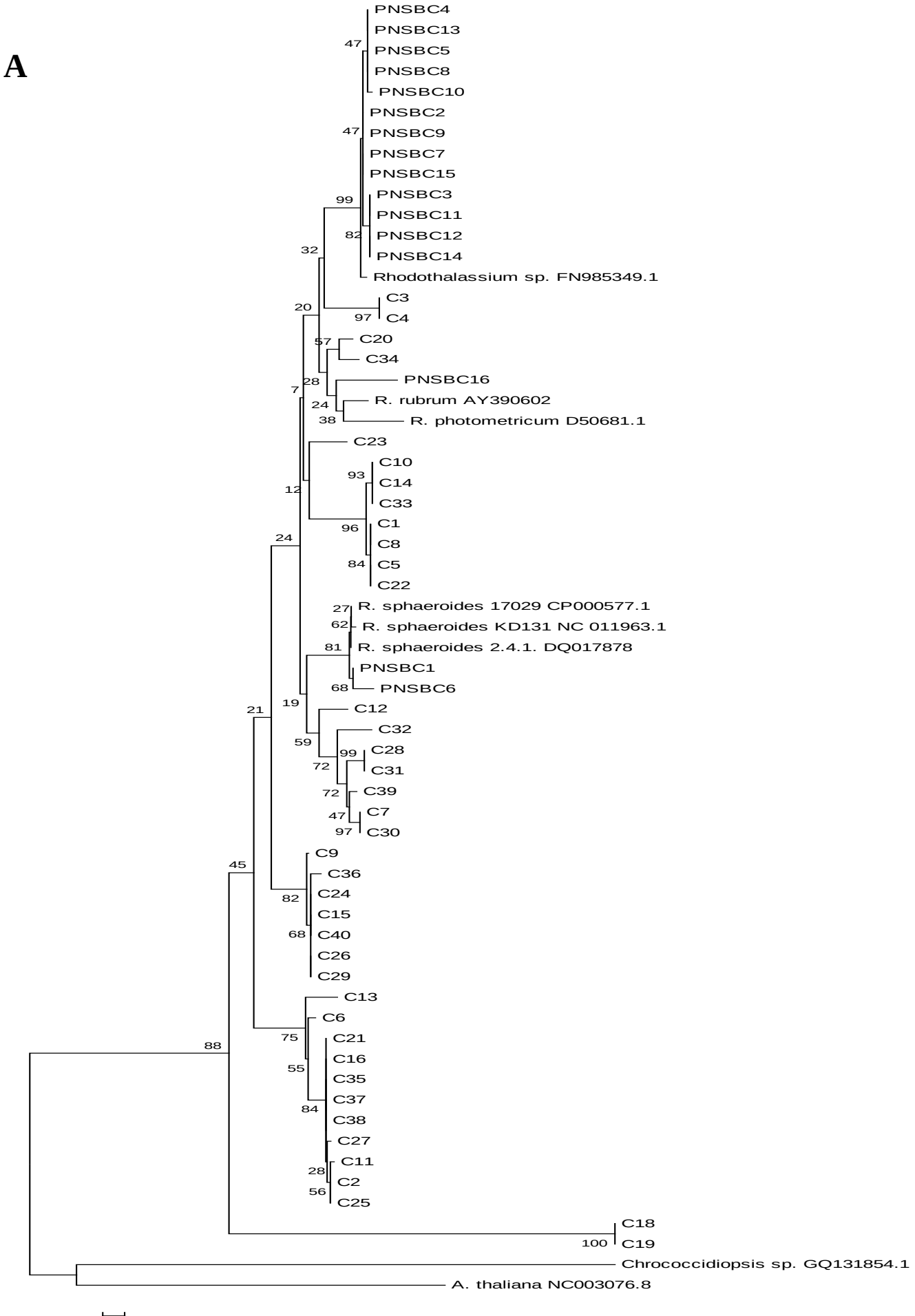


Figure 3.1 Amplificaction of the *pufM* gene in the clone libraries. The samples were run by electrophoresis in a 1.5% agarose gel, using 2Kb as a molecular marker. The gels A and B represent clones from Candelaria library and gels C and D representclones from Fraternidad library.

A


PNSBC4
PNSBC13
47
PNSBC5
PNSBC8
PNSBC10
PNSBC2
47
PNSBC9
PNSBC7
PNSBC15
99
PNSBC3
PNSBC11
82
PNSBC12
PNSBC14
32
Rhodothalassium sp. FN985349.1
C3
20
97
C4
57
C20
C34
28
PNSBC16
7
24
R. rubrum AY390602
38
R. photometricum D50681.1
C23
C10
93
C14
12
C33
96
C1
24
C8
84
C5
C22
27
R. sphaeroides 17029 CP000577.1
62
R. sphaeroides KD131 NC 011963.1
81
R. sphaeroides 2.4.1. DQ017878
PNSBC1
68
PNSBC6
19
21
C12
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C28
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C39
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C7
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C6
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C16
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C35
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C11
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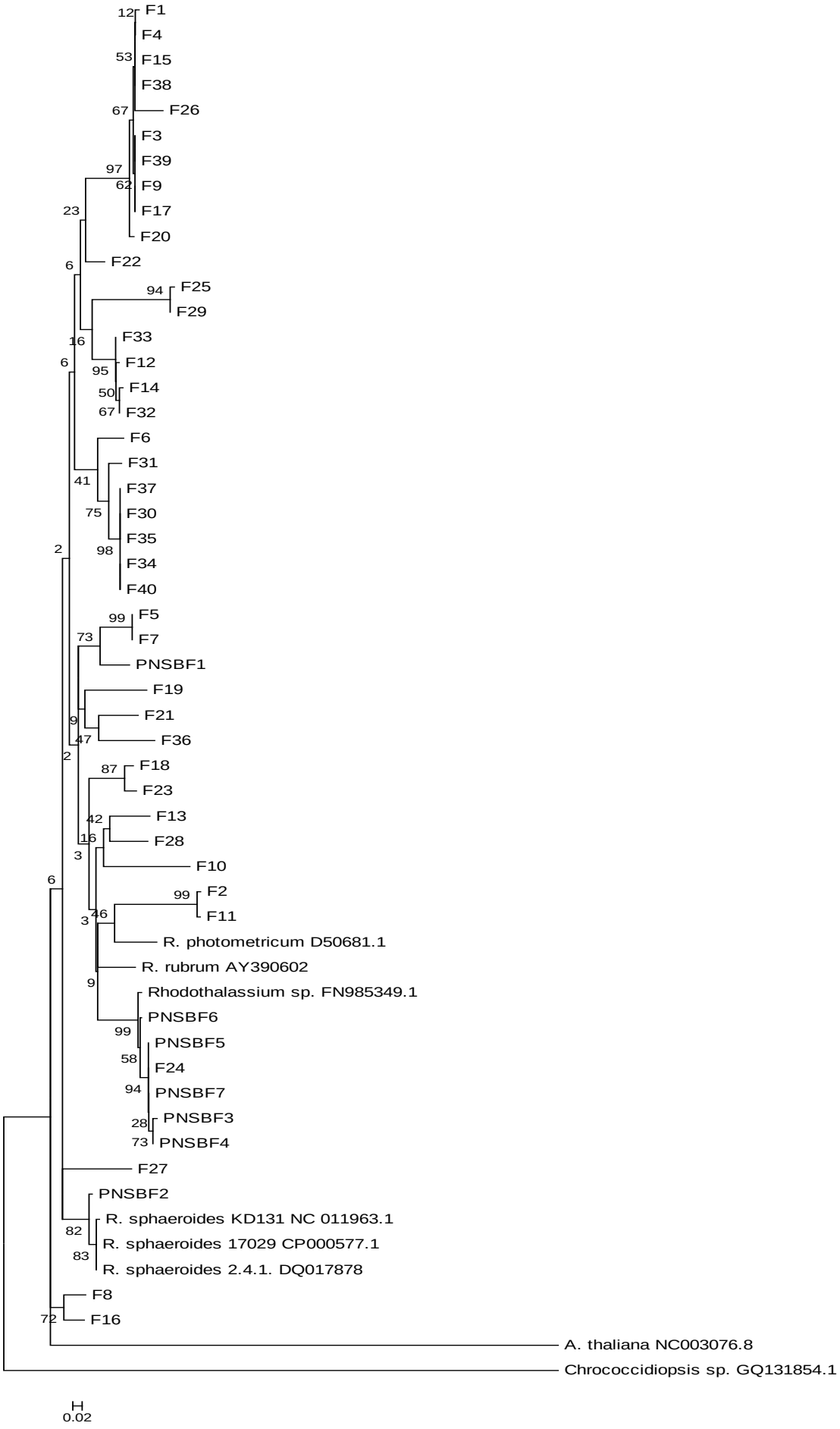
B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R. rubrum AY390602
Rhodothalassium sp. FN985349.1
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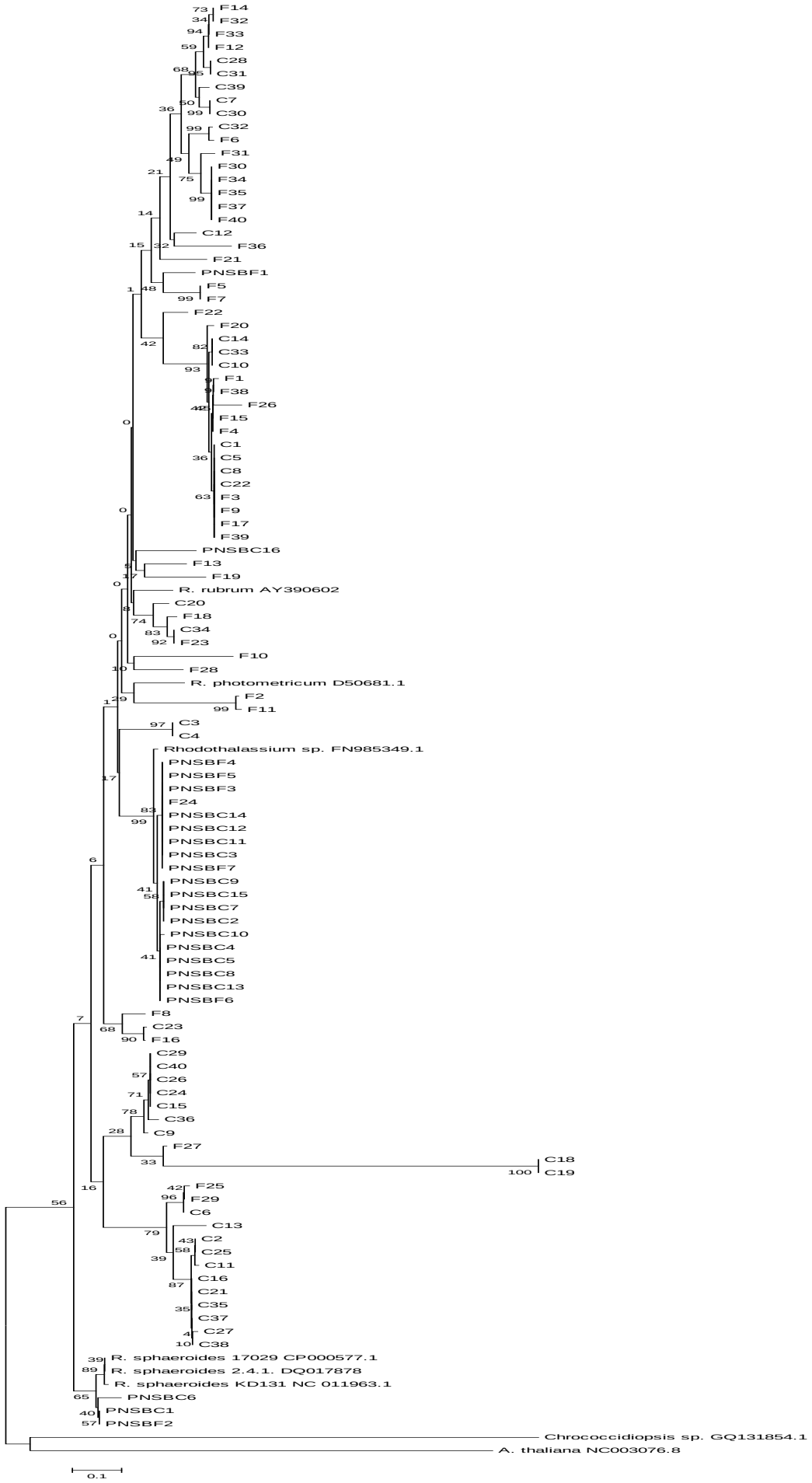
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Chrococcidiopsis sp. GQ131854.1
A. thaliana NC003076.8
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Figure 3.2 Phylogenetic analyses of the *pufM* gene clones libraries. The development of the concensus tree was made by the Neighbour-Joining 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 (Candelaria) the bar represents 0.05 subtitutions per nucleotide position, for the figure B (Fraternidad) 0.02 and for the figure C (Candelaria/Fraternida/Isolates) 0.1 sustitutions per nucleotide position. A total of 2000 repetitions based in pair wise arrangement were performed to produce both trees. For all trees *Chrococcidiopsis* GQ131854.1 and *Arabidopsis thaliana* NC003076.8 were used as out groups.

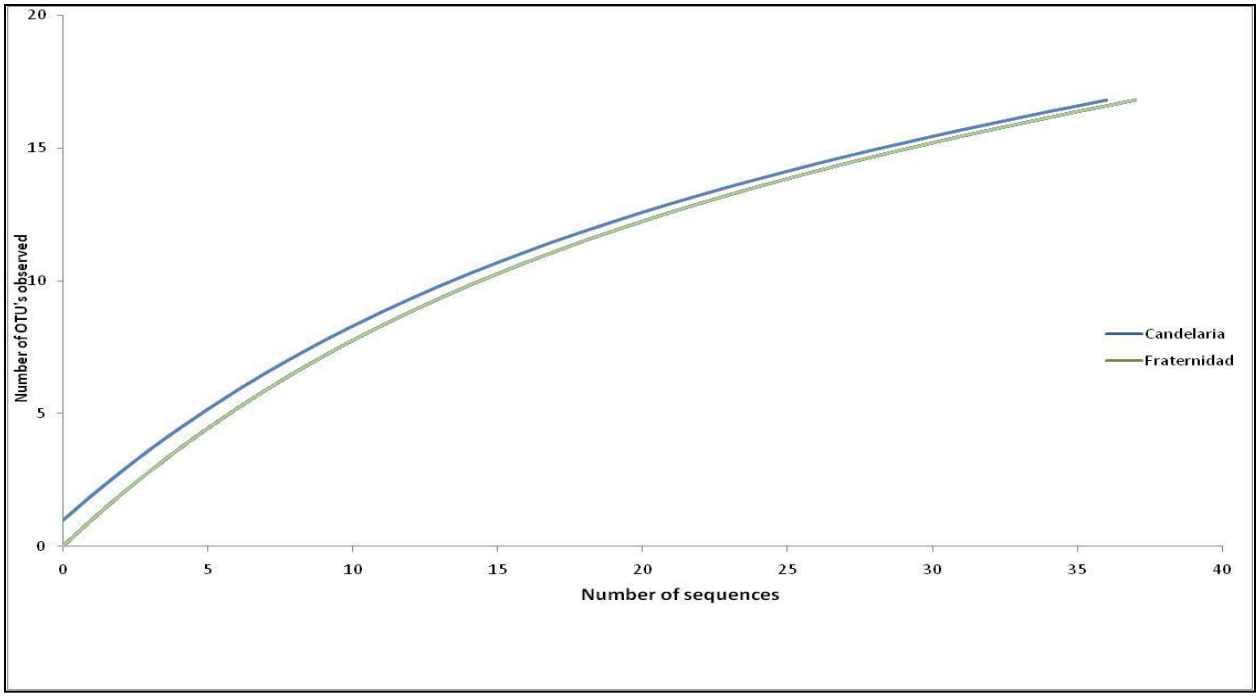

Figure 3.3Rarefaction curves for the *pufM* clones libraries from Candelaria and Fraternidad microbial mats. OTU'S were determined by ≥95% similarity. The rarefaction curve was done using the pyrosequencing pipeline (rarefaction tool) of the Ribosomal Data Base Project (http://rdp.cme.msu.edu/).

3.4 Discussion

Even though studies concerning microbial ecology and diversity have employedextensive use of culture independent techniques for assessing microbial communities,most of the work performed on purple non sulfur bacteria (PNSB) diversity are based on characterization of cultivable isolates obtained from diverse environments (Madigan, 2010). Because of insufficientknowledge about bacterial growth requirements, culture media for bacterial isolation is biased and many microorganisms enter a viable but not cultivable state, thus notreflecting the actual community structure and also limiting recuperation of organisms(Reiter and Sessitsch, 2006; Tholozan *et al*. 1999). Consequently, the culture independenttechniques employed in this study pretend to assess more broadly the presence of uncultivable purple non sulfur bacteria molecularly as in the two samples of microbial mats from the Cabo Rojo salterns. In order to confirm the presence of uncultivable PNSB, two clone libraries of the photosynthetic gene *pufM* were constructed from bulk DNA of the pink layer from the microbial mat samples of Candelaria and Fraternidad lagoons. Actually, all the *pufM* studies performed to date are base in the *pufM* cultivable anoxyphototrophic bacteria and only one study were published. In our study, a total of 872 clones were obtained from Candelaria mat and 1031 clones from Fraternidad mat. An overall of 40 clones with the right insert per library were sequenced. Clones were selected randomly to avoid any bias in our analysis. The *in silico* analysis confirms that all the sequenced samples code for *pufM*.

The first phylogenetic tree (Figure 3.2A) showed the phylogeny relationship between Candelaria clones and the cultivable *pufM* sequences from this sampling site. The tree suggested seven Operational taxonomic units (OTUs). The first OTU composed of thirteen sequences related to already describe genus of PNSB like *Rhodothalassium*. The second OTU showed a

relation between isolates (PNSBC16) and two Candelaria clones (C20, C34). The same relation can be observed in the four clusters, were two isolates were associated with seven clones sequence and the same time with the genus *Rhodobacter*. The other three OTUs are unique and only relate among them.

The second phylogenetic tree (Figure 3.2B) presented the phylogenetic relation between Fraternidad clones and the cultivable *pufM* sequences from this sampling site. Fraternidad clones in this tree were represented by eight OTUs, in which only two related the cultivable sequences (PNSBF1, PNSBF3-F7) with three clones (F5, F7, F24). In the phylogenetic tree the clone sequences were not related with sequence of already described purple non sulfur bacteria as in the case of Candelaria clones.

The final phylogenetic tree (Figure 3.2C) showed the complete phylogenetic relation between all the cultivable *pufM s*equences and the clone sequences of Candelaria and Fraternidad mats. A total of fifteen OTUs were obtained for this phylogenetic tree. Only three of the *pufM* OTUs were group with *pufM* sequences with the cultivable isolates, all of them from Fraternidad microbial mat. Surprisingly, none of the Candelaria cultivable *pufM* sequences were not represented in the *pufM* clone library. This result could be due to biased of the PCR *pufM* primers, low abundance of the cultivable PNSB community in the pink layer. The DNA extraction method performed was not as efficient extracting DNA from the cultivable PNSB. This also could be influence if the population of PNSB in the sample in low abundance. Due to the *pufM* gene size each different in base pairs will change completely the alignment. The *pufM* sequences also, can be influence with any mistakes in the process of editing the sequences.

The phylogenetic analysis of the *pufM* gene through an amplification of the gene in the isolated PNSB and the generation of a clone library suggested that the microbial mat samples of

both sampling locations (Candelaria and Fraternidad) are rich in diversity of purple non sulfur bacteria (there are more diversity than the cultivable analysis suggested). The phylogenetic relationship between the cultivable *pufM* sequences from Candelaria mat and the *pufM* clones libraries were not closely related. In contrast, all the cultivable PNSB community member from Fraternidad microbial mat were represented in the *pufM* library. Okubo and collaborators (2006) described the diversity of PNSB from microbial mats forming in wastewater ditch and showed a relation between the *pufM* clone library and the isolate purple non sulfur bacteria. In the study all the clones library were relate with *Rhodobacter* sp. and *Rhodopseudomonas* sp. In our study, the data only relate the genus *Rhodobacter* sp.

The diversity indexes and richness estimator were obtained using a rarefaction curve. The rarefaction analysis is useful to compare the relative complexity of two or more communities and to estimate the completeness of sampling of a community (Schloss and Handelsmann, 2004). The rarefaction analysis for forty *pufM* clone sequences from each sampling location (Candelaria and Fraternidad) suggests sixteen OTUs for Candelaria and 17 OTUs for Fraternidad analyzed clones (Figure 3.3). The OTUs were determine by >95% of similarity. The diversity indices of purple non sulfur bacteria in the studied microbial mat samples were not saturated, because the rarefaction showed that a range of 16-17 OTUs were obtained from both microbial mat samples. In environments like the microbial mats with the capability to provide the right ecosystem for this group of microorganism the diversity of purple non sulfur bacteria will be constituted an important part of the richness of the microbial mats.

The successful developments of a *pufM* clone library were used to describe the cultivable and uncultivable purple non sulfur bacteria present in the mat. Based on the *pufM* sequences, we are below the estimated PNSB on both microbial mats samples. Cultivable purple non sulfur

bacteria were represented in the clone library from both microbial mat sampling sites. The diversity of purple non sulfur bacteria in Candelaria and Fraternidad mat samples was higher than the ones that were studied in this research. These were represented with the rarefaction curve from both sampling sites. The assessments that were used to isolates purple non sulfur bacteria selected a group of them and a high number of these microorganisms were excluded and be represented in the clone library. During the isolation method, possibly the physical-chemical environment incubation conditions selected for one group and excluded other PNSB present on the mat.

Chapter 4
Conclusions, Recommendations and Literature Cited

4.1 Conclusions

- Seasonal changes and the kind of microbial mat (benthic or ephemeral) have a direct effect in the amount and diversity of the cultivable purple non sulfur bacteria (PNSB) present in the microbial mats.
- The range of bacteriochlorophyll and carotenoid pigments were limited in the purple non sulfur bacteria isolated from microbial mats samples (bacteriochlorophyll *a*and carotenoid pigments -spheroidene and spirilloxanthin).
- The purple non sulfur bacteria isolated from subtropical microbial mats from Cabo Rojo salters were classified as mesophilic, neutrophilic and halophiles.
- The presences of anoxygenic photosynthetic apparatus were confirmed through the use of *pufM* gene.
- The *in silico* analysis suggested the presences of *Rhodobacter* sp., *Rhodothalassium* sp., *Rhodovulum* sp., *Vibrio*sp. and several unidentified members of the *Rhodospirillacea* family.
- The PNSB across the microbial mats samples were under estimated, based on the rarefaction curve due to uncultivable PNSB in the sample.
- Most of the cultivable *pufM* sequences were represented in the total *pufM* library generated from total a DNA extraction.

4.2 Recommendations

- Performed a full length sequencing of the *16s rDNA*
- Performed the isolation using extreme halophilic NaCl concentration (15%-30%), and other physico-chemical parameters.
- Completed the taxonomical characterization using the recommendation to describe a novel PNSB, following the method describe by Imhoff and
- Analyze more *pufM* clones to complete the saturation of the rarefaction curve.

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