Identification and Biotechnological Potential of Novel Purple Non-Sulfur Bacteria (PNSB) from Aquatic Environments in Puerto Rico

By

Jesie Marie Rullán Cardec

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
IN
BIOLOGY

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2019

Approved by:

_____	_____
Dimaris Acosta Mercado, PhD. Member graduate committee	Date
_____	_____
Carlos J. Santos Flores, PhD. Member graduate committee	Date
_____	_____
Carlos Ríos Velázquez, PhD. President graduate committee	Date
_____	_____
Aikomarí Guzmán, PhD. Representative, Office of Graduate Studies	Date
_____	_____
Ana V. Vélez Díaz, M.S. Interim Biology Department Director	Date

Abstract

The anoxygenic photosynthetic purple non-sulfur bacteria (PNSB) have caught the attention of the scientific community due to their metabolic diversity. These microorganisms have the ability to adapt to fluctuations in oxygen levels and can use a great variety of carbon sources. In addition to their metabolic diversity, these microorganisms are widely distributed in nature and have different applications in the biomedical field, industrial processes, production of biofuels, among many others. Despite the promising and broad applications of these bacteria, there is only one study about the isolation of these microorganisms from Puerto Rico; however, their biotechnological potentials were not explored. The main focus of the current research was to identify PNSB isolates from different aquatic environments in Puerto Rico and to suggest possible biotechnological applications of these bacteria in the 1) biomedical field, 2) metal bioremediation, 3) hydrocarbon degradation, and 4) hydrogen production. For the identification of the PNSB isolates, the recommended standards for the description of new species of anoxygenic phototrophic species, in accordance with the International Code of Nomenclature of Bacteria, were used. For the study of potential biotechnological applications, the following were evaluated:1) bacterial growth under different concentrations of lead, copper, and mercury, 2) the use of phenanthrene and naphthalene as the sole carbon source under anaerobic conditions, 3) antimicrobial substance production, and 4) hydrogen production. In terms of the PNSB isolates, we found that 16 of the isolates are potentially new species of PSNB. Moreover, 2 of these bacteria were isolated from the *Heliconia* phytotelmata being the first PNSB isolated from this environment. Also, this is the first study to report whole genome sequences of PNSB from Puerto Rico. In terms of the biotechnological application, it was found that 100% and 50% of the studied isolates were able to grow in the presence of lead and copper, respectively. Similarly, 88% of the isolates produced

antimicrobial substances against *Bacillus subtilis*, and 38% were able to generate hydrogen under anaerobic conditions. These findings confirm the biotechnological potential of PNSB isolates from ecosystems in Puerto Rico. More studies about the capabilities of these isolates are strongly encouraged to have a clear understanding and profiles about other biotechnological and industrial applications (e.g. as producers of enzymes such as proteases, amylases, lipases, etc.). This will also promote the exploration and study of ecosystems in Puerto Rico as bioprospecting niches for the discovery of organisms with commercial applications.

Resumen

Las bacterias anoxigénicas fotosintéticas púrpuras no sulfurosas (PNS) han captado la atención de la comunidad científica por su diversidad metabólica. Estos microorganismos tienen la habilidad de adaptarse a fluctuaciones en los niveles de oxígeno al mismo tiempo de que utilizan una gran variedad de fuentes de carbono. Además, estos microorganismos están ampliamente distribuidos en la naturaleza y se han encontrado aplicaciones de estas bacterias en el campo de la biomédica, procesos industriales, producción de biocombustibles, entre otros. A pesar de las distintas aplicaciones de estos microorganismos, solo hay un estudio sobre el aislamiento de estas bacterias en Puerto Rico y no se exploró su potencial biotecnológico. El objetivo principal del estudio actual fue identificar las bacterias púrpuras no sulfurosas aisladas de distintos ambientes acuáticos en Puerto Rico y sugerir posibles aplicaciones en el campo de la biomédica, biorremediación de metales, degradación de hidrocarburos y producción de hidrógeno. Para identificar los aislados, se utilizaron los parámetros recomendados para la descripción de nuevas especies de bacterias púrpuras no sulfurosas de acuerdo al Código Internacional de Nomenclatura de Bacterias. Para explorar las posibles aplicaciones biotecnológicas de los aislados, se evaluó lo siguiente: 1) crecimiento del aislado en distintas concentraciones de plomo, cobre y mercurio, 2) el uso de fenantreno o naftaleno como única fuente de carbono bajo condiciones anaerobias, 3) producción de substancias antimicrobiales, y 4) producción de hidrógeno. En cuanto a la identificación de los aislados, encontramos 16 potencialmente nuevas especies de bacterias PNSB. Además, 2 de estas bacterias fueron aisladas de la fitotelma de *Heliconia*, siendo las primeras bacterias púrpuras no sulfurosas que se aíslan de este ambiente. Igualmente este estudio es el primero en reportar la secuenciación completa de genomas de bacterias púrpuras no sulfurosas aisladas de Puerto Rico. En cuanto al estudio de las posibles aplicaciones biotecnológicas de los

aislados, se encontró que un 100% y 50%, respectivamente, de los aislados fueron capaces de crecer en presencia de plomo. También se encontró que un 88% de los aislados produjeron substancias antimicrobiales contra *Bacillus subtilis* y 38% de los aislados generaron hidrógeno en condiciones anaeróbicas. Estos resultados confirman el potencial de las bacterias púrpuras no sulfurosas en ecosistemas de Puerto Rico. Es necesario realizar estudios adicionales con el fin de encontrar otras aplicaciones biotecnológicas no exploradas en este estudio y como productores de enzimas con aplicaciones industriales (ej. como productores de preoteasas, amilasas, lipasas, etc.). Esto fomentará la exploración y el estudio de estos ecosistemas como hábitats de organismos con aplicaciones comerciales.

Dedication

I want to dedicate this work to the person that taught me for the first time the meaning of science: my father Eduardo Rullán. Thank you for educating me and talking about science since I was a child. I know that even when I asked more questions that you could possibly answer, you never stopped me from asking and wondering about everything around. On the contrary, you encourage me to continue the pursuit of knowledge. Since I remember who I am, you always enforced me to overcome my fears and taught me that I'm able to do anything and that most of our obstacles are present only in our minds.

Acknowledgements

I feel very lucky and blessed by God for so many brave, humble and kind-hearted people that I have met through my life. Each of them has been a fundamental part of what I have become, I am, and want to be as a person and a professional. People whom I have shared laughs, sorrows, frustrations, and success. People that always have been on my side and have known the appropriate words to lift up my spirit. Thanks to all of you who have brought happiness to my life.

I want to start giving thanks to my family. Zoraida Cardec and Eduardo Rullán, everything that I am and have is because of you both. Thank you for caring and teaching me and my sister about values which have been fundamental for what we have become. Definitely, I could not have better parents than you. Also, I want to thank my sister for being my best friend. Thank you for your advice and being always by my side.

Sometimes special people come to your life unexpectedly, changing your world and the way that you see your future. Thank you, Alberto C. Rodríguez, for coming to my life and opening your heart to me. I'm going to be eternally grateful for all of your support even if it meant taking from our time together to work in the lab. Thank you for respecting and supporting my love for science.

I want to thank infinitely Dr. Carlos Ríos Velázquez, for giving me the opportunity of being a member of his lab. Being part of the B-266 family as an undergraduate and graduate student is one of the things that I must treasure in my life. Thanks to that, I met wonderful and special people that today are still my friends. Thanks for your guidance and for sharing with me your point of views about society and your ideal world. I know that sometimes our ways of thinking were too different but I really appreciate your advice and friendship.

To Dr. Dimaris Acosta and Dr. Carlos Santos; I greatly appreciate that without hesitation you accepted to be part of my thesis committee. Thank you for your availability, advice and recommendations on this manuscript.

Albin Cardona Correa, thank you for always being by my side. I admire and value you for the hardworking and good person that you are. More than a friend, for me you are part of my family. Thank you for all of your advice and for sharing with me this pathway of doing the master degree. I can't imagine how my life would be without your jokes and funny advice.

I want to thank infinitely Josué Malavé Orengo for accepting being my lab mentor. Thank you for believing in me, for your patience and your guidance in the laboratory. Also, I would never forget how many times you comforted me with your words and gave me hope in those moments where it seemed that all doors were closed for me. I owe to you a big part of the scientist that I am today.

Kristina Soto and Irimar Zapata, thank you for being so caring with me those first days at the laboratory and for being willing to take of your time to talk about your lab work. Those words were part of the sparks that lighted my interest in research.

Thanks to all of my graduate and undergraduate lab-friends for your company during this adventure. Thank you Lizahira, Frank, Laura, Robert, Wilmer, Edgar, Luis and Moisés for all of the laughs in the lab. Moisés, you are one of the kindest people that I have ever met and I feel very fortunate about having you as a friend.

Kiara M. Rodríguez and Ángel M. Rubio, I want to express how fortunate I feel that you choose me for being your lab mentor. Thank you for all the help and laughs. Each of you is a fundamental part of this work. I feel very proud of the scientists that you have become and for all of your success.

From the Department of Biology, I want to thank Magaly Zapata for all her willingness to help in providing materials, knowledge and for the opportunity that she gave me as a microbiology instructor. Likewise, I want to thanks José Almodovar for his work and assistance with the SEM analyses during this work. Thanks to Dr. Rafael Montalvo for his advice and to his students Jéysica Zayas and Rubén Sánchez for their help. I want to thanks to Dr. Carlos Rodríguez Minguela for all of his advice related to DNA sequences and analyses. Also thanks to Mary Jiménez y Vilmarie for all your help.

From the Biotechnology Program, I want to thank Lizzy Muñiz and Deyka Y. López. Even after I graduated from the Industrial Biotechnology program you both still make me feel part of it with all of your support and caring.

Also, I want to recognize the work of Félix Caro from the Purchase Department at the UPRM for his availability and for giving the extra mile to guarantee that I received in time key material used during this work.

From the Department of Chemistry, I want to thanks Dr. Samuel Hernández and Dr. Juan López Garriga's laboratory teams for their disposition in letting me use their lab equipment for the absorbance analyses.

Also, I want to thank my Abbvie-family from the Technical Operation Laboratory for all their support and caring. Thanks to all for your consideration, support, and advice. Particularly, I want to thank Ritsdeliz Pérez for believing in my capabilities in continuing graduate studies.

List of Figures

List of Tables

Table of Content

Chapter 1: Isolate Identification of Potential Purple Non-Sulfur Bacteria (PNSB)

1.1 Introduction

Purple non-sulfur bacteria (PNSB) are phototrophic members of the alpha and beta Proteobacteria. Their name was given by their distinctive red to purple pigmentation and the previous belief that, under photoautotrophic conditions, these bacteria could not use hydrogen sulfide as an electron donor (Basak & Das, 2007). PNSB have been studied before because of their metabolic diversity and, hence, for their biotechnological and biomedical applications. Due to their metabolic diversity, these phototrophs are ubiquitous in nature. Their growth is favored in aquatic environments with low oxygen levels and infrared radiation for photosynthesis (Belila et al., 2013). Paddy soils, stagnant waters, mangroves, lakes, and ponds are some examples of places where these bacteria have been isolated (Ramana et al., 2006; Kar et al., 2014; Demin et al., 2002; Hougardy et al. 2000). Moreover, PNSB have been found in extreme environments such as hypersaline and alkaline soda lakes, microbial mats in solar salterns, and peat bogs (Madigan et al., 2000; Kompantseva et al., 2010; Soto-Feliciano, 2011; Rosenberg et al., 2012a; Srinivas et al., 2014; Kulichevskaya et al., 2017). Despite their abundance and wide distribution, there are environments, such as bromeliad and *Heliconia* phytotelmata, that had not been studied for the presence of PNSB. Given that phytotelmata have been described as micro aquatic environments with shared physicochemical characteristics to other water bodies such as ponds and lakes from where PNSB have been isolated successfully, we expect that these bacteria can inhabit phytotelmata as well (Killick S. et al., 2014).

Since 2001, the Laboratory of Microbial Biotechnology and Bioprospecting (MBB) at UPRM has studied and isolated PNSB in various ecosystems in Puerto Rico. Among the inventory of their collection, there are isolates from bromeliads, *Heliconia* and bamboo phytotelmata. Since there is no other study about the isolation of PNSB from phytotelmata, then there is a possibility

that the isolates in the MBB Laboratory, isolated from this microenvironment could represent novel species of PNSB.

In this chapter, we described the procedures to confirm that the isolates from water reservoirs, bromeliad and *Heliconia* phytotelmata in the collection of MBB were PNSB. In addition, carbon source utilization was used as an initial criterion to address differences among the isolates. Identification of the isolates and its biotechnological potential is described in Chapter 2 and Chapter 3, respectively.

1.2 Literature Review

1.2.1 PNSB Photosynthesis

PNSB have been widely studied because of their metabolic diversity and versatility (Figure 1.1). These bacteria can grow as chemoorganotrophs, photoorganotrophs, and as photolithioautotrophs (Overmann et al., 2013). Contrary to oxygenic photosynthesis, PNSB performs anoxygenic photosynthesis in which organic compounds (i.e. acids, alcohols, carbohydrates) are used as electron donors instead of water, therefore oxygen is not produced.

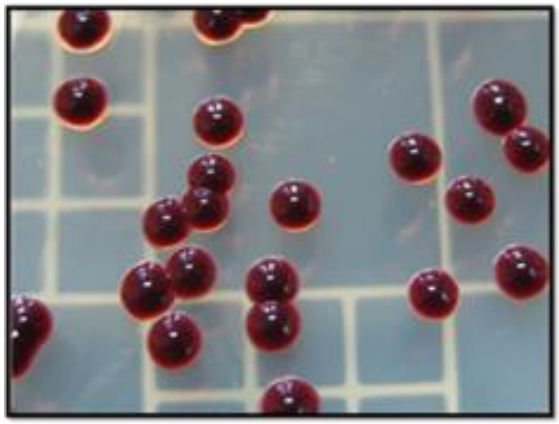


Figure 1.1: Colonies of Purple Non-Sulfur Bacteria (PNSB): Under anaerobic conditions in the presence of light, PNSB produced bacteriochlorophyll and carotenoid pigments to harvest light during photosynthesis. The presence of these pigments give their characteristic pigmentation and, therefore, their name.

In PNSB, the synthesis of the photosynthetic apparatus is regulated mainly by oxygen levels (Imhoff et al., 2004). Although their synthesis is not activated by light, the number of photosynthetic complexes in the cells is inversely proportional to light intensity (Gregor et al.,

1999). There are several studies about PNSB photosynthesis, whose information was used for the generation of models about the synthesis of the photosynthetic machinery in members of PNSB. The most studied and complete model is that of *Rhodobacter capsulatus*. It explains coding genes, composition, and structure of the mechanisms involved in the synthesis of the photosynthetic apparatus (Figure 1.2).

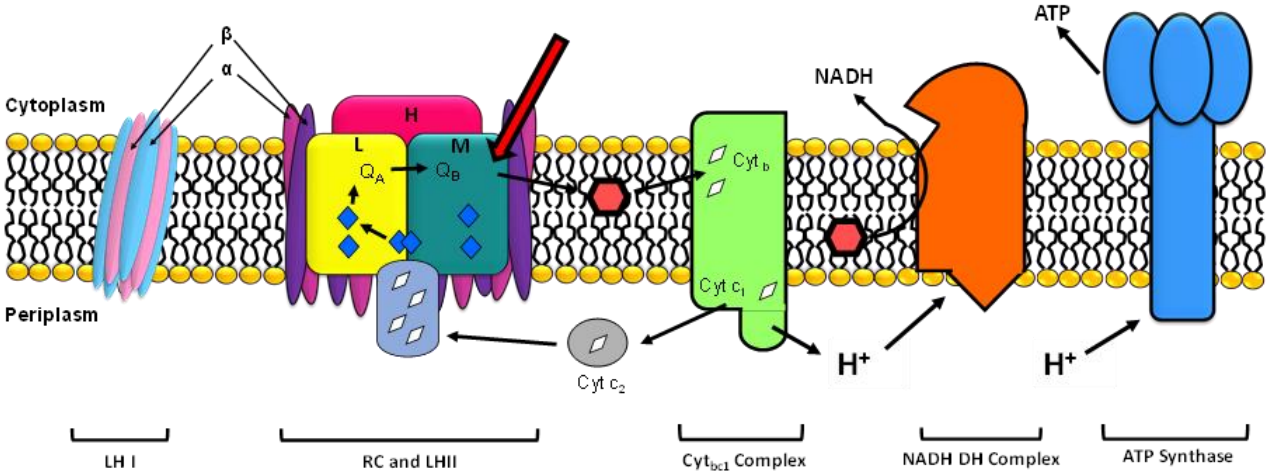


Figure 1.2: Photosynthetic Apparatus of Purple Non-Sulfur Bacteria. The photosynthetic apparatus is located in invaginations of the cytoplasmic membrane. In anaerobic conditions, the light is collected in the Light Harvesting complex LHI or LHII composed of α and β polypeptides. The energy is transferred to bacteriochlorophylls (◆) in the reaction center and is used to release an electron for the reduction of a quinone. After the quinone is reduced twice by the same process (⬢), it captures protons from the cytoplasmic space to the membrane where the cytochrome bc_1 complex transfers the electrons (▱) to the Cytochrome c_2 (●) while protons (H+) are released to the periplasmic space. Finally, Cyt c_2 reduces the bacteriochlorophyll or other electron donors in the RC and the process is repeated. The protons accumulated in the periplasmic space are used by the ATP-synthase to generate ATP. The red arrow point out the protein encoded by the *pufM* gene (Adessi et al., 2014). Figure adapted from Vega-Sepúlveda, (2009). "Bacterias fototróficas púrpuras no-sulfurosas en la fitotelmata de bromelias en diversos bosques de Puerto Rico". University of Puerto Rico at Mayagüez, Mayagüez, PR.

Proteins and pigments of the photosynthetic complex are encoded by the *puf* and *puc* operons (Gregor et al., 1999). The reaction center, where light energy is converted to electrochemical energy, is composed of the proteins subunits L, M, and H, encoded by the *pufL*, *puf*M and *puhA* genes, respectively (Figure 1.2) (Nagashima et al., 1997). This reaction center is surrounded by the light harvesting complex I (LHI) which has α and β polypeptides encoded by the *pufA* and *pufB* genes, respectively, to which bacteriochlorophyll (BChl) *a* or *b* and carotenoids are bound. Light harvesting complex II (LHII) is also formed of α and β polypeptides but encoded

by the *pucBA* operon (Overmann et al., 2013). It is important to mention that, in addition to confirm the presence of genes related to photosynthesis in bacteria, the *pufM* gene has been used to study diversity and phylogenetic relationships among anoxic phototrophs due to similar results when compared to 16SrDNA analysis (Nagashima et al., 1997; Zeng et al., 2007; Hirose et al., 2012).

By spectroscopic analysis, it is possible to identify the type of bacteriochlorophyll and carotenoids pigments in the cells. Whole cells absorption maxima at 375, 590, 805 nm and 830-911 nm are indicative of bacteriochlorophyll *a* while absorption maxima at 400 nm, 605 nm, 835-850 nm, and 986-1035 nm reveal the presence of bacteriochlorophyll *b* (Rosenberg et al., 2012b). The presence of peaks between 400 and 450 nm is indicative of the presence of carotenoids. The identification of these pigments serves to confirm the identity of possible PNSB as members of this group.

1.2.2 PNSB in Puerto Rico

The Laboratory of Microbial Biotechnology and Bioprospecting (MBB) at UPRM has a collection of PNSB isolated from different aquatic environments. As the preparation of this work, we were unable to find in the literature any other study or culture collection with PNSB isolates from Puerto Rico. The collection is composed of: 11 isolates from water reservoirs (i.e. Guajataca, Dos Bocas, La Plata, Carraízo, Lucchetti, Guayo, Guayabal, Toa Vaca, Carite and Patillas reservoirs), 23 from hypersaline microbial mats, and, 26, 8 and 13 isolates from bromeliad, *Heliconia* and bamboo phytotelmata, respectively. By using 16SrDNA, it was found that the isolates from microbial mats and bromeliad phytotelmata are related to the genera *Rhodothalassium*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodobacter* and *Rhodovulum* (Vega-Sepúlveda, 2009; Soto-Feliciano et al., 2010; Soto-Feliciano, 2011). Despite that the genera of these isolates were known, the species and their biotechnological potential are still unknown. Also,

for some of the isolates, their 16SrDNA sequences were not similar to other sequences in the databases, suggesting novel members of PNSB. In addition, the identity of the isolates from water reservoirs and *Heliconia* phytotelma remained unknown.

1.2.3 Phytotelmata as Habitat for Purple Non-Sulfur Bacteria

Phytotelma is referred to as the aquatic environment developed by the accumulation of water in a plant. Tree holes, bamboo nods, pitcher plants traps, bromeliad leaves and *Heliconia* bracts filled with water, are some examples of phytotelmata. Depending on the plant, the physicochemical characteristics of the phytotelma varies and consequently their microbial composition. For this reason, each phytotelma is a unique environment with its own conditions and community. In addition, the organisms that inhabit the phytotelma are subjected to physiological pressure due to rapid changes in temperature and pH (Richardson et al., 2000; Carmo et al., 2014). Moreover, this microenvironment has been described as ecological models for the study of aquatic communities and interactions (Belila et al., 2013). Since PNSB have been isolated from different aquatic environments, then the isolation of these bacteria from phytotelma could be possible as well.

Bromeliad phytotelmata are formed by the accumulation of rainwater in the axils of the plant leaves (Figure 1.3). This aquatic environment provides acidic, anaerobic conditions with a high content of organic matter and nitrogen. Therefore, bromeliad phytotelmata provide a niche for the development of organisms with these physiological requirements (Goffredi et al., 2011). Most studies about organisms in bromeliad phytotelmata are focused on animals, fungi, and protist communities (Richardson, 1999; Richardson et al., 2000; Silva et al., 2013; Roberto et al., 2014; Sousa et al., 2017). The scarce studies about prokaryotic communities and their ecological roles in bromeliad phytotelmata do not mention the presence of PNSB. In one of these studies, by using

16SrDNA and RFLP analysis, members of the Alphaproteobacteria, Betaproteobacteria, Acidobacteria, Planctomycetes, Bacterioidetes, and Firmicutes were found to be part of the bromeliad phytotelmata microbial communities (Goffredi et al., 2011). Also, members of the genera *Aeromonas*, *Pseudomonas*, *Xanthomonas*, *Vibrio*, *Rahnella*, *Erwinia* and *Bacillus* have been found (Brighigna et al., 1992). In another study, enzymatic degradation of hydrolyzed casein, gelatin, starch and cellulose by heterotrophic bacteria and coliforms isolated from bromeliad phytotelmata was studied (Carmo et al., 2014). From the MBB culture collection, PNSB isolates belonging to the genera *Rhodopseudomonas*, *Rhodospirillum*, and *Rhodomicrobium* were identified (Soto-Feliciano et al., 2010). Moreover, it was suggested that some of the isolates belong to new genera of PNSB (Vega-Sepúlveda, 2009). These studies provide insight about not only the diversity of microorganisms in bromeliad phytotelmata but also about their biotechnological potential for industrial applications.

Figure 1.3: *Heliconia* and bromeliad phytotelma. **A.** The *Heliconia* phytotelma is formed by the accumulation of water and nectar in the bracts of the flowers. **B.** Bromeliad phytotelma is formed by the accumulation of water between the leaves of this plant (white arrows show the phytotelma of each plant).

Unlike bromeliad, the accumulation of rainwater, nectar and plant secretions in the bracts that protect the flowers against herbivores forms the *Heliconia* phytotelmata (Bronstein, 1986; Wootton, 1990; Missagia et al., 2016) (Figure 1.3). In comparison to bromeliad, *Heliconia*

phytotelmata contain higher concentrations of calcium, phosphorus, sodium, magnesium, potassium, sulfur, and chlorine; and lower concentrations of iron and aluminum (Richardson et al., 2000). In terms of studies about PNSB in *Heliconia* phytotelmata, these are fewer than those for bromeliads. Most are focused on the insect communities that inhabit the *Heliconia* phytotelmata. There is only one study about the description of the novel PNSB bacterium *Asaia krungthepensis* isolated from the flower of a *Heliconia* in Thailand (Yukphan et al., 2017). However, is not clear if this bacterium was isolated from the phytotelmata. For this reason, the PNSB in the MBB culture collection isolated from *Heliconia* phytotelmata could be the first bacteria isolated from this environment. Our samples were isolated from *Heliconia bourgaeana*, *H. pssittacorum*, *H. longiflora*, and *H. maraca*. Therefore, this study will be the first to provide awareness about the members of the bacterial community in this not well-studied environment.

1.3 Objectives

The main objective of this section was to confirm that the isolates from water reservoirs, *Heliconia* and bromeliad phytotelmata in the culture collection of the MBB laboratory are members of PNSB. The following was assessed: 1) colony pigmentation under aerobic and anaerobic conditions with light, 2) presence of the *pufM* gene, 3) types of bacteriochlorophylls, and 4) 16SrDNA sequence analysis. Also, similarities between the isolates were be established by their ability to use different carbon sources.

1.4 Methods

The PNSB used in this study belong to the microbial collection of the Laboratory of Microbial Biotechnology and Bioprospecting (MBB) at UPRM. For this investigation, we took into consideration the isolates from water reservoirs, bromeliad, and *Heliconia* phytotelmata (Section 1.2.2).

All tests (if not specified otherwise) were performed using the medium described by Lakhmi et al. (2009). This medium has the following composition per L: 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.4 g NaCl, 0.6 g NH_4Cl, 0.05 g $CaCl_2.2H_2O$, 0.3 g sodium pyruvate, 0.3 g yeast extract, 5 mL ferric citrate solution (0.1% w/v) and 1 mL of trace element solution SL7. Trace element solution SL7 has the following composition per L: 1 mL HCl 25% solution, 70 mg $ZnCl_2$, 100 mg $MnCl_2.4H_2O$, 60 mg H_3BO_3, 200 mg $CoCl_2.6H_2O$, 20 mg $CuCl_2.H_2O$, 20 mg $NiCl_2.6H_2O$ and 40 mg $NaMoO_4.2H_2O$ (Biebl and Pfening, 1981). The following modification was done to the medium: sodium pyruvate (carbon source) was omitted and the followings were added per L of the medium: 4 g succinic acid, 0.1 g glutamic acid and 0.04 g aspartic acid.

1.4.1 Evaluation of Phototrophic and Chemotrophic Mode of Growth by the Isolates

Streaks of the isolates were done in the medium described above to evaluate phototrophic growth. Then, the isolates were incubated for 4 d under anaerobic conditions with illumination of 2500 lux (provided by an incandescent light bulb) and a temperature of 32 to 35 ^oC. For the evaluation of chemotrophic growth, the same procedure was performed with the exception that the incubation was done under aerobic conditions in the dark. After the incubation time, the presence or absence of pigments in the colonies under phototrophic and chemotrophic growth was evaluated. Also, the colony morphology was addressed (i.e. colony diameter, form, elevation, margins, opacity, and texture) (Woolverton et al., 2016).

1.4.2 Amplification of *pufM* gene

The presence of the *pufM* gene, related to anoxygenic photosynthesis, was determined by PCR and confirmed by gel electrophoresis. A streak plate of the isolates were performed and incubated under anaerobic conditions at 32 to 35 ^oC with incandescent light with illumination of 2500 lux until isolated pigmented colonies were observed. To extract the DNA, 3 colonies of the

isolate were picked with a micropipette tip, suspended in 10 µL of PCR buffer and incubated at 94 ^{o}C for 5 min. The PCR reaction for the amplification of *pufM* was done as follow per 25 µL of reaction: 9.5 µL of sterile distilled deionized water, 1 µL of DNA, 12.5 µL Green Taq Master Mix (Promega) and 1 µL of primers at 10 pmol [forward 557F (5'-CGC ACC TGG ACT GGA C-3') and reverse 750R (5'-CCC ATG GTC CAG CGC CAG AA-3'] (Achenbach et al., 2001; Vega-Sepúlveda, 2009; Soto-Feliciano, 2011). PCR parameters were the following: first denaturalization at 94 ^{o}C for 3 min, followed by 30 cycles with a denaturalization of 94 ^{o}C at 1 min, annealing at 55 ^{o}C for 1 min and extension at 72 ^{o}C for 1 min, and a final elongation at 72 ^{o}C for 10 min. The PCR products were confirmed by gel electrophoresis with agarose 1.5% prepared with TAE1X.

1.4.3 Determination of Bacteriochlorophyll Type

The types of bacteriochlorophyll in the cells was determined by absorbance of *in vivo* cell suspension in sucrose. The cells were grown in 6 mL vials (filled with media to achieve anaerobic conditions) with light until the change in coloration in the media (also called "bloom"), due to the production of pigments by the cells, was observed. Next, 2 mL of cells were centrifuged and washed twice with a solution of 0.85% NaCl. Then, the cells were suspended in 1 mL of 60% sucrose solution and the absorption spectra of the cells from 370 to 1020 nm were obtained with a spectrophotometer (Biebls & Drews, 1969) (Imhoff et al., 2004). The peaks of the absorption spectra were analyzed and compared to published spectra of bacteriochlorophylls in cells, to determine the type of bacteriochlorophyll produced by the isolates. The same suspensions were also used to determine the color of the cell suspension of the isolates using the Munsell Color chart (Soto-Feliciano, 2011).

1.4.4 Partial Identification of Isolates Using 16SrDNA

16SrDNA gene was amplified and sequenced to partially identify the isolates,. The cells were incubated under anaerobic conditions in light until pigmented colonies were observed (approximately 3 days). For 16SrDNA amplification, 3 colonies of each isolate were suspended in 10 µL of PCR buffer and were incubated at 94 ^{o}C for 5 min to extract the DNA. Then 1 µL of the suspension was used for the PCR reaction containing the following per 25 µL of reaction: 9.5 µL of sterilized and distilled deionized water, 12.5 µL Green Taq Master Mix (Promega), 1 µL of forward primer 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and reverse primer 1492R (5'-GGT TAC CTT GTT AGG ACT T-3') (Weisburg et al., 1991). The parameters of the PCR were the following: first denaturalization at 94 ^{o}C for 3 min, followed by 30 cycles with a denaturalization of 94 ^{o}C at 30 secs, annealing of 48 ^{o}C for 30 secs and elongation at 72 ^{o}C for 1.5 min and a final elongation at 72 ^{o}C for 10 min. PCR product was confirmed by gel electrophoresis with agarose at 1% prepared with TAE1X. The PCR product was sent for cleanup and sequencing to McLabs (www.mclab.com). The sequenced partial 16SrDNA of each isolate was ensembled using the program BioEdit (http://www.mbio.ncsu.edu). *In silico* analysis of the 16SrDNA was done using EZTaxon server (https://www.ezbiocloud.net/) (Chun et al., 2017).

1.4.5 Differentiation of Purple Non-Sulfur Bacteria Isolates by Carbon Source Utilization

Carbon/electron sources utilization by the isolates under phototrophic conditions were evaluated to identify similarities between these bacteria. For the determination of carbon source/electron donor used by the isolates, media was prepared substituting succinic acid, L-glutamic acid and L-aspartic acid for the carbon source to be tested at 0.35%. Also yeast extract concentration was lowered to 0.1% w/v (Kumar et al., 2013). The following compounds were tested as carbon sources: butyrate, formate, fumarate, glucose, glycerol, glycolate, malate,

propionate, succinate, valerate, acetate and pyruvate. These carbon sources were used previously in the description of *Rhodopseudomonas* species. (Ramana et al., 2012; Kumar et al, 2013a). These tests were conducted in triplicate in 2 mL-96 wells plates with 500 µL of medium per well and 20 µL of inoculum of a 4-day incubation culture prewashed twice with a 0.85% NaCl solution. The inoculated 96-wells plates were incubated in the presence of light, with illumination of 2500 lux for seven days at approximately 32 to 35 ºC. After the incubation time, the isolates able to grow under photoheterotrophic conditions with the tested carbon sources were identified by observing the change in color of the media. Carbon test usage and 16SrDNA partial identification results were used to differentiate the PNSB isolates.

1.5 Results and Discussion

1.5.1 Evaluation of Phototrophic and Chemotrophic Mode of Growth by the Isolates

The capability of the isolates to grow under chemotrophic and phototrophic conditions in light was used as a criterion to confirm that the isolates were members of the PNSB. From the collection of PNSB of the Laboratory of Microbial Biotechnology and Bioprospecting (MBB) at UPRM, a total of 33 bacteria, 9 from water reservoirs and 19 and 5 from bromeliad and *Heliconia* phytotelmata, respectively, were used for this study. These isolates grew under aerobic and anaerobic conditions in the presence of light. The colonies of the isolates were punctiform, opaque, butyrous, round, smooth, convex, with entire margins (Figure 1.4).

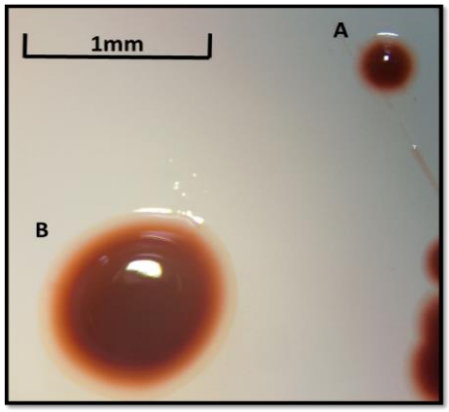


Figure 1.4: Colony morphology of PNSB isolated from Bromeliad and *Heliconia* phytotelmata after incubation under anaerobic conditions with light for 3 days in the media described by Lakhmi et al. (2009): All LMB isolates colonies are opaque, smooth, round, convex, with entire margins. **A**. Bacterial colony of PNSB isolate from water reservoir with a diameter of less than 1 mm. **B**. Bacterial colony of a PNSB isolate from *Heliconia* phytotelmata with a diameter of approximately 1 mm.

Under anaerobic conditions, the colonies were pigmented with color ranging from pink to red except for two of the isolates from the phytotelma of *Heliconia* which were green in color. The color green changes to red when exposed to oxygen (Figure 1.5). In addition, these two isolates were slightly pink when grown under aerobic conditions in contrast to all the other isolates which grew white under the same conditions. Also, one of the isolates from water reservoirs released a pigment into the media (Figure 1.5). In an effort to find information about pigments produced by PNSB, different from bacteriochlorophyll or carotenoids, we found only one study in which an unknown water-soluble pigment was produced by some members of the PNSB (van Niel, 1944). The difference in pigmentation in colonies grown under anaerobic and aerobic growth conditions is characteristic of PNSB. It has been found that the transcription of proteins and pigments related to photosynthesis in PNSB is repressed by the presence of oxygen (Bazire et al., 1957; Sganga et al.,1992; Imhoff, 2006).

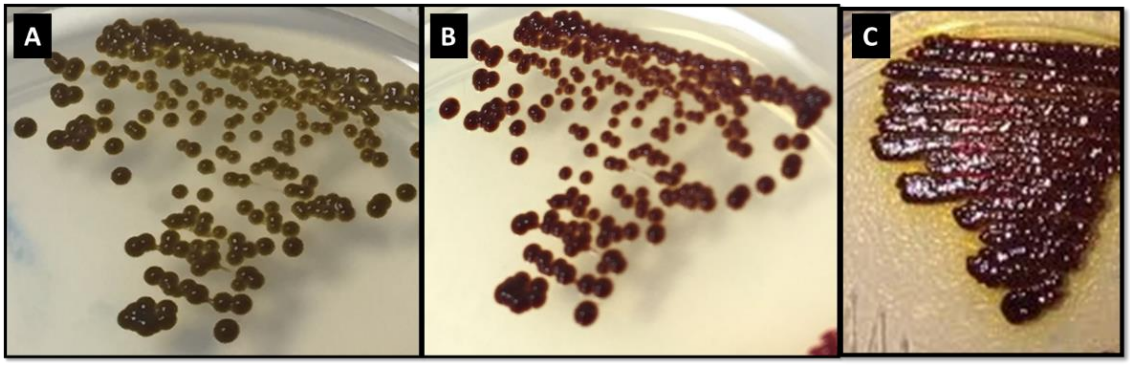


Figure 1.5: Colonies of isolates from *Heliconia* phytotelmata and water reservoir collected from Puerto Rico. A. Pigmentation of colonies under phototrophic conditions. B. Change in pigmentation after 24h exposed to oxygen. C. Unknown pigment secreted by an isolate from water reservoirs.

1.5.2 Amplification of *pufM* Gene

The presence of a photosynthetic apparatus in the isolates was addressed using molecular techniques. Specific primers for the amplification of the *pufM* gene (229 bp) were used. This gene encodes for the M subunit of the reaction center in the photosynthetic apparatus (Achenbach et al., 2001). Sequences between 200 and 300 bp were obtained for all the isolates (Figure 1.6). Therefore, the isolates had genes related to photosynthesis metabolism.

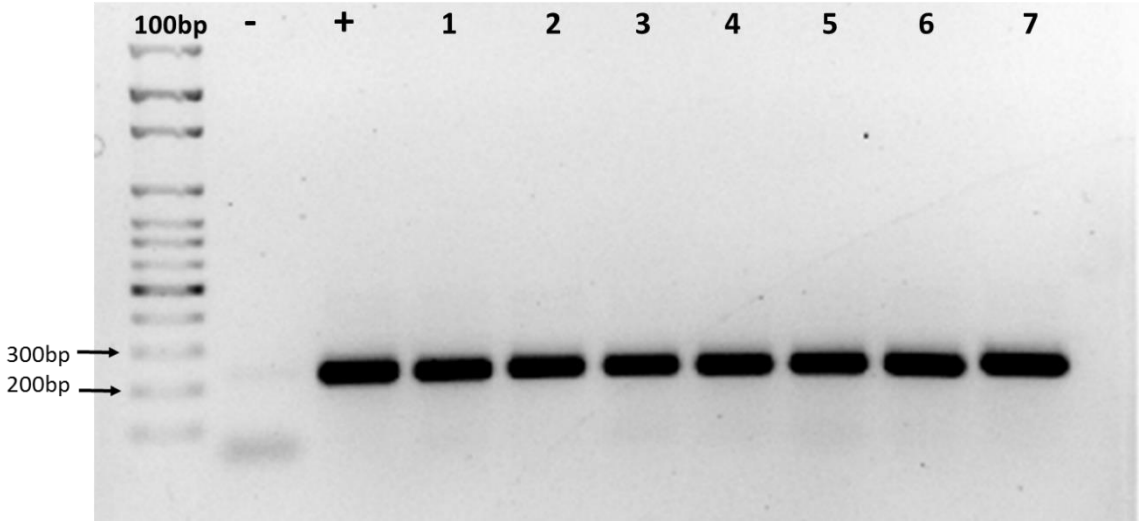


Figure 1.6: Electrophoresis of pufM PCR product (1.5% TAE agarose gel). Amplicons between 200 and 300bp were obtained after the amplification of pufM in all the isolates of possible PNSB. Axygen 100bp ladder was used as marker. Positive and negative control denoted by + and –, respectively. Amplification of *pufM* from isolates of water reservoirs and bromeliad and *Heliconia* phytotelmata in wells 1-3, 4-6 and 7, respectively.

1.5.3 Determination of Bacteriochlorophyll Type and Color of Cell Suspensions

A whole cell absorption spectra analysis was performed to confirm that the isolates colonies color was a result of photosynthetic pigments production and to determine the type of bacteriochlorophylls in the cells. In the spectra of all the isolates peaks at approximately 375, 590, 805 nm, and between 830 and 911 nm, were observed which indicate the presence of bacteriochlorophyll a. The presence of bacteriochlorophyll *a* or *b* is characteristic of PNSB (Rosenberg et al., 2012b). Also, peaks between 400 and 450 nm were observed which are indicative of carotenoid pigments (Figure 1.7).

In previous studies, the color of PNSB cells suspensions, has been reported based only in the investigator's perception,. Terms such as pink, red, brown-red, and green have been used in the description of PNSB. However, this approach makes difficult to relate isolates with described species due to the variations of color perception from each observer. In an effort to reduce this variability, the color of cell suspension in sucrose and the Munsell Color Chart were used in our study to describe the color of the isolates. The color of the isolates varies from green, red and brown-red. In Munsell color codification, the cell suspensions were: 2.5GY6/10, 10R2/8 and 2.5YR 2/8, respectively (Figure 1.8).

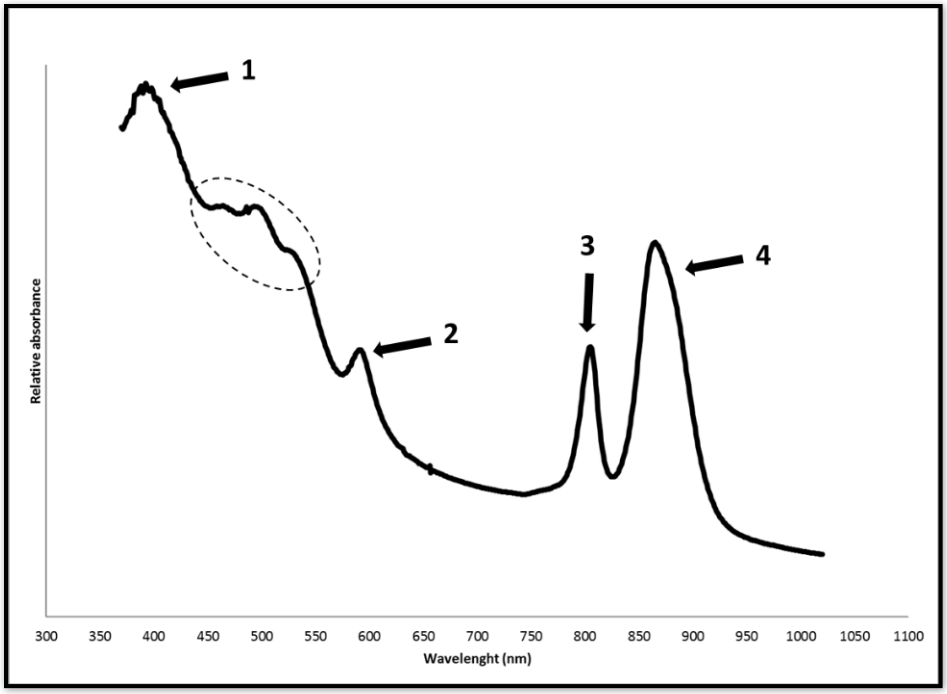


Figure 1.7: Relative absorbance of in vivo cell suspensions in sucrose (60%). Bacteriochlorophyll *a* [absorption maxima at 375 nm (arrow 1), 590 nm (arrow 2), 805 nm (arrow 3), and 830-911 nm (arrow 4)] and carotenoids pigments [absorption maxima between 400-550 nm (region in the oval)] were identified in all the isolates.

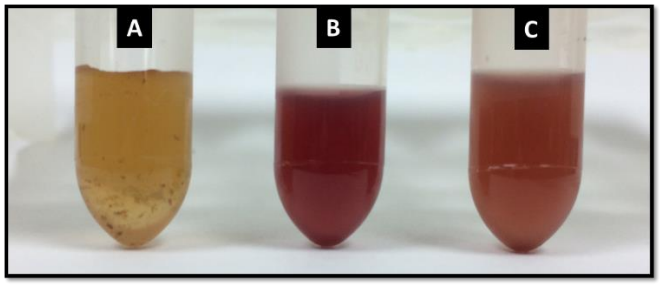


Figure 1.8: Color of PNSB isolates cell suspensions in 60% sucrose. Munsell color codifications: **A.** 2.5GY6/10, **B.** 10R2/8 and **C.** 2.5YR2/8.

1.5.4 Partial Identification of Isolates Using 16SrDNA

Assessment of the 16SrDNA partial sequence was done using the following databases: EZTaxon server (Chun et al., 2017), NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) and NCBI Reference RNA sequences (refseq_rna), and Silva (https://www.arb-silva.de/). It was

found that 30 of the 33 isolates had over 97% of 16SrDNA similarity with bacteria in the genus *Rhodopseudomonas (Rps.).* These bacteria are *Rps. palustris, Rps. pentothenatexigens, Rps. harwoodiae* and *Rps. thermotolerans.* The other isolates 16SrDNA had similarities of more than 97% with bacteria in the genus *Rhodobacter* (*Rba*.). These bacteria are *Rba. johrii*, *Rba. sphaeroides*, *Rba. megalophillus*, *Rba. azotoformans* and *Rba. ovatus*. PNSB from the MBB culture collection belonging to *Rhodopseudomonas* were isolated from bromeliad and *Heliconia* phytotelmata, and water reservoirs. However, members of the genus *Rhodobacter* were only found in the *Heliconia* phytotelmata.

The presence of members of the genus *Rhodopseudomonas* in phytotelmata was expected since they are known to be ubiquitous in nature (Larimer et al., 2004). Vega-Sepúlveda (2009) reported the presence of *Rhodospirillum* spp., and *Rhodomicrobium* spp. (3 isolates) in bromeliad phytotelmata. However, we were not able to confirm this information using 16SrDNA. According to our study, these isolates reported as *Rhodomicrobium* spp., are also *Rhodopseudomonas* spp. Vega-Sepúlveda (2009) also mentioned that one of the isolates could be a possible new genus of PNSB but more related to *Enterobacter* than PNSB genera. Streaks of this isolate after incubation under phototrophic conditions showed pigmented colonies (characteristic of PNSB) in addition to unexpected white colonies. It is important to mention that these white colonies were not evident when grown under anoxyphototrophic conditions. We confirmed the presence of these colonies by letting the streaks under oxygenic conditions (aiming to favor the growth of any other organism if present). The presence of the unknown organism could have been the cause in the mistaken partial identification of PNSB isolates as more related to *Enterobacter* spp.

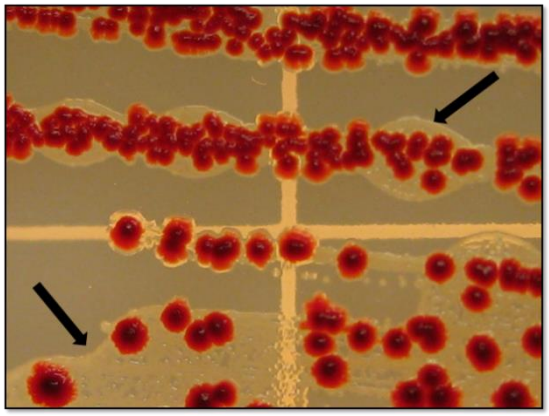


Figure 1.9: Contamination in cultures of PNSB grown under anaerobic conditions with light in media described by Lakhmi et al. (2009): The presence of contamination (pointed by the black arrows) was evident after growth of the PNSB isolates under aerobic conditions for 3 d. Before the incubation under aerobic conditions, the isolates were grown under anaerobic conditions with light until pigmented colonies were observed.

1.5.5 Differentiation of PNSB Isolates by Carbon Source Utilization

Carbon source usage comparison between the PNSB isolates was performed in an effort to identify possible same species among these bacteria and also to compare with described PNSB. Given that, most of the PNSB isolates were more related to *Rhodopseudomonas* spp., we used carbon sources references previously known for the description of novel species of this genus (Demin et al., 2002; Hougardy et al., 2000; Ramana et al., 2012; Kumar et al., 2013a; Hiraishi et al., 2017). Although all of the isolates were able to use acetate, as all described *Rhodopseudomonas* spp., none of the isolates had the same usage profile as any member of this genus for the other tested carbon sources. In addition, the 2 isolates related to the genus *Rhodobacter* used similar compounds as *Rba. johrii* (only known PNSB to produce endospores) (Girija et al., 2010). However, unlike *Rba. johrii*, our isolates were not able to grow with pyruvate but with propanol as a carbon source. Also, one of these isolates, HE0M, could use propionate unlike the other *Rhodobacter* related isolate (HE0K) and *Rba. johrii*. These differences between the isolates and the described PNSB could mean that the isolates are new species of PNSB.

When combining carbon usage, the color of cell suspensions and 16SrDNA similarities, we grouped the PNSB isolates into 16 different groups. For the identification of the isolates as new

species in Chapter 2 and its biotechnological potential in Chapter 3, one isolate representing each group was used.

1.6 Conclusion

The purpose of this study was to confirm that the isolates from the microbial culture collection of the Microbial Biotechnology and Bioprospecting (MBB) laboratory were members of the PNSB. PNSB are known for their metabolic diversity, specifically their phototrophic mode of growth under anaerobic conditions. This characteristic of PNSB is used favorably for the isolation of these organisms and was the method employed for the isolation of the isolates used in this study (Soto-Feliciano et al., 2010; Del Socorro et al., 2013; Kar Soon et al., 2014). However, because anoxygenic photosynthesis is not exclusive of PNSB, further analyses were performed to confirm the isolates as PNSB members.

During this study, in addition to identify anoxygenic photosynthetic metabolism by the isolates, the production of photosynthetic pigments and proteins were also confirmed. Also, 16SrDNA analysis partially identified the isolates as members of the PNSB genera *Rhodopseudomonas* and *Rhodobacter*. The fact that most bacteria in this study were isolated from not well-studied environments, and the differences between their carbon source usage with those reported in the literature, suggest the presence of novel members of the PNSB. Also, despite the fact that all of the 16SrDNA sequences of the isolates shared similarities of more than 97% with described species of PNSB, that does not imply necessarily that they are the same species (Imhoff et al., 2004). The recommended criteria for the description of anoxygenic phototrophic bacteria will be used in Chapter 2 to evaluate the possibility that the isolates represent new species of the PNSB.

Chapter 2: Species Identification of Purple Non-Sulfur Bacteria (PNSB) from Bromeliad and *Heliconia* Phytotelmata and Water Reservoirs in Puerto Rico

2.1 Introduction

The isolation and study of purple non-sulfur Bacteria (PNSB) have contributed to the understanding of their metabolic diversity, extend in nature, diversity and biotechnological applications. However, it is estimated that less than 3.5% of the members in this group are recognized and their ecological roles are not well understood (Rosenberg et al., 2012b; Overmann et al., 2013).

Since 2001, the Laboratory of Microbial Biotechnology and Bioprospecting (MBB) at UPRM has built a microbial culture collection of isolates from different environments in Puerto Rico. In Chapter 1 of this work, we were able to confirm the isolates of this collection as members of the PNSB. These PNSB were isolated from different aquatic environments from Puerto Rico such as water reservoirs, microbial mats, and bromeliad and *Heliconia* phytotelmata. As we understand, the PNSB isolates in the culture collection of the MBB are the first to be isolated from Puerto Rico. Furthermore, there is no other study about the isolation of PNSB from bromeliads or *Heliconia* phytotelmata.

In the previous chapter, the partial identification of the PNSB isolates from the water reservoirs, and bromeliad and *Heliconia* phytotelmata was addressed (Chapter 1). Although it was found that the isolates are related to the *Rhodopseudomonas* (*Rps*.) and *Rhodobacter* (*Rba*.) genera, the species of these bacteria are still unknown. Moreover, when addressing the carbon usage of the isolates, we were unable to find similarities with any described PNSB species in these genera.

Because of the uniqueness of the environments were these bacteria have been isolated (Chapter 1), the fact that there are no other studies about the isolation of these bacteria in Puerto Rico and the differences in carbon usage of the isolates from described members of the PNSB, the isolates in the MBB could represent novel species of PNSB.

In this chapter, we aim to identify the species of the PNSB isolated from water reservoirs and bromeliad and *Heliconia* phytotelmata. If novel species are described, this study would be the first to describe new species of PNSB isolated from environments in Puerto Rico. Moreover, it would be the first to report new species of PNSB from bromeliads and *Heliconia* phytotelmata. The description of novel PNSB from these environments will contribute to the understanding of these organisms ecological roles, metabolic diversity, and biotechnological applications.

2.2 Literature Review

2.2.1 Description of Novel Species of Purple Non-Sulfur Bacteria

Before molecular biology advancement, the identification of prokaryotes was based on phenotypical traits of the organisms. It is not until the proposal of 16rDNA as a tool to address phylogeny that the phylogenetic relations between bacteria was apparent. However, the systematics of prokaryotes has been always a debate in the scientific community and currently there is not an official classification for prokaryotes. In an effort to addressed the challenge of prokaryotes classification, the International Committee on the Systematics of Prokaryotes has established different subcommittees that define the parameters that must be taken into consideration to publish and describe new species (http://www.the-icsp.org/taxonomic-subcommittees). For the description of novel members of PNSB, the methods described by the subcommittee for the description of phototrophic bacteria have to be followed (Imhoff et al., 2004). These methods were defined taken into consideration a polyphasic approach where phenotype and genotype traits such as morphology (i.e. cell and colony size and morphology etc.), chemical composition (i.e. fatty acid analysis, type of bacteriochlorophyll, DNA-DNA hybridization, 16SrDNA, etc.) and physiology (i.e. carbon, nitrogen and sulfur usage, temperature and pH range and optimum, etc.) have to be evaluated.

2.2.2 Physiology of Purple Non-Sulfur Bacteria

When addressing phylogenetic relations of described members of the PNSB, it is found that some PNSB are more related to non-phototrophic bacteria than to other members among the PNSB and the only trait shared by all species is the ability to make photosynthesis, the result of horizontal gene transfer (Kawasaki et al., 1993). Consequently, these organisms are physiologically diverse and hence ubiquitous in nature, inhabiting different environments such as pond water, marine environments and soils (Srinivas et al., 2006; Okamura et al., 2009; Kumar et al., 2013; Hiraishi et al., 2017). Moreover, isolation of PNSB from saline soda lakes and Antarctic microbial mats demonstrate the ability of this group to also inhabit extreme environments (Madigan et al., 2000; Kompantseva et al., 2010). Although most PNSB are mesophilic, some members such as *Rhodoferax antarcticus* are psychrophilic, with an optimal growth temperature range of 0 to 25 ^{o}C (Madigan et al., 2000). Regarding salt requirements, some PNSB do not require salt for growth but other members are halophilic, such as *Rhodovulum steppense*, with 1-5% NaCl optimum growth range (Kompansteva et al., 2010). The pH growth requirements also varied among members of PNSB; most of them are neutrophiles but other members, such as *Rhodovolum steppense* have an optimal growth at pH of 8.5 (Kompansteva et al., 2010). On the contrary, *Rhodoblastus acidophilus* (formerly known as *Rhodopseudomonas acidophila*) can grow at a pH of 5.2 (Pfenning, 1969). The fact that these organisms can withstand such conditions provides a reference to the extent and applications of these bacteria for biotechnological applications such as the source of enzymes with different temperature and pH optima for industrial use (Sánchez-Porro et al., 2003; Sarethy et al., 2011).

Although their preferable mode of growth is photoheterotrophic, most PNSB can grow under microaerobic and aerobic conditions (Garrity et al., 2005; Soto-Feliciano, 2011). They have

different carbon sources such as inorganic (CO_2) and organic (including hydrocarbons) during phototrophic and chemotrophic modes of growth (Zhao et al., 2011; Merugu et al., 2012). These bacteria can also grow as fermenters using different organic sources such as sugars and pyruvate under anaerobic conditions in the dark (Imhoff et al. 2004). PNSB can derive nitrogen from different sources as well; for instance, ammonium chloride, aspartate, glutamate and urea (Girija et al. 2010; Ramana et al. 2012; Hiraishi et al., 2017). Moreover, it is well known that members of the PNSB, such as *Rhodobacter* spp. and *Rhodopseudomonas* spp., fix nitrogen (Basak et al., 2007; Larimer et al., 2004). Moreover, 3 different nitrogenases (involved in nitrogen fixation) were found in *Rhodopseudomonas palustris* (Larimer et al., 2004; Oda et al., 2005). Before this finding, the presence of 3 different functionally nitrogenases was only found in *Azotobacter* sp. (Larimer et al., 2004; Hales et al. 1986). These enzymes are key in the biotechnological application of PNSB as hydrogen producers. During nitrogen fixation, hydrogen is released as a bioproduct of the reduction of nitrogen to ammonium catalyzed by nitrogenases (Melniki et al., 2008; McKinlay et al. 2010; Wang et al. 2016). Despite the physiological capabilities and hence the possible applications of the PNSB, it is estimated that less than 3.5% of this group has been described (Rosenberg et al., 2012b).

2.2.3 The Genera *Rhodopseudomonas* and *Rhodobacter* of The Purple Non-Sulfur Bacteria

As concluded in Chapter 1 of this work, most isolates in this study belonged to the genus *Rhodpseudomonas* (*Rps.*). However, the species of these isolates are unknown. Until 2012, *Rps. harwoodiae, Rps. parapalustris*, *Rps. pseudopalutris, Rps. palustris* (Ramana et al., 2012), *Rps. faecalis* (Demin et al., 2002), and *Rps. rhenobacensis* (Hougardy et al., 2000) were the only valid members of *Rhodopseudomonas* (Kumar et al., 2013b). In 2013, the number of species in the genus *Rhodopseudomonas* increased to 8 with the description of *Rps. pentotenatexigens* and *Rps.*

thermotolerans (Kumar et al., 2013b). However, in 2017, in the description of *Rps. telluris* as a new species of the PNSB, the species description of *Rps. thermotolerans* and *Rps. pentothenatexigens* was invalidated since no significant differences were found between the type strains of these bacteria (Hiraishi et al., 2017). Additionally, the reconsideration of *Rps. parapalustris* as a novel species of PNSB was recommended due to the absence of the type strain in all culture collections. Also the description of *Rps. harwodiae* and *Rps. pseudopalustris* was invalidated and amended description of these species is going to be published by Hiraishi and Okamura (2017). It is noticeable the necessity of more studies aiming to identify and describe new species of PNSB which could provide a better understanding of the extent and member composition of this group of photosynthetic bacteria.

2.3 Objectives

The objective of this chapter is to identify the species of the 16 PNSB isolates (Chapter 1) from the MBB culture collection isolated from water reservoirs and bromeliad and *Heliconia* phytotelmata according to the recommended standards for the description of phototrophic bacteria (Imhoff et al., 2004). The full description of the isolates in the MBB culture collection will represent the first attempt to describe PNSB from *Heliconia* and bromeliad phytotelmata. In addition, it will provide an insight into the microbial diversity, the physiology of the microorganisms inhabitants in these environments and their exploration as niches of PNSB with biotechnological applications (Chapter 3).

2.4 Methods

2.4.1 Phylogenetic Relations of the Isolates Using 16SrDNA

16SrDNA was used to address the phylogenetic relations of the PNSB in the MBB collection (isolated from water reservoirs and bromeliad and *Heliconia* phytotelma) and described members of the PNSB. The phylogenetic analysis was done using Phylogeny.fr platform (http://www.phylogeny.fr/) (Dereeper et al., 2008). Sequences were aligned using Muscle v3.8.31 with default settings (Edgar et al., 2004). Ambiguous regions were removed using G-blocks v0.091b with the following parameters: 1) minimum length of a block after gap cleaning of 10, 2) no gaps positions were allowed in the final alignment, and 3) all segments with contiguous non-conserved positions bigger than 8 were rejected and with a minimum number of sequences for a flank position of 85%. The reconstruction of the phylogenetic tree was done using the maximum likelihood method in the PhyML v3.1/3.0 aLRT program. The maximum likelihood method use a mathematical model to build the phylogenetic tree. It constructs different candidate trees and based on probability calculations select the tree that best accounts for the variation in the data set (Shimodaira, 2002). This method had shown to recover correct tree topologies by computer simulation studies and have been used before in the description of PNSB (Kumar et al., 2013). The default substitution model was selected assuming an estimated proportion of invariants sites (of 0.571) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma= 0.558). Internal branch reliability was addressed using the bootstrapping method (100 replicates). Finally, the graphical representation of the phylogenetic tree was performed using TreeDyn (v198.3) (Chevenet et al., 2006).

2.4.2 Determination of NaCl Requirements, pH and Temperature Range and Optima

For the determination of pH growth range and optimum, medium as described in Section 1.4 was prepared with pH ranging from 5.5 to 9.0 in 0.5 unit increments. To determine if NaCl was required for growth, the same medium was prepared without the addition of NaCl. These tests were conducted in triplicate in 2 mL-96 wells plates (VWR) with a working volume of 500 µL and 20 µL of inoculum of a 3 d of incubation culture prewashed twice with a 0.85% NaCl solution and adjusted to an absorbance of 0.020 at 600 nm. The inoculated plates were incubated in the presence of light with illumination of 2500 lux for 7 d at approximately 32 to 35 ºC. Change in color of the media to red (Section 1.5.3) was recorded as growth. Also, absorbance at 600 nm was measured. For the determination of temperature range and optima, the medium described in Section 1.4 was used at pH of 7.0. VWR borosilicate 6 mL crystal vials were filled with media (to achieve anaerobic conditions during incubation). Then the medium was inoculated with a 3 d incubated culture prewashed twice with a 0.85% NaCl solution and adjusted to an absorbance of 0.020 at 600 nm. The tubes were incubated for 4 d in light at the desired temperatures; 10, 15, 20, 25, 30, 35, 40 and 45 ºC. After incubation, absorbance at 600 nm was measured to measure cell growth.

2.4.3 Nitrogen, Sulfur and Vitamin Requirements

Ammonium chloride (Section 1.4) was substituted in the media with urea, glutamate or glutamine, each at a final concentration of 0.06% as recommended by Imhoff et al. (2004) to determine the utilization of nitrogen sources by the isolates. For the determination of vitamin requirements, yeast extract was substituted by one of the followings: 0.1 mg/L biotin (vitamin B7), 0.3 mg/L thiamin HCl (vitamin B1), 1 mg/L nicotinic acid (vitamin B3). To determine sulfur sources, magnesium sulfate was substituted in the media by one of the followings: 0.2 mM ammonium sulfate, 2 mM thioglycolic acid, 5 mM sodium thiosulfate or 0.5 mM sodium sulfide.

These tests were conducted in triplicate in 2 mL-96 wells plates with a working volume of 500 µL and 20 µL of inoculum of a 3 d incubated culture prewashed twice with a 0.85 % NaCl solution, and diluted until reaching an absorbance of 0.020 at 600 nm. The inoculated plates were incubated under anaerobic conditions in the presence of light with illumination of 2500 lux for seven days at approximately 32 to 35^{o}C. A change in color of the media (to red) was recorded as growth and absorbance at 600 nm was measured to confirm cell growth.

2.4.4 Evaluation of Photolithotrophy, Chemolithoautotrophy and Fermentative Mode of Growth by Purple Non-Sulfur Bacteria Isolates

To determine photolithoautotrophy, chemolithoautotrophy, and fermentative growth, the media used were prepared without succinic acid, L-glutamic acid, and L-aspartic acid. Sodium sulfide (0.5 mM), sodium thiosulfate (2 mM) and sodium bicarbonate (0.1%) were added to the media for photolithoautotrophy tests. For chemolithoautotrophy tests, sodium bicarbonate (0.1%) and sodium thiosulfate pentahydrate (1 mM) where added to the media whereas pyruvate and glucose (0.3%) were used for fermentative growth. Photolithoautotrophy tests were performed in triplicate in 2 ml-96 wells plate with 500 µL of the corresponding media and 20 µL inoculum of a 3 d culture prewashed twice with a 0.85% NaCl and diluted to an absorbance of 0.020 at 600nm. Incubation was done at 32 to 35 ^{o}C with light for 4 d. For chemolithoautotrophy tests, 3 mL of media were added to 6 mL borosilicate bottles. An inoculum of a 3-day culture, prewashed twice with 0.85% NaCl, was added to obtain an initial absorbance of 0.020 at 600 nm. Incubation was done in the dark at 32 ^{o}C for 4 d. Fermentation tests were done in 6 mL bottles filled completely with media to achieve anaerobic conditions. An inoculum of a 3 d culture, prewashed twice with 0.85% NaCl, was added to obtain an initial absorbance of 0.020 at 600nm. The incubation was performed at 32 ^{o}C without light.

2.4.5 Motility, Gram and Endospore Stain

Slides, for all stains, were previously washed and stored in 3% hydrochloric acid and 95% ethyl alcohol solution for a minimum of 4 d (Leifson, 1951). Motility, endospores, and Gram staining were addressed after growing the isolates in 6 mL VWR borosilicate bottles filled with media to achieve anaerobic conditions. Incubation was done at 32 - 35 ^{o}C in the presence of light for 3 d. The motility of the cells was addressed using wet mount preparation. For Gram and endospore staining, the protocols published by the American Society of Microbiology were used (Hussey, 2016; Smith et al., 2016).

2.4.6 Scanning Electron Microscopy (SEM)

The morphology of the isolates was confirmed by scanning electron microscopy (SEM). The isolates were grown in media until turbidity was observed under phototrophic conditions at 32 ^{o}C (approximately 3 d). About 4 mL of culture were centrifuged at 1.8 x g for 3 min to form a cell pellet. Then, 1 µL of 4% glutaraldehyde in Sorenson's phosphate buffer pH 7.2 was added to the pellet and incubated for 24 hrs at 4 ^{o}C. After incubation, the supernatant was discarded and 1 mL of Sorenson's phosphate buffer pH 7.2 was added to the pellet and incubated at 25 ^{o}C for 10 min (this step was repeated 3 times). To dehydrate the cell pellet, 1 mL of ethanol solutions ranging from 10 to 100% (in 10% increments) was added to the pellet with an incubation time of 10 min at 25 ^{o}C for each solution. The dehydration step with 100% alcohol was repeated 3 times. The samples were taken to the Microscopy Center at the Biology Department of the University of Puerto Rico at Mayagüez, where critical point drying for dehydration and coating with palladium-gold was done per their established parameters (https://microscopico.wordpress.com/mer/).

2.4.7 Orthologous Average Nucleotide Identity (OrthoANI)

Since 16SrDNA similarities between the isolates and previously described members of the PNSB were above 97%, DNA-DNA hybridization is mandatory to elucidate to species level

(Imhoff & Caumette, 2004). Although DNA-DNA hybridization has been used for prokaryotes species differentiation, the emergence of sequencing technologies and, thus, the affordability of genome sequencing has encouraged the development of genome relatedness indices algorithms such as Orthologous Average Nucleotide Identity (OrthoANI) (Lee et al., 2016). Moreover, this technique is known to be more reliable than DNA-DNA hybridization and has been used for the description of different species (Moore et al., 2010). For all these reasons, Orthologous Average Nucleotide Identity (OrthoANI) was performed instead of DNA-DNA hybridization.

For the OrthoANI analysis, 4 isolates were selected. The selection was based taken into consideration differences among the isolates (i.e. pH and temperature ranges, carbon, sulfur, and nitrogen usage, etc.) and phylogenetic position (see Figure 2.1). Streaks in plate of the isolates were done in the media previously described in Section 1.4. Incubation was done under anaerobic conditions (achieved by the use of an anaerobic jar) in light until pigmented colonies were observed (approximately 4 days). Then, a substantial amount of cells mass was resuspended in lysis buffer (40 mM Tris-acetate pH 7.8, 20 mM sodium acetate pH 8.0, 1.0 mM EDTA pH 8.0 and 1% SDS), sodium chloride 5 M and RNAse (20 µg/µL). After incubation for 30 min at 37 ºC, two organic extractions using chloroform were performed followed by DNA precipitation using absolute ethanol (Malavé Orengo, 2011). Finally the DNA was resuspended in EB buffer (10 mM Tris-HCl pH 8.5 without EDTA) and its concentration measured with NanoDrop. The DNA was sent to Microbes NG (https://microbesng.uk/) for full genome sequencing using Illumina platforms. Finally, OrthoANI was done using the OAT software (Lee et al.; 2015). The following reference genomes were used in the analyses: *Rhodopseudomonas palustris* BisA53 (NC008435), *Rps. palustris* BisB18 (NC007925), *Rps. palustris* CGA009 (NC005296), *Rps. palustris* TIE-1 (NC011004), *Rps. pseudopalustris* (jgi.1102342.1)**.** OrthoANI values below 95~96% between

genomes are indicative of different species (Lee et al., 2015). The PNSB isolates selected for the ANI and OrthoANI evaluation were WA056, BR0C11, BR022, and BR0G17. The NCBI Accession number for these sequences are SAMN09658591, SAMN09658594, SAMN09658593, and SAMN09658592, respectively.

2.5 Results and Discussion

It is important to mention that the following analysis was done using the results in the published descriptions of the mentioned *Rhodopseudomonas* species. However, an amended description of *Rps. pentothenatexigens, Rps. thermotolerans*, *Rps. pseudopalustris, Rps. harwoodiae* is going to be published by Hiraishi and Okamura (Hiraishi et al., 2017). Also, due to the lack of an *Rps. parapalustris* type strain in any culture collection, its taxonomical validity is under reconsideration.

2.5.1 Phylogenetic Relations of the Isolates Using 16SrDNA

In Chapter 1, it was concluded that the isolates of this study were members of the PNSB, specifically related to the genera *Rhodopseudomonas* (*Rps.*) and *Rhodobacter* (*Rba.*). Isolates HE0M and HE0K are in the same cluster as *Rba. johrii* and *Rba. aflagellatum*. In the phylogenetic tree, it can be observed how the isolates cluster between described species of these genera. Isolates BR0C12 and WA056 are more related to *Rhodopseudomonas harwoodiae*, whereas BR0M22 is in another branch within this group. This suggests that BR0M22 could be a novel species of PNSB. The other isolates formed a distinct clade suggesting that these are new species of PNSB. However, in order to describe these isolates as new species of PNSB, DNA-DNA hybridization (or OrthoANI) is mandatory, since the 16SrDNA similarities of these bacteria to previously described members of *Rhodopseudomonas* and *Rhodobacter* are above 97 % (Imhoff et al., 2004) (Section 2.5.7).

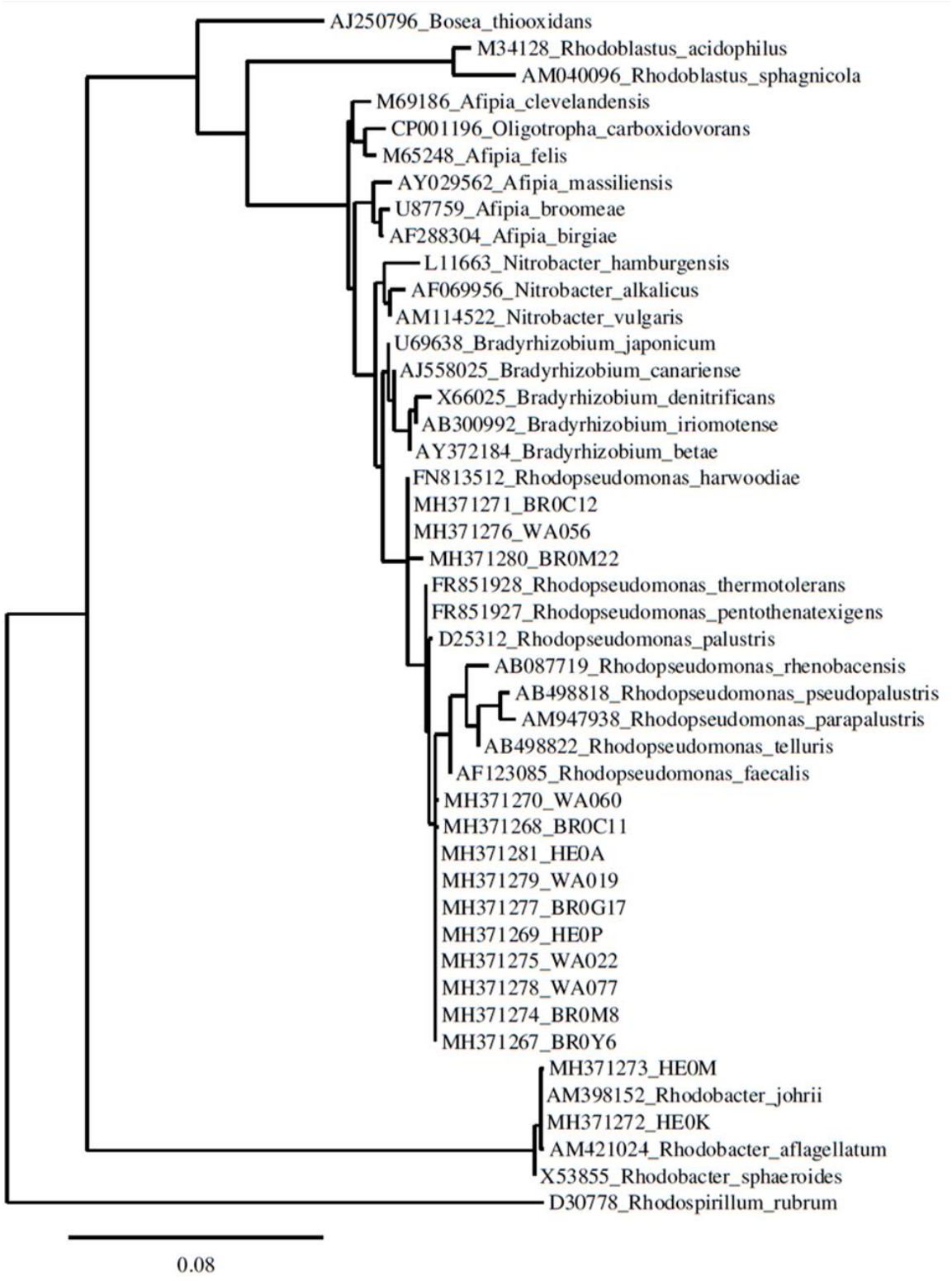


Figure 2.1: Phylogram showing phylogenetic relations among the PNSB isolates, described members of this group, and other chemotrophs using 16SrDNA. NCBI accession numbers accompanied by the name of each strain.

Names without accession number represent the studied PNSB isolates. *Rhodospirillum rubrum* was used as an outgroup to root the tree. The bar represents 8 nucleotide substitutions per 100 nucleotides.

2.5.2 NaCl Requirements, pH and Temperature Range and Optima

In terms of NaCl, none of the 16 isolates of PNSB required NaCl in the media for growth. However, 9 of the isolates were able to grow with 1% or higher concentration of NaCl in the media (Table 2.1).

From the 14 isolates related to the genus *Rhodopseudomonas*, the isolate WA0A was able to grow up to 6 % of NaCl in the media. When compared to the described species in the genus *Rps. penthotenatexigens* and *Rps. thermotolerans* are the species with the highest NaCl tolerance, of 3 % (Hiraishi & Okamura, 2017). This suggests that the isolate WA0A is a new halotolerant species of *Rhodopseudomonas*. The isolates HE0K and HE0M were able to grow in 7 % of NaCl in the media with an optimal NaCl concentration of 1 - 2 %. Both of these isolates were similar in terms of 16SrDNA to *Rhodobacter johrii*, which has an optimal growth at 3 % of NaCl (tolerance of this strain was not reported in its description) (Girija et al., 2010).

In terms of pH, none of the 14 isolates related to the *Rhodopseudomonas* had the same pH range and optima than described members of the genus. Moreover, 15 of the 16 isolates were able to tolerate a pH above 9.0 (Table 2.1). Specifically, 3 of these 15 isolates were able to grow at a pH of 12. *Rps. telluris*, *Rps. harwoodiae*, *Rps. palustris* (ATCC17001), *Rps. pseudopalustris*, *Rps. pentotenatexigens* and *Rps. thermotolerans* are the *Rhodopseudomonas* species with tolerance to a pH of 9.0 (Ramana et al., 2012; Hiraishi et al., 2017). Moreover, *Rhodovulum steppense* and *Rhodovulum tesquicola*, PNSB isolated from soda lakes in Siberia and Central Asia, respectively (high pH extreme environment), had a pH maxima of 10.0 (Kompantseva et al., 2017). It is interesting that the PNSB in our study can withstand such alkaline pH, particularly because of the ecosystems from where they collected (water reservoirs and bromeliad and *Heliconia*

phytotelmata). However, we guess that the pH tolerance of the isolates permits these bacteria to thrive with the phytotelmata water fluctuations and thus the change in pH of this environment. The isolate BR0C11, isolated from bromeliad phytotelmata is able to grow up to a pH of 11.5, despite that its optimum pH for growth is 5.0. When compared to the described *Rhodopseudomonas* species, the isolate BR0C11 has the lowest pH optimum of 5.0. It is important to mention that a precipitate in the media was observed at pH above 7.5. This precipitate increased with pH. Therefore, it is unknown if the ability of the isolates to grow at higher pH is inhibited by the increasing pH or by the precipitation of components in the media.

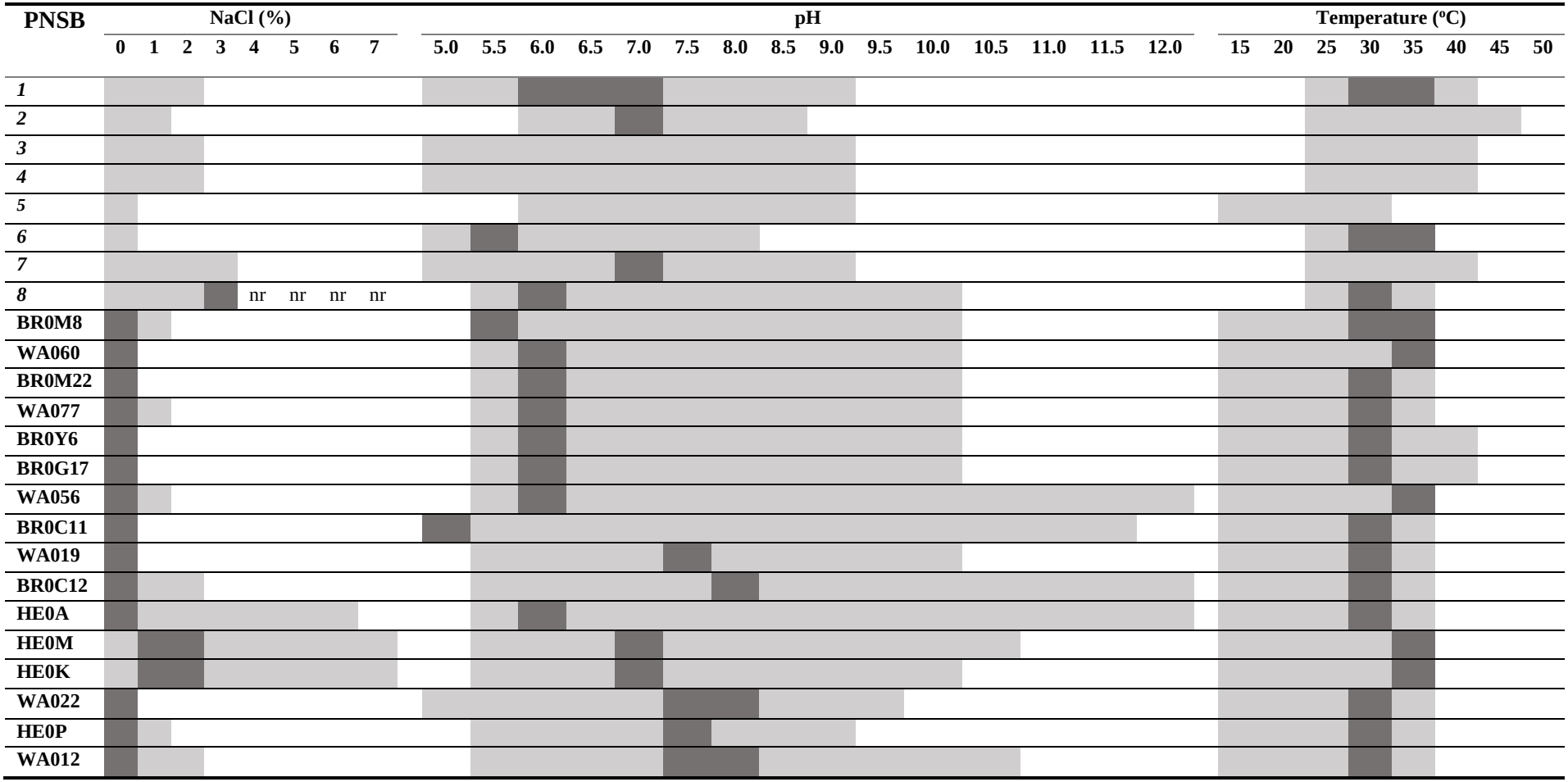
In terms of temperature, all of the studied isolates have a different temperature range from previously described species of *Rhodopseudomonas* and *Rhodobacter* (Table 2.1). All of our isolates were able to grow at temperatures as low of 15 ^{o}C. The only known *Rhodopeudomonas* known to be able to grow at 15 ^{o}C is *Rps. pseudopalustris* (Hiraishi et al., 2017). These differences in growth temperature ranges between our isolates and described species of *Rhodopseudomonas* and *Rhodobacter* suggest that the isolates could be new species of PNSB.

The differences in NaCl, pH, and temperature parameters for optimal growth of the isolates as compared to similar described species of PNSB suggest that these isolates could represent new species of PNSB. Moreover, these organisms provide an insight into the physiology of organisms inhabitant of the water reservoirs and bromeliad and *Heliconia* phytotelmata. In an effort to contrast and compare the physiological characteristic of the isolates with their environment of origin we found that studies addressing the physicochemical characteristics of Bromeliad or *Heliconia* phytotelmata are almost inexistent. It is known that organisms in phytotelmata are subject to quick changes in pH, temperature and solute concentrations (Jalinsky et al., 2014). This

could explain the wide range of pH and temperature tolerance from the PNSB isolated from bromeliad phytotelmata.

Also, these results provide an insight about the microorganisms that inhabit the phytotelmata as a source of enzymes that can withstand different pH, salt concentrations and temperatures, which are highly valuable for industrial processes (Sánchez-Porro et al., 2003). Due to the high yield, low production costs and safety of microbial enzymes processes, these enzymes are widely used in the industry specially for food and detergent industrial processes (Sarethy et al., 2011). In a previous study, 90 % of bacteria isolated from bromeliad phytotelmata had the enzymatic activity for the hydrolysis of at least one or more of the followings: casein, gelatin, starch and cellulose hydrolysis (Carmo et al., 2014). Because of this, we strongly recommend to explore the capabilities of the isolates not only for industrial applications but also for other biotechnological applications in bioremediation and the biomedical field (Chapter 3). The lack of studies about bacterial communities in phytotelmata (Chapter 1), and the physiology of our isolates, evidence the necessity of more studies to understand the inhabitants of these environments and their possible biotechnological potential.

Table 2.2: NaCl, pH and temperature range and optima of PNSB isolates and members of the genera *Rhodopseudomonas* and *Rhodobacter*.



1- *Rps. telluris*, 2- *Rps. faecalis*, 3- *Rps. harwoodiae*, 4- *Rps. palustris* ATCC17001, 5- *Rps. pseudopalustris*, 6- *Rps. rhenobacencis*, 7- *Rps. thermotolerans* and *Rps. pentothenatexigens*, 8- *Rba. johrii*. Gray, white and black spaces represent growth, no growth, and optimum, respectively. The data of the described strains (1-8) were obtained from published descriptions (Hougardy et al., 2000; Demin et al., 2002; Girija et al., 2010; Venkata Ramana et al., 2012; Vinay Kumar et al., 2013a; Hiraishi & Okamura, 2017). nr- Data were not reported in the description of the species.

2.5.3 Nitrogen, Sulfur and Vitamin Requirements

In terms of nitrogen sources (Table 2.2), 6 of the isolates grew without any added nitrogen source to the media. It is well known that some members of the PNSB are able to fix nitrogen when no other nitrogen source is available. *Rhodopseudomonas palustris* (Larimer et al., 2004; Oda et al., 2005), *Rps. rhenobacensis*, and *Rps. harwoodiae* (Hougardy et al., 2000) are some examples of *Rhodopseudomonas* strains able to fix nitrogen. Moreover, *Rhodopseudomonas palustris* is the only known bacteria, in addition to *Azotobacter* sp., with genes encoding for 3 different nitrogenases (Larimer et al., 2004). The isolates that do not exhibit diazotrophic growth were able to use one or more of the following compounds as a nitrogen source: NH_4Cl, aspartate, urea, and glutamate. The isolates, HE0M and HE0K, isolated from *Heliconia* phytotelmata and belonging to the genus *Rhodobacter*, were not able to use any of the tested compounds as nitrogen sources. Contrary the isolates, *Rba. johrii*, the PNSB most related to these bacteria in terms of 16SrDNA, can utilize NH_4Cl and glutamate as a nitrogen source. These differences in nitrogen sources usage between the isolates and *Rba.johrii* could be because are different species.

In terms of sulfur sources (Table 2.2), 9 of the isolates belonging to the genus *Rhodopseudomonas* were able to use sulfate, thioglycolate, thiosulfate, and sulfide. This was expected since this sulfate usage profile was previously reported for other members of *Rhodopseudomonas* (i.e. *Rps. thermotolerans, Rps. pentothenatexigens, Rps. harwoodiae,* and *parapalustris*) (Ramana et al., 2012; Kumar et al., 2013b). However, WA12, also from the genus *Rhodopseudomonas*, did not use any of the sulfur sources tested. This suggests that this isolate can be a new species of the genus *Rhodopseudomonas*.

In terms of vitamin requirements, it was found that all the 14 isolates belonging to the genus *Rhodopseudomonas* were able to grow without the addition of vitamins or growth factors in

the media (Table 2.2). This was not expected since well-known and common characteristics among most PNSB are their vitamin and growth factor requirements. This is the reason why most of the media developed for the isolation and culture of these organisms have as part of its constituents vitamins or yeast extract as a source of growth factors (Hunter et al., 2009). Bacteria in the genus *Rhodopseudomonas* require one or a combination of growth factors such as yeast extract, p-aminobenzoic acid, thiamin, etc. for growth (Hougardy et al., 2000; Shuang-jiang, 2017; Ramana et al., 2012; Kumar et al., 2013b). Moreover, *Rps. pentothenatexigens* was named due to its pantothenate (Vitamin B5) requirements for growth (Kumar et al., 2013a). The only described species of *Rhodopseudomonas* which not requires growth factors is *Rps. pseudopalustris*, isolated from surface water and mud (Hiraishi et al., 2017). The lack of growth factor requirements by the isolates and the differences with *Rps. telluris* in terms of carbon, nitrogen and sulfur sources suggest that these bacteria could be new species of *Rhodopseudomonas*. The isolates HE0M and HE0K, related to the genus *Rhodobacter*, were found to require biotin as a growth factor. This was expected since other members of this genus, such as *Rba. johrii* and *Rba. vinaykumarii*, required biotin as a growth factor (Girija et al., 2010).

While we found some similarities in terms of carbon, sulfur, nitrogen or growth factor requirement between our isolates and some of the described species of *Rhodopseudomonas* or *Rhodobacter* none of our isolates had the same usage combination as the described species. These differences suggest that our isolates could be novel species of PNSB.

2.5.4 Purple Non-Sulfur Bacteria Isolates Photolithiotrophy, Chemolithioautotrophy and Fermentative Mode of Growth

Photolithoautotrophy by the isolates with Na_2S (0.5 mM), $Na_2S_2O_3$ (2 mM) and $NaHCO_3$ (0.1%) in the media was not observed in any of the isolates. This mode of growth is not common among species of *Rhodopseudomonas*. Only *Rhodopseudomonas palustris* and *Rhodopseudomonas faecalis* are able to fix CO_2 with an inorganic electron donor such as hydrogen, thiosulfate or sulfide (Demin et al., 2002; Larimer et al., 2004). In addition, to the inability of the isolates to grow under photolithoautotrophic conditions, none of the isolates could grow as chemoautotrophs either. The only species of *Rhodopseudomonas* for which chemoautotrophy has been reported is *Rhodopseudomonas palustris* (Larimer et al., 2004). In terms of fermentation, none of the previously described members of *Rhodopseudomonas* can grow under this metabolic mode of growth (Hougardy et al., 2000; Shuang-jiang, 2017; Ramana et al., 2012; Kumar et al., 2013b). However, our isolates belonging to the genus *Rhodopseudomonas* grew with glucose and pyruvate under anaerobic conditions in the dark. This ability to ferment suggests that these bacteria could be novel species of PNSB.

Table 3.2: Nitrogen and sulfur usage of PNSB isolates and species of the genera *Rhodopseudomonas* and *Rhodobacter*.

Purple Non-Sulfur Bacteria	Nitrogen source				Sulfur source				
	Diazotrophic growth	Ammonium chloride	Aspartate	Urea	Magnesium sulfate	Ammonium sulfate	Thioglycolic acid	Sodium thiosulfate	Sodium sulfide
Rps. telluris	+	+	nr	+	nr	nr	nr	nr	nr
Rps. pentothenatexigens	nr	+	+	-	+	+	+	+	+
Rps. thermotolerans	nr	+	+	-	+	+	+	+	+
Rps. harwoodiae	+	n/a	n/a	n/a	+	+	+	+	+
Rps. parapalustris	-	+	+	-	+	+	+	+	+
Rps. pseudopalustris	nr	nr	nr	nr	nr	nr	nr	nr	nr
Rps. palustris	+	n/a	n/a	n/a	n/a	n/a	n/a	nr	n/a
Rps. faecalis	+	n/a	n/a	n/a	+	+	nr	nr	nr
Rps. rhenobacensis	+	n/a	n/a	n/a	nr	nr	nr	-	nr
Rhodobacter johrii	nr	+	nr	nr	nr	nr	nr	nr	nr
BR0M8	-	+	+	-	+	+	+	+	+
WA060	+	n/a	n/a	n/a	+	+	+	+	+
BR0M22	-	+	+	+	+	-	+	+	+
WA077	+	n/a	n/a	n/a	+	+	+	+	+
BR0Y6	-	+	+	+	+	+	+	+	+
BR0G17	-	+	+	-	+	+	+	+	+
WA056	-	+	+	+	+	+	+	+	+
BR0C11	-	-	+	-	-	+	+	+	+
WA019	-	+	+	-	+	+	+	+	+
BR0C12	+	n/a	n/a	n/a	-	+	+	+	+
WA0A	+	n/a	n/a	n/a	-	+	+	+	+
HE0M	-	-	-	-	-	-	-	-	-
HE0K	-	-	-	-	-	-	-	-	-
WA022	+	n/a	n/a	n/a	+	+	+	+	+
HE0P	-	+	+	-	+	+	+	+	+
WA012	+	n/s	n/a	n/a	-	-	-	-	-

The symbols + and - represent growth and no growth, respectively. The data of the described strains were obtained from published descriptions (Demin, Zhang Huifang, Yang Zhiyong, 2002; Girija et al., 2010; Hougardy et al., 2000; Venkata Ramana et al., 2012; Vinay Kumar et al., 2013a; Hiraishi & Okamura, 2017). NR-Data were not reported in the description of the species.

1

2.5.5 Motility, Gram and Endospore Stain

All members of the genus *Rhodopseudomonas* are motile by polar flagella (Hougardy et al., 2000; Shuang-jiang, 2017; Ramana et al., 2012; Kumar et al., 2013b). Motility is also observed in some species of *Rhodobacter*, all with polar flagella (Girija et al., 2010). It was found that all the PNSB isolates were motile. Although Leifson Flagella Stain was performed to identify the type of flagella in the isolates, it was not possible to obtain clear results. Thus, in order to determine the type of flagella in the isolates, Electronic Microscopy after negative stain is recommended. This method has been used previously and clear results about the presence and position of the flagella had been obtained (Hougardy et al., 2000; Shuang-jiang, 2017).

In terms of Gram Staining, it was found that all the isolates were Gram-negative (Figure 2.2). These results were expected since all PNSB that have been described are Gram-negative. In terms of morphology, the 14 isolates belonging to the genus *Rhodopseudomonas* were rods and the two isolates belonging to the genus *Rhodobacter* have an ovoid cell morphology. The cell morphology of the isolates was also confirmed by Scanning Electron Microscopy (SEM) (Figure 2.3). These results agree with literature since previously described *Rhodopseudomonas* spp. were rods with the exception of *Rhodopseudomonas faecalis* with a vibrio cell morphology (Demin et al., 2002). Also, the ovoid morphology of the isolates confirms that these bacteria are members of the genus *Rhodobacter* since cell morphology of described species in this genus ranges from rods, spherical, rod-shaped to ovoid cells.

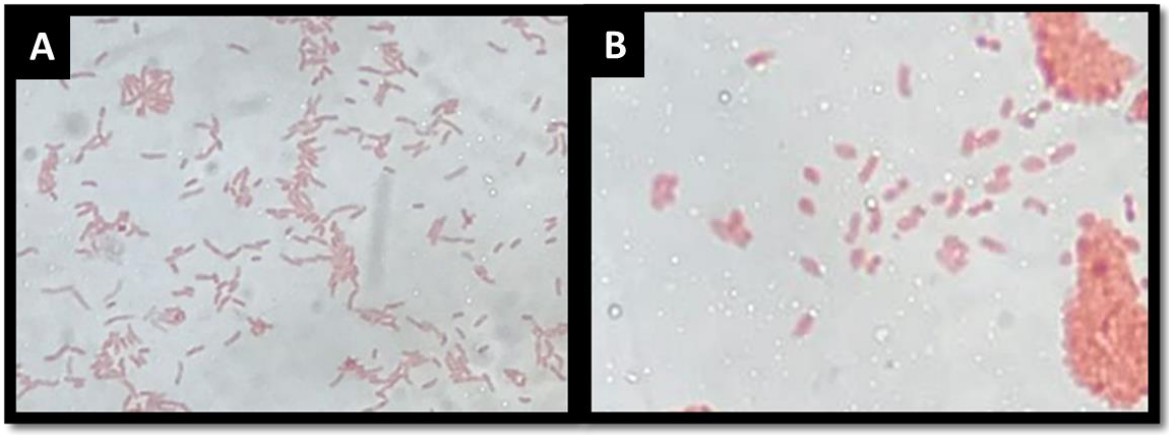


Figure 2.2: Gram Stain Results of PNSB isolates. As previously described PNSB species, all of the isolates were Gram-negative. A: Gram-stain results of the isolate BR0M22 (isolated from bromeliad phytotelmata). BR0M22 and all the other isolates related to the genus *Rhodopseudomonas* were Gram-negative bacilli. B: Gram stain of isolate HE0K (isolated from *Heliconia* phytotelmata). This bacteria and isolate HE0M, also from *Heliconia* phytotelmata and belonging to the genus *Rhodobacter*, have Gram-negative ovoid cells.

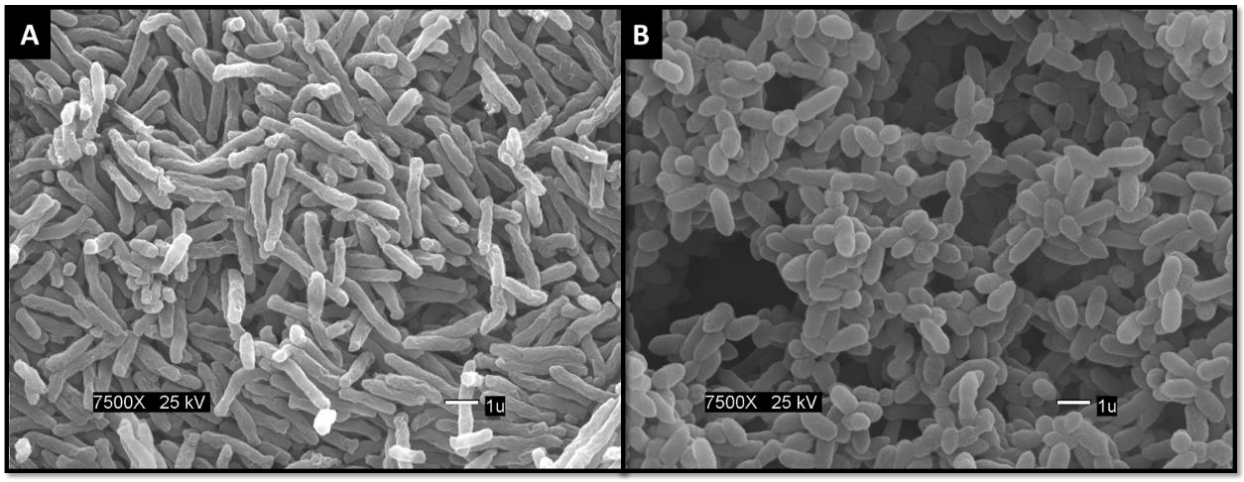


Figure 2.3: Scanning Electron Microscopy of PNSB isolates. A: The cells of the isolates belonging to the *Rhodopseudomonas* genus where rods. Mean cells average length and width were 2 µm and 0.4 µm, respectively. B: Isolates belonging to the genus *Rhodobacter* had ovoid cells with a mean length and width of 1.3 µm and 0.5 µm, respectively.

In terms of endospore formation, the 14 isolates related to the genus *Rhodopseudomonas* are not endospore producers as all described species in this genus. It was believed that members of the PNSB were not endospores producers. However, in 2010, *Rhodobacter johrii*, the first known endospore producer PNSB was described (Girija et al., 2010). This bacterium was isolated in India, specifically from a pasteurized wet rhizosphere soil sample where sorghum (*Gramineae* family) was cultivated. From the 16 PNSB of this study, HE0M and HE0K, isolated from *Heliconia* phytotelmata, belonging to the genus *Rhodobacter,* and most similar to *Rhodobacter johrii* by 16SrDNA, were endospore producers. Until our study, there is no other report about the isolation or description of endospore-forming PNSB from other environments. Therefore, this is the second study that reports the existence of PNSB endospore producers.

Even thou HE0M and HE0K are similar to *Rba. johrri* in terms of physiology, DNA-DNA hybridization or ANI is recommended to confirm the species of these isolates. If this is confirmed, then it will be the second record of the isolation of *Rba. johrii*.

Understanding the aspects that support the growth of *Rba. johrii* in these environments (i.e. rhizosphere soil and *Heliconia* phytotelmata) would be interesting since it could provide information about common factors between these environments and mechanisms related to endospore formation in this only known endospore producer PNSB. However, since there is no additional information about the physiochemistry of the rhizosphere soil sample from which *Rba. johrii* was isolated, in addition to the fact that *Heliconia* phytotelmata studies are scarce, we were unable to compare these environments.

2.5.7 Orthologous Average Nucleotide Identity (OrthoANI)

Since 16SrDNA similarities between the isolates and described species of PNSB were above 97%, OrthoANI was performed to determine if the isolates were new species of this group

(Imhoff et al., 2004). To our knowledge, all previous PNSB descriptions have been based on traditional DNA-DNA hybridization. Therefore, this is the first study that an in silico technique instead of DNA-DNA hybridization was used for the identification and description of PNSB species.

It was found that OrthoANI values among the PNSB isolates (WA056, BR0C11, BR0G17, and BR0M22) and the *Rhodopseudomonas* spp. are below 93% (Figure 2.4). Using the ANI value threshold of 97% for species delimitation, it was found that these isolates are not *Rhodopseudomonas palustris*. This bacteria was more similar to isolates BR0C11 and BR0G17 by anayses of phylogenetic relationships based on 16SrDNA (Section 2.5.1). Another important finding was that OrthoANI values below 97% were also found among the used reference genomes reported as belonging to *Rps. palustris*. Only *Rps. palustris* CGA009 and *Rps. palustris* TIE-1 genomes have an OrthoANI value > 97%. This is not the first time that organisms in culture collections deposited as *Rps. palustris* has been found to be different species. For example, a recent study concluded that *Rps. palustris* ATCC17005 phenotypic and genotypic characteristics are different from *Rps. palustris* ATCC1701 and other *Rhodopseudomonas spp.* (Hiraishi et al., 2017). These errors in classification may be due to the techniques employed earlier were phenotypic properties were only taken into account for the description of bacteria. Also, Hiraishi and Okamura (2017) did not find any phylogenetic or phenotypic difference between *Rps. pentotenatexigens* and *Rps. thermotolerans*. These results are consistent with our 100% OrthoANI value (not shown in Figure 2.4) between the genomes of these bacteria, which means both are the same species. For this reason, we propose that whole genome sequencing should be part of the description of new species of bacteria. The availability of these sequences in public databases, will help to accelerate

the description and study of prokaryotes by using genome relatedness indices algorithms such as OrthoANI.

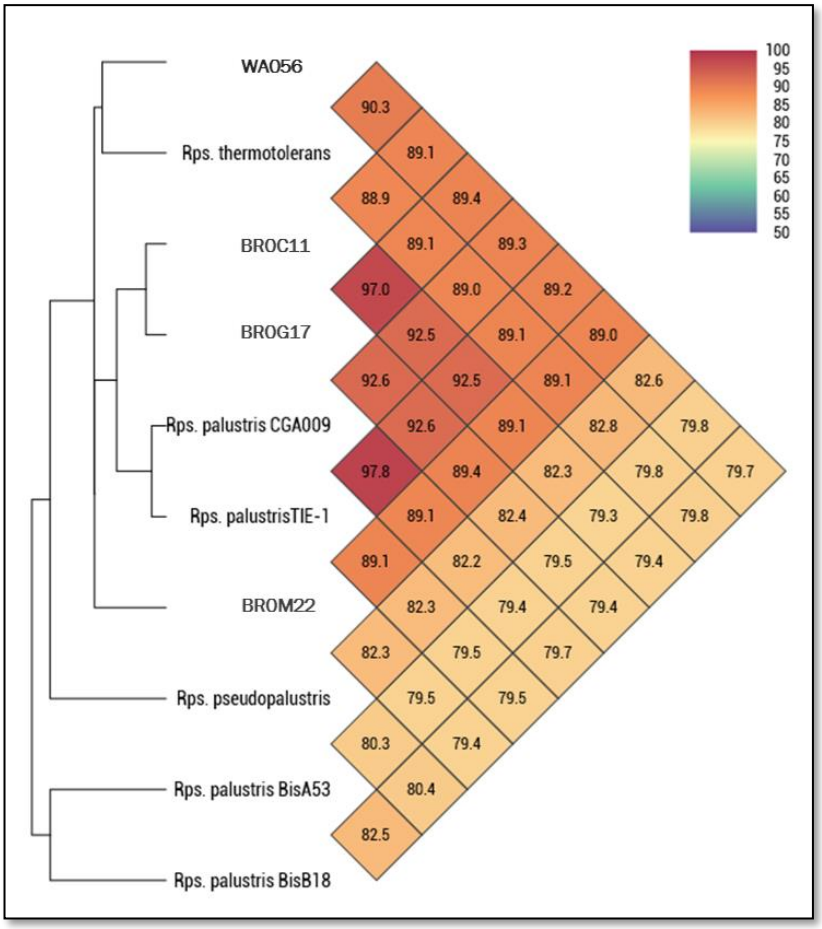


Figure 2.4: Heatmap generated with OrthoANI values calculated from the OAT software. As depicted in the figure, the OrthoANI values between the isolates and described *Rhodopseudomonas* spp. are below 93%. This means that these isolates are different and not the species *Rhodopseudomonas palustris*. Also, it was found that the PNSB isolates BR0C11 and BR0G17 are the same species.

In terms of the isolates BR0C11 and BR0G17, it was found that OrthoANI values were above 96% between their genomes. Regardless of phenotypic differences, we conclude that these bacteria are the same species and probably different subspecies. To elucidate the relationship of these bacteria at the subspecies level, additional genome analysis such as pan-genome analysis should be performed (Fouts et al., 2012). However, these isolates are different species from *Rhodopseudomonas palustris,* sharing OrthoANI values below 94% with *Rps. palustris* strains.

Regarding the isolates WA056 and BR0M22, we can also conclude that these are not *Rps. palustris*; however, it is mandatory to analyze genome relatedness of these isolates with *Rps. harwoodiae*. At the time of this study, *Rps. harwoodiae* was not available from neither of the culture collections where it was deposited. The KCTC type strain was not available for selling at the time of our request and the NBRC type strain was also not available due to contamination. Because of the absence of *Rps. harwoodiae* type strain in any public culture collection, we were not able to perform genomes relatedness analysis between this bacterium and our isolates. The presence of these contaminants in *Rps. harwoodiae* type strains were also reported by Hiraishi and Okamura (2017). Also, inconsistencies in phenotypic results of this strain from the original description were reported; therefore, an amended description will be published soon (Hiraishi et al., 2017).

2.6 Conclusion

Taking into consideration all the results of the polyphasic approach used in this study for the identification of PNSB isolates, we conclude the following:

1- The isolates HE0M and HE0K isolated from the phytotelmata of *Heliconia,* are related to the genus *Rhodobacter*. The morphology and physiology are similar to *Rba. johrii*, the first described endospore-producer phototrophic proteobacterium (Girija et al., 2010). Moreover, the fact that the isolates HE0M and HE0K were endospore producers strongly suggest that these bacteria are *Rhodobacter johrii*.

2- The isolates BR0C11 and BR0G17 were not *Rhodopseudomonas palustris* in terms of genome relatedness. However, we were unable to conclude that are not different species since the available description of *Rps. palustris* was invalidated by Hiraishi and Okamura (2017). In order to describe these organisms as new species of *Rhodopseudomonas,* we would need the

amended description of *Rhodopseudomonas* spp. In addition, genome relatedness with other *Rhodopseudomonas* species has to be done since 16SrDNA analysis was over 97%.

3- Because of the differences found in the physiology (i.e carbon, nitrogen and sulfur usage, pH and temperature ranges, NaCl requirements etc.) and OrthoANI values (i.e. below the threshold of 96% for species delimitation) between the isolates WA056 and BR0M22 we were able to conclude that these isolates are not *Rps. palustris*. In order to identify the species of these isolates it is necessary to perform genome relatedness analysis such as OrthoANI between WA056, BR0M22, and *Rps. harwoodiae,* since 16SrDNA similarities between these bacteria, is over 97% (Imhoff et al., 2004; Auch et al., 2010).

In addition, it is very important to address the challenges with the parameters used for the description of species. It seems that the recommended standards are not aligned with the development of technology. For this reason, we urge the International Committee on the Systematics of Prokaryotes to reevaluate the criteria used for the description of PNSB. We also encourage the establishment of detailed methodology and standardization of experiments for the analysis of these organisms. This would minimize errors in the taxonomic analysis which contribute to the lack of understanding and taxonomical information about these group of bacteria.

Chapter 3: Biotechnological Potential of Purple Non-Sulfur Bacteria (PNSB) Isolated from Aquatic Environments in Puerto Rico

3.1 Introduction

According to the United States Census Bureau (2016), 9 billion people will be inhabiting Earth by 2050. This overpopulation represents a challenge due to the lack of natural resources and, thus, the potential inability to sustain the population and natural reserves. Also, the world economy is based on oil, an unrenewable resource that will be depleted during the next 30 to 40 years causing eventually food shortage by the use of food crops to produce biofuels (Pimentel, 2012; Sakpirom et al., 2016). Therefore, there is an urgency to find other sources of energy as soon as possible. Also, the management of crude oil is bound to spills, which contaminates soils and water bodies, resulting in disastrous effects on the ecosystems.

In addition to the depletion of natural resources, the overpopulation also increases the amount of waste produced worldwide. In 2013, the amount of solid waste generated in the United States was 254 million tons, of which only 34.3% was recycled or composted (EPA, 2016). Another aspect that is negatively impacted by overpopulation is health. In 2014, the World Health Organization (WHO) officially declared that as an effect of antibiotic overuse and misuse, antimicrobial resistance is a current threat. Furthermore, it was acknowledged that most antibiotics available today have lost their effectiveness due to the development of antimicrobial resistance by bacteria. In 2013, the Center for Disease Control and Prevention (CDC) estimated that each year 2 million people are infected by antibiotic-resistant bacteria of whom 23,000 dies of these infections.

Diverse efforts had been employed to find eco-friendly alternatives to overcome the challenges and effects of the continuously growing population. One of the alternatives is biotechnology or the use of biological entities or bioproducts to solve a problem. For example, phytoremediation, bioremediation, and phycoremediation have been explored with promising

results (Kensa, 2011; Luo et al., 2016; Ansari et al., 2017; Ijoma et al., 2017; Sanz-Fernández et al., 2017). Thus, there is an urgency to study new organisms with biotechnological potential and with the ability to cope with different environmental conditions.

In previous studies, members of the PNSB bacteria have shown promising biotechnological applications in the biomedical field, bioremediation and biofuels production (Sasikala et al., 1995; Vincenzini et al., 1997; Larimer et al., 2004; Basak et al., 2007; Panwichian et al., 2010; Merugu et al., 2012). The versatility of Purple Non-Sulfur Bacteria in terms of their metabolism and physiological diversity (Chapter 1 and 2) makes them promising in biotechnological applications. There is one study on the presence of *Rhodobacter* sp. in a swine waste lagoon and its relationship with the decrease in the volatile organic compounds concentration such as acetic acid, propionic acid, butyric acid, valeric acid, phenol, among others (Do et al., 2003). Also, members of PNSB have been found consistently growing favorably in polluted environments where organic matter predominates (Belila et al., 2013).

Although the potential of some PNSB for biotechnological applications is known, most of the studies nowadays are focused on PNSB hydrogen production and its optimization. It is necessary to explore other biotechnological potentials of PNSB and their application to overcome the challenges linked to overpopulation. Also, in Puerto Rico PNSB diversity or biotechnological potential have not been fully explored. There is only one study, also from the Microbial Biotechnology and Bioprospecting (MBB), where it was reported that PNSB isolates use biodiesel as the sole carbon source under anaerobic conditions (Vega-Sepúlveda, 2009). This confirms the necessity of research about PNSB ecosystems of Puerto Rico. These studies can result in the discovery of novel members of PNSB and, therefore, new opportunities for biotechnological applications and industrial development in Puerto Rico.

Because of the lack of studies in Puerto Rico about PNSB and thus their biotechnological potential, in this chapter we explored the biotechnological potential of the PNSB isolates for possible use in metal bioremediation, biofuels production, hydrocarbon degradation and as producers of antimicrobial substances.

3.2 Literature Review

3.2.1 Biotechnological Potential and Applications of Purple Non-Sulfur Bacteria (PNSB)

Due to PNSB widespread distribution in nature and high growth rate by the utilization of different organic sources, these phototrophic bacteria have been proposed strongly as suitable organisms for bioremediation and other biotechnological applications (Idi et al., 2015). In addition, unlike most organisms used in bioremediation, PNSB could growth under anaerobic conditions, therefore their use is not limited to aerobic zones. In addition to oxygen concentrations, another factor that limits bioremediation is the possible production of toxic substances by the microorganisms employed (Vidali, 2001). PNSB, on the contrary, produce non-toxic byproducts such as biopolymers, biofuels, carotenoids, vitamins, and hydrogen when used in bioremediation processes (Idi et al., 2014). For these reasons, PNSB has been proposed as good candidates for waste water treatment, heavy metal removal, and biomedical applications among many others (Idi et al., 2014).

3.2.2 Purple Non-Sulfur Bacteria (PNSB) and Metal Bioremediation

The presence of metals in the environment is of natural occurrence with concentrations depending on the geology of the site. However, because of human activities, non-natural toxic metal concentrations are frequently found, being aquatic environments the most impacted. The presence of these pollutants in water sources leads to health hazards because of their accumulation along the food chain. The United States Environmental Protection Agency (EPA) has established

different regulations to control drinking water contamination by metals. For example, the Lead and Copper Rule (LCR) established in 1991 aims to reduce drinking water contamination by these metals.

Different physicochemical techniques have been employed for the removal of metals from the environment, such as oxidation-reduction, filtration, chemical precipitation, evaporation, ion-exchange, reverse osmosis, and electrochemical treatment (Buccolieri et al., 2005). However, most of these methods are expensive, the employed materials are not durable and in most cases result in toxic byproducts. As a result, bioremediation had been proposed and used as a tool to remove heavy metals from soils and water. Different organisms have been used for heavy metals bioremediation such as plants (phytoremediation), fungi (mycoremediation), algae (phycoremediation) and bacteria (Luo et al., 2016; Malaysiana et al., 2016; Kwarciak-koz, 2014).

Different general mechanisms have been proposed and identified in bacteria providing these organisms with the ability to survive in the presence of metals in the environments. These mechanisms are metal exclusion by permeability barrier, active transport of the metal, intracellular sequestration by protein binding, extracellular sequestration, the transformation of metal to a less toxic form by enzymes and reduction of metal sensitivity of cellular targets (Bruins et al., 2000). Different studies have been done about metal resistance by members of the PNSB and the capability for these bacteria to remove these contaminants is known (Idi et al., 2015). For example, it was found that *Rhodobacter sphaeroides* R26.1 has tolerance to the ions Ni^{2+}, Co^{2+}, CrO_4^{2-} and MoO_4^{2-} (Buccolieri et al., 2005). Also, *Rhodobacter sphaeroides* was able to remove 97.92% and 97.76% of Cd and Zn, respectively, from waste water and was successfully used in bioremediation of lead-contaminated soils (Li et al., 2016 a-b). In addition, *Rhodobacter sphaeroides* KMS24 and *Rhodobium marinum* NW16 were able to grow in the presence of Cu^{2+}, Pb^{2+}, and Zn^{2+} (Panwichian

et al., 2011). In another study, *Rhodobacter capsulatus* was able of zinc biosorption from the media (Magnin et al., 2014; Li et al., 2016a). Chromosome analysis of this bacterium showed the presence of *znt*A1 and *znt*A2 genes which encode for a P-type ATPase involved in Zn efflux. Moreover, a plasmid of 130 kb that enhance the biosorption of Zn but is not required for Zn resistance was also found in this bacterium (Magnin et al., 2014). In another study, *Rhodopseudomonas palustris* was used to recover gold from wastewaters by biosorption (Colica et al., 2012). At the end of this experiment, gold was recovered after incineration of the cells. In a more recent study, members of PNSB reduced the concentration of copper, lead, cadmium, and zinc from metal contaminated shrimp ponds up to 20, 39, 7, and 5%, respectively (Panwichian et al., 2010).

In Puerto Rico, during EPA 2016 Puerto Rico Quality Assessment, the presence of copper, lead and mercury was reported in water bodies in Puerto Rico, some with intended use for recreation and drinking water supply (EPA, 2017). Copper and lead reached drinking water supplies by erosion of natural deposits and corrosion of plumbing systems, while industrial discharges have been identified as sources of mercury in drinking waters (EPA). For this reason, EPA has established maximum contaminant levels (MCL) for copper, lead, and mercury in drinking water as 1.3, 0.015, and 0.002 mg/L, respectively (https://www.epa.gov/ccl).

3.2.2.1 Copper in the Environment and Purple Non-Sulfur Bacteria (PNSB)

Copper is a reddish solid heavy metal with a density of 5 g/cm^3 and an atomic number of 29. It has three oxidation states: Cu^o, Cu(I) (reduced state) and Cu(II) (oxidized state) (Flemming et al., 1989). Because of its properties such as malleability and conductivity, copper has been used for construction, power generation, electronic devices, wiring, plumbing systems, telecommunications devices, heating and cooling systems (Doebrich, 2009). Although copper is found naturally in the environment at approximately 50 ppm in soil (ATSDR, 2004), its demand

is causing a rise in environmental copper concentrations and consequently a threat to ecosystems. In 2000, approximately 6.4 x 10^8 kg of copper were released into the environment by industries (ATSDR, 2004), ending in water bodies used for recreation, fishing and drinking water. In addition to EPA, different studies have reported the presence of this metal in different water bodies in Puerto Rico such as estuarine lagoons, like Jobos and Guanica Bay (Acevedo-Figueroa et al., 2006; Aldarondo-Torres et al., 2010; Whitall et al., 2014).

Although copper has toxic effects, it is classified as an essential metal for its role as an enzyme cofactor or structural component, and, thus cell survival resides in its ability to maintain copper homeostasis. In humans, the Wilson Disease disorder is characterized by high copper concentrations that lead to hepatic failure, neurological defects and psychiatric symptoms (de Bie et al., 2007). On the other hand, Menkes disease, characterized by low concentrations of copper, is a lethal disorder leading to hypopigmented skin, aneurysm, bladder diverticula and cognitive underdevelopment (Tumer et al., 2010).

Bacteria have also developed different mechanisms to maintain copper homeostasis and survive under unfavorable copper concentrations (Figure 2.5). Three mechanisms have been identified in bacteria (Figure 3.1): 1) CopA- Copper Transport P-ATPase as in *Pseudomonas aureginosa,* 2) CusCFBA system- Multicomponent copper efflux system of the Resistance Nodulation Cell Division (RND) as in *E. coli* , and 3) CueO- Multicopper oxidase (MCO) also in *E. coli*. The copper transport ATPase CopA exports copper from the cytoplasm to the periplasm of the cell (Petercen & Moller, 2000), CusCFBA system forces out copper from the periplasm to the exterior of the cell, and CueO oxidizes Cu^+ to Cu^{2+} (less toxic form)(Bruins et al., 2000; Franke et al., 2003; Hobman et al., 2015) .

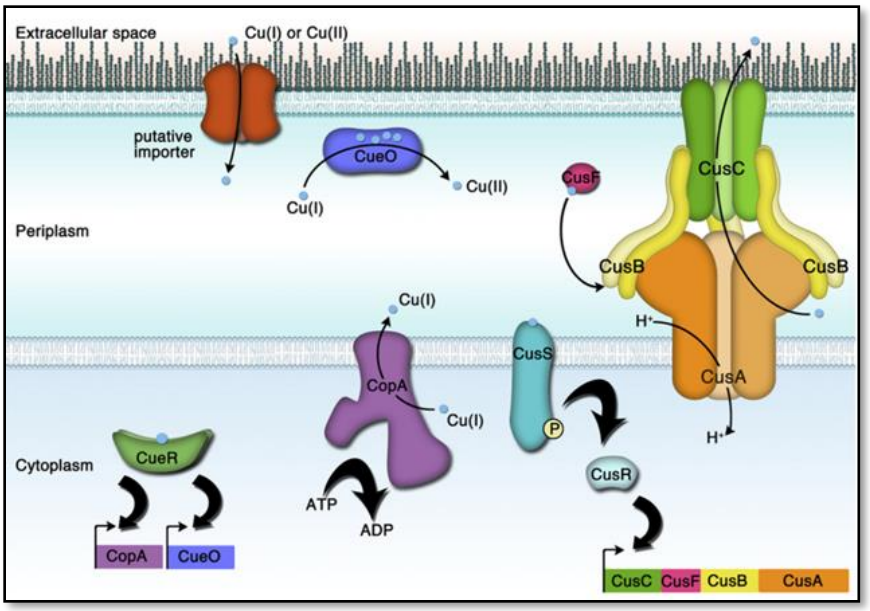


Figure 3.1: Copper Homeostasis Mechanisms. When copper is sensed by CusS in the periplasm, it phosphorylates CusR which activates the transcription of *cusCFBA*. The CusCFBA system force out copper from the periplasm to the exterior of the cell. CueR activates the transcription of *copA* and *cueO*. The copper transport ATPase CopA exports copper (Cu+) from the cytoplasm to the periplasm and CueO oxidise Cu^{+} to the less toxic form Cu^{2+}. Reprinted from "Metal Resistance and its Association with Antibiotic Resistance" by Pal et al., 2017, Advances in Microbial Physiology, 10, p.261. Copyright (2017) with permission from Elsevier.

Purple non-sulfur bacteria also have mechanisms for copper resistance; however, studies about these mechanisms are not well understood (Redemacher et al., 2012). In a previous study, it was found that *Rhodobacter sphaeroides* mutants for the *copA* gene were resistant to copper suggesting the presence of novel copper resistant mechanism (Peuser et al., 2011). In addition, *Rhodobacter sphaeroides* and *Rhodomicrobium marinum* produce exopolymeric substances (EPS) which bind copper allowing these bacteria to survive (Panwichian et al., 2011). Incidentally, because of the production of EPS and their ability to bind to heavy metals, these bacteria have been proposed as bioprospects for copper and other metals bioremediation.

3.2.2.2 Mercury in the Environment and Purple Non-Sulfur Bacteria (PNSB)

Mercury (Hg) is a liquid metal at room temperature with an atomic number of 80. Although mercury can be found naturally in the environment, most of its presence in the ecosystem is related to anthropogenic activities. In 2016, mercury mine production was estimated to be 4,500 metric tons with uses in the manufacturing of batteries, fluorescent lamps, thermometers, thermostats, amalgam for dental fillings, electronics, skin creams, and jewelry among many others (George, 2017). Exposure to mercury can result in brain, heart, kidneys, lungs and immune system damage, thus different efforts have been established to reduce its use and search for substitutes of this metal. For example, in 2013, the Minamata Convention on Mercury was established with the support of over 100 countries in order to reduce mercury pollution globally (Parties et al., 2013).

Most of the exposure to mercury is related to the ingestion of fish with high levels of methyl mercury, thus the abundance of this metal in aquatic ecosystems is of great concern (Tchounwou et al., 2003; EPA, 2017). Methyl mercury is the most common form of mercury found in ecosystems in addition to mercury in its elemental (Hg^0) and oxidized (Hg^{2+}) forms. As a result of its high affinity to sulfur atoms in the cysteine residues, methylmercury and Hg^{2+} interfere with the function and structure of proteins (Møller et al., 2013). In order to survive in the presence of organic and inorganic mercury compounds, bacteria have developed the ability to transform these compounds to Hg^0 which is volatile and less reactive (Figure 3.2) (Barkay et al., 2003). However, once in the environment, Hg^0 can be remethylated (Tchounwou et al., 2003). Bacteria are able of methyl mercury demethylation to Hg^0, due to the presence of the *mer* operon (Figure 3.2). The *mer* operon is found in bacteria such as *Shigella flexneri*, *Serratia marcescens*, and *Pseudomonas aureginosa* and consists of 5 to 6 genes: *merT*, *merC*, *merP*, *merB*, *merR*, *merA* (Bruins et al., 2000). The *merR* and *merT* genes encode for a trans-acting repressor/activation protein that

regulates the mer operon. *merC* encodes for a cytoplasmic membrane Hg^2 transport protein, *merP* for a periplasmic Hg_2 transport protein and *merB* for an organomercurial lyase enzyme. This enzyme cleavages C-Hg bonds of organomercury compounds resulting in Hg^2. Finally, *merA* encodes for the mercuric reductase which converts Hg^2 to Hg^0 (Kane et al., 2016; Tchounwou et al., 2003).

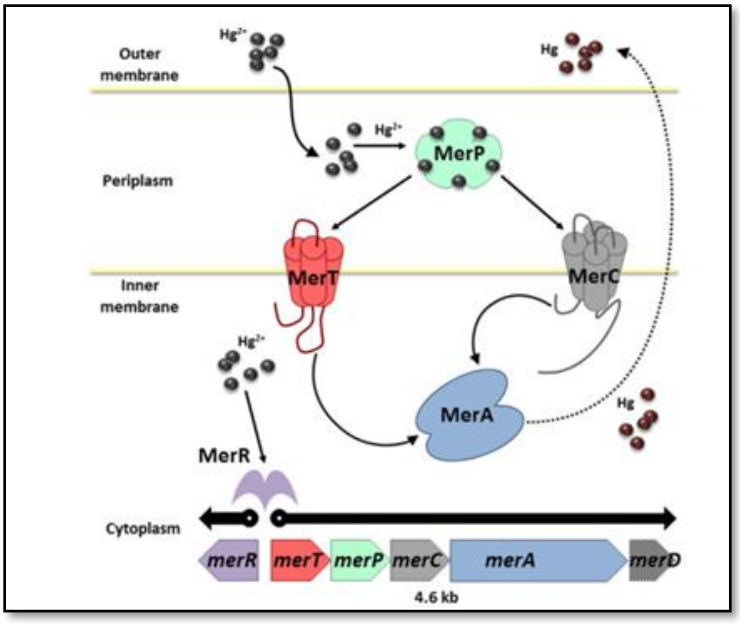


Figure 3.2: Gram-negative bacteria *mer* operon. Hg^{2+} enters into the periplasm and binds to MerP cysteine residues. Then the ions are passed to MerT or MerC for Hg^{2+} transport to the cytosol where it is reduced to Hg^0 by MerA. Hg^0 leaves the cell as mercury vapor. In response to ionic mercury, transcription of *mer* operon is activated by MerR and its regulated secondarily by MerD . Reprinted from "Metal Resistance and its Association with Antibiotic Resistance" by Pal et al., 2017, Advances in Microbial Physiology, 10, p.261. Copyright (2017) with permission from Elsevier.

PNSB mercury resistance studies are scarce. Although, in one of these studies a physiological role for mercury was reported for the first time. It was found that *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, and *Rhodopseudomonas palustris* were able to reduce Hg^2 to Hg^0. Also, this known toxic metal was used physiologically by these PNSB to maintain cell homeostasis by using Hg^2 as an electron acceptor (Grégoire et al., 2016). In another study, *Rhodovulum sulfidophilum* and *Afifella marina* were also able to reduce Hg^2 to Hg^0 (Mukkata,

2016). All of these studies confirm the need for more research to elucidate the potential diversity of mechanisms of mercury resistance in PNSB and their possible use in bioremediation.

3.2.2.3 Lead in the Environment and Purple Non-Sulfur Bacteria (PNSB)

As mercury and copper, lead is also found naturally in the environment, and its concentration in the environment is also increasing due to anthropogenic sources. In 2015 it was estimated a production of 4,710 metric tons of lead worldwide (Guberman, 2016). Lead can be found in fuels, paints, plumbing materials, batteries, and cosmetics, among many other products. Lead is not an essential metal and can bioaccumulate in the body causing neurotoxicity, kidney damage, anemia, coordination problems, short-term memory loss and death (Rensing et al., 1998; Li et al., 2016a; EPA, 2017). Contrary to copper or mercury, specific mechanisms related to Pb resistance by bacteria are not well studied. ZntA and CadA, which are P-type cation-translocating ATPases, have been identified as Pb(ii) pumps providing Pb resistance to some bacteria such as *Echerichia coli* and *S. aureus* (Rensing et al., 1998). The only known bacteria with specific Pb resistance genes or the *pbr* operon is *Cupriavidus metallidurans*, a beta-proteobacterium resistant to high concentrations of more than 20 metals (Janssen et al., 2010; Jarosławiecka et al., 2017). This *pbr* operon is regulated by PbrR; *pbr*A encodes for a Pb(ii)-efflux ATPase, *pbr*T for a Pb(II)-uptake protein, *pbr*B for a pro-lipoprotein, *pbr*C for a prolipoprotein signal peptidase, and *pbr*D for a putative Pb(II)-sequestration protein (Taghavi et al., 2009). In terms of PNSB, to our understanding, there is only one study of lead resistance by members of the PNSB where *Rhodobacter sphaeroides* was used for bioremediation of lead-contaminated soils (Li et al., 2016a). This also confirmed the necessity of research about the mechanisms involved in metal resistance by members of the PNSB and their capabilities and applications as bioremediation tools.

3.2.3 Hydrogen production by Purple Non-Sulfur Bacteria (PNSB)

In recent years, biohydrogen has been considered as a clean substitution for fossil fuels. It is renewable, environmentally friendly and many organisms are able to produce this biofuel in controlled environments contributing to higher yield (Wang et al., 2016a). PNSB have been extensively studied by their ability to produce hydrogen by photofermentation (Figure 3.3) (Muzziotti et al., 2016). *Rhodopseudomonas palustris, Rhodobacter capsulatus, Rhodobacter sulfidophilus* and *Rhodospirillum rubrum* are some examples of PNSB able to produce hydrogen (Tsygankov et al., 1998; Koku et al., 2002; Larimer et al., 2004; Basak et al., 2007). *Rhodobacter sphaeroides* is the most studied PNSB in the production of biohydrogen (Wang et al., 2016a). In this bacterium, hydrogen evolves as a byproduct of nitrogen fixation by the activity of the hydrogenases or nitrogenases, both active under anaerobic conditions (Basak et al., 2007; Muzziotti et al., 2016). Due to this capability of PNSB, recent efforts are geared towards the development and optimization of procedures for large scale hydrogen production (Tsygankov et al., 1998; Adessi et al., 2014; Mckinlay, 2014).

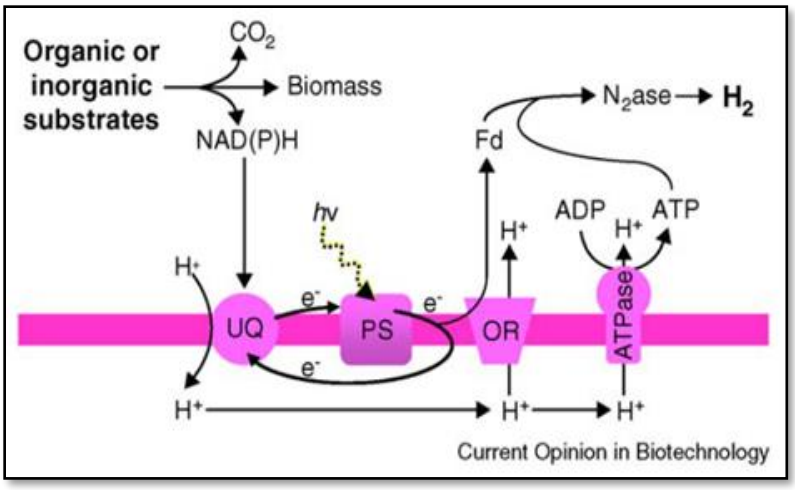


Figure 3.3: PNSB hydrogen production. Organic compounds are oxidized to CO_2 and biomass. Ubiquinone (UQ) transfers electrons to photosystem (PS) where they are energized by light. A proton gradient is produced due to repeatedly energized and cycled electrons through the photosynthetic electron transport chain. Energy from the proton gradient is used to transfer electrons from the photosynthetic electron transport chain to ferredoxin via oxidoreductases (OR) and also for ATP generation. Ferredoxin and ATP are then used for the generation of H_2 via nitrogenase (N_2ase). Reprinted from "Photobiological production of hydrogen gas as a biofuel" by J.B. Mc Kinlay and C.S. Harwood, 2010, Current Opinion in Biotechnology, 21, p.244. Copyright (2010) with permission from Elsevier.

In addition to the optimization of hydrogen production, a multiproduct procedure for hydrogen and the biopolymer poly-β-hydroxybutyrate (PHB) production has been explored in PNSB (Vincenzini et al., 1997). PHB is synthesized by these bacteria as carbon and energy storage compound under low concentration of oxygen, nitrogen, phosphate or sulfate and high concentrations of carbon source (Chen et al., 2012). Since PHB is biodegradable, it has great economic value as a substitute for petrochemical plastic (Koku et al., 2002; Khosravi-Darani et al., 2013). These products are also examples of the importance of PNSB and their biotechnological potential still to be discovered (Vincenzini et al., 1997). For this reason, it is necessary to explored different environments for the isolation of PNSB that could be used for this multiproduct procedure.

3.2.4 Hydrocarbon Degradation

It is estimated that 38 to 94 x 10^6 L of crude oil are spilled each year as a result of its transport, production, and storage; thus, threatening health, drinking waters, air quality and wildlife

(EPA, 2016). In marine ecosystems, ubiquitous aerobic bacteria play an important role in the degradation of hydrophobic molecules (i.e. saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes) which make up between 50 – 98 % of crude oil (Zhu et al., 2001; Kostka et al. 2011). These bacteria require oxygen to degrade hydrocarbons, consequently, their activities are limited to the water surface. However, hydrocarbons are also present in the subsurface where oxygen is limited due to its low solubility (Holliguer et al., 1997; Widdel et al., 2001). For this reason, the research of hydrocarbon degradation under anaerobic conditions by different species of bacteria such as *Tolumonas auensis*, and *Thauera aromatica* has been explored (Ramana et al., 2006; Widdel et al., 2001).

Due to its metabolic diversity and different usage of carbon sources for metabolism, PNSB are among the studied bacteria for hydrocarbon bioremediation. For example, the PNSB *Rubrivivax benzoatilyticus* is known to degrade a variety of hydrocarbons compounds such as benzoate, 2-aminobenzoate, 4-aminobenzoate, 4-hydroxybenzoate, 2-carboxybenzoate, cyclohexanone, among many others (Ramana et al., 2006). Similarly, *Rhodopseudomonas palustris* was able to degrade phenanthrene, a carcinogenic Polycyclic Aromatic Hydrocarbon (PAH) under anaerobic conditions (Zhao et al., 2011). This is a dramatic example since PAH's are difficult to degrade due to their structure, composed of multiple aromatic rings (Widdel et al., 2001; Zhao et al., 2011). Given that most of the hydrocarbons present in the subsurface are monocyclic aromatic compounds (e.g. benzene, toluene) and polycyclic aromatic hydrocarbons (PAHs) (Holliguer et al., 1997), there is an urgency to evaluate bacteria that could degrade such compounds and the creation of databases with such environmental conditions that will facilitate their degradation. The PNSB photocatabolic pathways involved in the degradation of aromatic compounds and PAHs still remains unknown (Sasikala et al., 1995; Idi et al., 2015).

3.2.5 Biomedical Applications of Purple Non-Sulfur Bacteria (PNSB)

PNSB have also valuable applications in the biomedical field (Sasikala et al., 1995; Sasaki et al., 2005; Merugi et al., 2012). Examples of molecules with biomedical value produced by members of the PNSB, but not limited to, are: vitamin B12, used in the treatment of anemia and as health supplement; coenzyme Q10, for the treatment of heart diseases and also food supplement, and porphyrin for the treatment of liver conditions (Pollich et al., 1995; Sasikala et al., 1995; Sasaki et al., 2005). *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* produce 5-aminolevulinic acid (ALA) used for cancer treatment and as a herbicide (Sasaki et al., 1987; Liu et al., 2014). Also, *Rhodobacter capsulatus* produces *cis*-vaccenic acid, an antiviral substance with activity against poliovirus, Sindbis virus, various fish viruses and coliphages without damage to the host cell (Hirotani et al., 1991). Similarly, *Rba. capsulatus*, *Rba. sphaeroides*, and *Rps. palustris* were able to produce an antimicrobial substance that inhibits the growth of the Gram-positive bacterium *B. subtillis* (Kaspari et al., 1977). Also, *Rba. sphaeroides* produces a thermoestable and intracellular compound able to inhibit the growth of *Vibrio harveyi* and *Allivibrio fischerii* (formerly known as *Vibrio fisherii*), known to be shrimp pathogens and thus, causing the source of substantial loses in the aquaculture industry (Chandrasekaran et al., 2011).

Despite the biomedical potential of the PNSB, actual studies are scarce. The last publication in which biomedical applications of this bacterial group were mentioned was Merugu et al. (2012). The reason for the lack of studies about the application of PNSB in the biomedical field could be related to the urgency in finding environmental friendly protocols to manage fossil fuel wastes which have been the topic in most and latest publications about PNSB biotechnological potentials. However, recent viral outbreaks, such as Ebola in 2014, Chikungunya in 2015, and Zika in 2016, and the emerging antimicrobial resistance by microorganisms, support the urgency of

finding new and accessible strategies for the development of new antivirals and antimicrobial substances such as antibiotics.

3.2.6 Biotechnological Potential of Purple Non-Sulfur Bacteria from Puerto Rico

As mentioned previously in previous Chapters, studies about PNSB in Puerto Rico are scarce. There is only one study about the biotechnological potential of PNSB isolates from bromeliad phytotelmata in Puerto Rico. In this study, the ability of such PNSB revealed that out of 26 PNSB isolates, 2 were capable of using biodiesel as a carbon source under anaerobic conditions. Phylogenetic analysis suggested that these isolates were novel genera of PNSB (Vega-Sepúlveda, 2009). Due to the scarce studies of PNSB, and biotechnological potential in Puerto Rico, our study will contribute knowledge about the applications of possible novel members of PNSB.

3.3 Objectives

The objective of this chapter was to explore possible biotechnological potentials of PNSB isolated from aquatic environments in Puerto Rico. Metal resistance to copper, mercury and lead, hydrogen and antimicrobial substances production, and hydrocarbon degradation (phenanthrene and naphthalene) under anaerobic conditions by the PNSB isolates were explored.

3.4 Methods

3.4.1 Resistance of PNSB to Copper, Mercury, and Lead

To determine if the isolates could grow in the presence of copper, mercury, and lead, media was prepared as described, in section 1.4, with the following compounds: 30 mg/L $CuCl_2$, 5 mg/L $HgCl_2$ and 60 mg/L $PbNO_3$ (Nies, 1999; Panwichian et al., 2010). The media was transferred to 2 mL- 96 wells plates with 500 µL working volume and 20 µL of a 4 d culture previously washed twice with 0.85% NaCl and adjusted to an absorbance of 0.020 at 600 nm. The plates were

incubated at 32 - 35 ºC under incandescent light for 5 d and cell growth was estimated at an absorbance of 600 nm. Also, the minimum inhibitory concentration (MIC) of the isolates was determined by increasing the tested metal concentration in the media by 5 mg/L increments until no bacterial growth was detected at 600 nm.

Genes related to copper and mercury resistance among the PNSB isolates, were detected by amplifications of copper P-ATPases (*cop*-like) and *merA* genes. Streaks on plates of the isolates were performed and these culture were incubated under anaerobic conditions at 32 to 35 ºC under incandescent light until discrete pigmented colonies were observed. To extract the DNA, 3 colonies of the isolate were picked with a micropipette tip, resuspended in 10 µL of PCR buffer and incubated at 94 ºC for 5 min. The PCR reaction for the amplification of *copA* was done as follow per 25 µL reaction: 9.5 µL of sterilized and distilled deionized water, 1 µL of DNA, 12.5 µL Green Taq Master Mix (Promega) and 1 µL of primers (10 pmol) copAUF (5'-GGT GCT GAT CAT CGC CTG-3') and copAUR (5'-GGG CGT CGT TGA TAC CGT-3') (Iglesia et al., 2010). The parameters of the PCR were: first denaturalization at 94 ºC for 5 min, followed by 25 cycles with a denaturalization of 94 ºC at 45 s, annealing at 57 ºC for 1 min and extension at 72 ºC for 2 min and a final elongation at 72 ºC for 7 min. The PCR products (~733 to 766 bp) were confirmed by gel electrophoresis with agarose 1.5% prepared with TAE1X.

For the amplification of *merA* the previously described PCR reactions were used. The used primers were A1-(5'-ACC ATC GGC GGC ACC TGC GT-3') and A5-(5'-ACC ATC GTC AGG TAG GGG AAC AA-3') (Liebert et al., 1997). The parameters for the PCR were: first denaturalization at 95 ºC for 2 min, annealing at 64 ºC for 2 min and 72 ºC for 3 min, followed by 29 cycles with a denaturalization of 95 ºC at 1 min, annealing at 64 ºC for 2 min and extension at

72 ºC for 3 min and a final elongation at 72 ºC for 5 min. The PCR products (~1,238 bp) were confirmed by gel electrophoresis with agarose 1% prepared with TAE1X.

3.4.2 Purple Non-Sulfur Bacteria Hydrogen Production

To evaluate hydrogen production by PNSB isolates, Hydrogen Production medium (RPP), described by (Bianchi et al., 2010), was used. RPP medium has the following composition per L of the medium: carbon source (4.0 g malic acid, 3.6 g lactic acid, 3.6 g acetic acid or 3.6 g succinic acid), 0.5 g K_2HPO_4, 0.3 g KH_2PO_4, 0.4 g $MgSO_4.7H_2O$, 0.4 g NaCl, 0.075 g $CaCl_2.2H_2O$, 0.005 g ferric citrate. For the tests, the following modifications were done to the medium: NH_4Cl instead of Na-glutamate was used as a nitrogen source, and the pH was adjusted to 7.0 with KOH instead of NaOH. The test was done in 6 mL borosilicate glass bottles filled completely with the RPP medium. A Durham tube was placed inside the bottle to detect gas production by the isolates. As inoculum, 100 µL of a 3 d culture of the isolate grown in the medium described in Section 1.4 was used. The bottles were incubated under incandescent light at 32 to 35 ºC for 1 wk. To confirm that hydrogen was the gas produced by the PNSB isolates, a 200 mL bottle was filled with medium. In order to collect more volume of the gas produced by the isolates, an essay tube was added to the bottle instead of a Durham tube. After one week of incubation, the opening in the tube with the collected gas was exposed to a Bunsen burner. A chirping noise suggests that the gas produced in the tube is hydrogen gas (Shah, et al. 2017).

3.4.3 Antimicrobial Substances Production by PNSB

The assessment of antimicrobial substance production by the PNSB isolates against *Escherichia coli ATCC8739, Staphylococcus aureus ATCC 6538, Bacillus subtilis ATCC 6633, Klebsiella pneumoniae ATCC 3882* and *Pseudomonas aeruginosa ATCC9721* were evaluated following Kaspari and Kleme (1976). Patching of the PNSB isolates was done in Petri dishes

containing Sistrom medium (Sistrom, 1962). The plates were incubated for 4 d under aerobic conditions at 32 ^{o}C. Then 5 mL of top Agar at 45 ^{o}C was mixed by vortex with 100 µL of an overnight culture of the target, previously washed and diluted with NaCl 0.85 % to a 0.05 McFarland. Next, the mixed top agar and target culture were poured carefully over the 4 d PNSB patching. Incubation was done at 37 ^{o}C and observed at 24, 48 and 72 h of incubation for the development of an inhibition zone.

3.4.4 Naphthalene and Phenanthrene Degradation by PNSB

The medium described in Section 1.4 was prepared with naphthalene or phenanthrene as the sole carbon source, at a final concentration of 0.1 % to determine the capabilities of the isolates to degrade hydrocarbons. Then, 500 µL of the medium with the hydrocarbon were transferred to each well of a 2 mL-96 wells plate. As inoculum, 20 µL of a previously grown 4 d culture of the PNSB isolate washed twice and adjusted with NaCl 0.85% to an absorbance of 0.020 at 600nm were used. The plates were incubated under incandescent light for 4 days. Absorbance measurements at 600 nm were performed to confirm growth.

3.5 Results and Discussion

3.5.1 PNSB Copper, Mercury and Lead Resistance

After evaluating the capabilities of the isolates to grow in the presence of copper, it was found that 8 out of 16 PNSB isolates (4 from bromeliad phytotelmata, 2 from *Heliconia* phytotelmata and 2 from water reservoirs) were able to grow in the presence of 30 mg/L of $CuCl_2$ in the medium. From these isolates, 2 could grow in up to 100 mg/L of $CuCl_2$. Similarly, Panwichian et al. (2011) were able to grow PNSB isolates up to 1.94 mM (260 mg/L) of Cu^{2+} in the medium. The latter is the only report assessing copper resistance capabilities of PNSB

members; therefore, our study confirms the findings of Panwichian et al. (2011) and also encourage for further studies of PNSB members and their resistance to copper.

After PCR amplification of the isolates using specific primers for Copper P-type ATPase, it was interesting that only 3 of the isolates (grew up to 40, 70 and 80 mg/L of $CuCl_2$) had amplification after confirmation by gel electrophoresis (Figure 3.4). In silico analysis revealed that the most similar sequence in the NCBI nucleotide collection to the amplified gene was a copper-translocating P-type ATPase from *Rhodopseudomonas palustris*. The function of copper transport ATPases such as CopA is to export copper from the cytoplasm to the periplasm (Pal et al., 2017). Since none of the other isolates resistant to copper had amplification for copper P-type ATPase, further studies about the identification of the mechanisms involved in the copper resistance by these isolates could end in the discovery of new mechanisms for bacterial metal resistance. In a previous study, it was found that *Rps. palustris* lacks of known motifs important in copper binding, suggesting novel genes or mechanisms related to copper homeostasis (Bird et al., 2013). In order to identify novel genes related to copper resistance by the PSNB isolates, transposon mutagenesis can be performed (Simon et al., 1983). Recently, this technique was used successfully for the identification of genes related to hydrogen production in *Rhodobacter sphaeroides* (Wang et al., 2018). After performing transposon mutagenesis, the isolates that had lost their ability to resist copper in the media are identified. If these isolates have the transposon in genes related to copper resistance, thus the genes can be identified later by sequencing. In an effort to find information about copper regulation in PNSB we found that there are no studies about copper regulation mechanisms in PNSB.

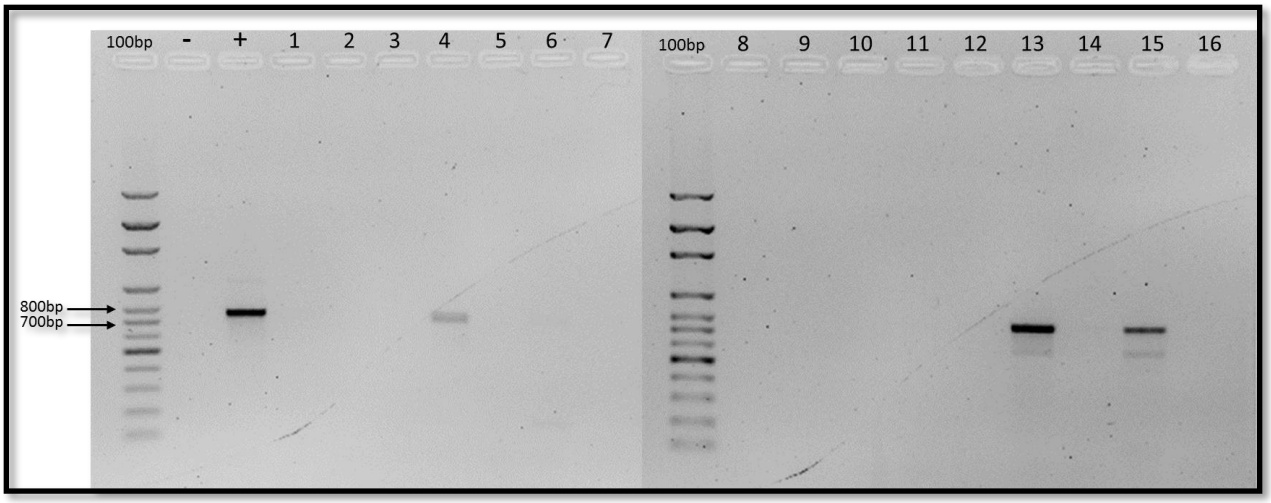


Figure 3.4: PCR amplification of *copA* genes. PCR amplification was confirmed by gel electrophoresis in agarose at 1.5% in TAE1X Buffer. Azygen Biosciences 100 bp ladder (100 bp to 3000 bp) was used as DNA marker. Negative and positive controls for the amplification of *copA* genes are denoted as – and +, respectively, in the figure. Numbers represent the PNSB isolates as follows: 1- WA012, 2-WA019, 3-WA022, 4-WA056, 5-WA060, 6-WA077, 7-HE0A, 8-HE0K, 9-HE0M, 10-HE0P, 11-BR0C11, 12-BR0C12, 13-BR0G17, 14-BR0M8, 15-BR0M22, and 16-BR0Y6.

It is important to mention that the isolates took between 2 - 3 additional days to grow in the presence of copper in the medium when compared to the isolates growing in the medium without the metal. This low grow rate must be related to a negative effect of copper on the cells. However, the fact that despite the presence of copper in the medium the cells were able to grow could be related to the presence of exopolymeric substances released by the isolates. As previous studies demonstrate, PNSB isolated from metal contaminated shrimps produce exopolymer substances for the biosorption of the metal, consequently lowering its concentration in the medium (Panwichian et al., 2011). If we assumed that our isolates are able to produce exopolymeric substances, we understand that as the culture develops, the exopolymeric substances increase and, thus, the biosorption of copper. This increment of copper biosorption helps the development of the culture in later stages and thus it could explain the low growth at the beginning of the culture. However, we would need to confirm exopolymeric substances production by the isolates. In

addition, an experiment evaluating the relation between cell growth and copper concentration over time must be performed.

In terms of mercury resistance, none of the isolates were able to grow in the presence of $HgCl_2$ at a concentration of $\geq$ 5 mg/L. In previous studies, it was found that $HgCl_2$ in concentrations over 4 mg/L was bactericidal to the PNSB *Rhodovilum sulphidophilum* and *Afifella marina* (Mukkata, 2016). Data from the mentioned study suggest that Hg binds to motifs in the photosynthetic apparatus of the PNSB leading to cell death. However, the same test can be performed under aerobic conditions to evaluate whether there are significant differences in Hg resistance by PNSB isolates between aerobic and anaerobic conditions.

After PCR amplification of *merA* genes, PCR products of variable sizes were obtained for most of the PNSB isolates. Amplicons of approximately 1500 bp were obtained for the isolates WA012, BR0C12, and WA056 from water reservoirs and bromeliad and *Heliconia* phytotelmata, respectively (Figure 3.5). In addition, other amplicons below 1000 bp were present in 15 of the 16 isolates. All of these amplicons are not the expected length for *MerA* genes and might mean putative genes related to mercury resistance. More studies to elucidate the identity and roles of these genes are strongly recommended. This could lead to the discovery of new genes related to mercury resistance and their possible use in recombinant DNA for mercury bioremediation.

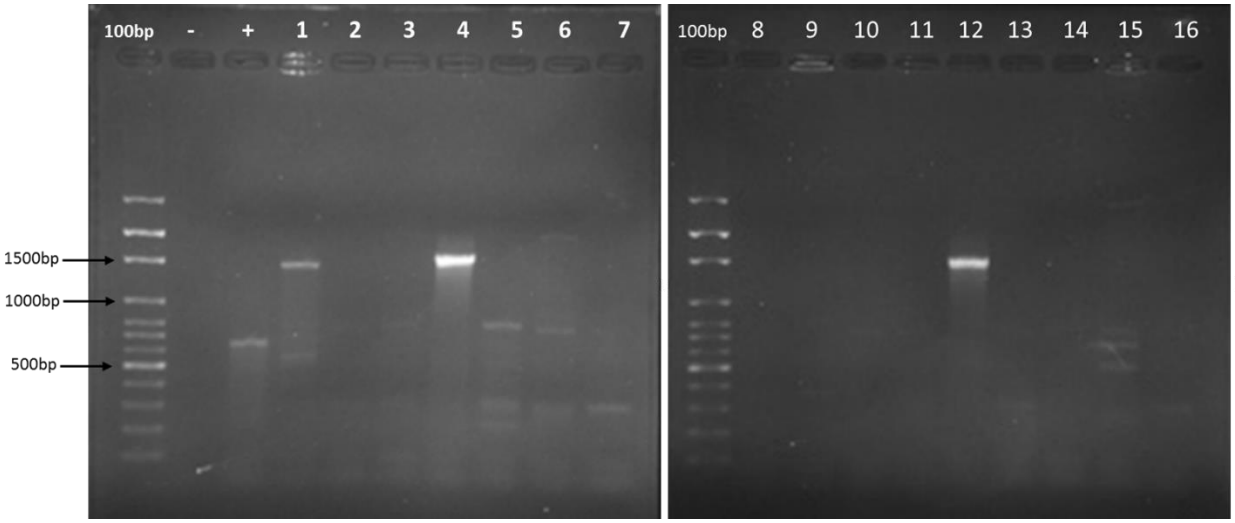


Figure 3.5: PCR amplification of *merA* genes. PCR amplification was confirmed by gel electrophoresis in agarose at 1.5% in TAE1X Buffer. Azygen Biosciences 100bp ladder (100bp to 3000bp) was used as DNA marker. Negative and positive controls for the amplification of *merA* genes are denoted as – and +, respectively, in the figure. Numbers represent the PNSB isolates as follows: 1- WA012, 2-WA019, 3-WA022, 4-WA056, 5-WA060, 6-WA077, 7-HE0A, 8-HE0K, 9-HE0M, 10-HE0P, 11-BR0C11, 12-BR0C12, 13-BR0G17, 14-BR0M8, 15-BR0M22 and 16-BR0Y6.

In terms of lead resistance, all the isolates were able to grow in the presence of 60 mg/L of $PbNO_3$ in the medium. Moreover, 11 of the 16 isolates could grow at a concentration of 130 mg/L in the medium. It is important to mention that sulfate in the medium precipitate with Pb, forming $PbSO_4$ (Li et al., 2016a). In our case, a precipitate was formed by the addition of $PbNO_3$. This precipitate was more evident as the concentration of $PbNO_3$ increased in the media. In order to confirm that the precipitate was formed as a result of the interaction of $PbNO_3$ and sulfate, the previous was added to media without sulfate. However, the precipitate was also formed. Apparently, the formation of the precipitate is related to $PbNO_3$ interaction with other components in the media. Nevertheless, the potential application of these isolates should not be dismissed. Further analysis to identify the precipitate in the media and also the accumulation, distribution of the metal in the cells (membranes, periplasm, cell wall, and cytoplasm) and the action of exopolymeric substances (EPS) should be addressed. For example, as done by Panwichian et al. (2011), lead biosorption capabilities of PNSB EPS can be tested by adding the EPS (previously

extracted from the culture) to an aqueous solution with the metal). By this method, it was found that 96 - 98% of the initial amount of lead in the aqueous solution was removed by the EPS extracted from the PNSB (Panwichian et al., 2011). In an effort to compare our results with literature related to PNSB and lead resistance, we found that there are no other studies in addition to Panwichian et al. (2011). This also confirms the necessity of further studies to further explore the capacities of these organisms to resist metals and the mechanisms involved. Also it is important to mention that some of the isolates minimal inhibitory concentration (MIC) for copper and lead were above the maximum contaminant level allowed by the Environmental Protection Agency (Table 3.1), therefore there is a potential for the use of these microorganisms in metal bioremediation of contaminated waters.

Table 4.1: Maximum contaminant levels (MCL) in drinking water and minimal inhibitory concentration (MIC) of the tested metals for PNSB isolates

Metal	MCL [1] (mg/L)	MIC [2] (mg/L)
Copper	1.3	100
Lead	0.015	130
Mercury	0.002	0

1-MCL is the maximum contaminant level allowed by EPA in drinking water. 2- The highest minimal inhibitory concentration observed in PNSB isolates in this study.

3.5.2 PNSB Hydrogen Production

Due to the necessity of finding alternate sources of energy, one of the well-studied potentials of PNSB is the production of hydrogen under phototrophic conditions. Moreover, due to its versatility, PNSB have been preferred over other microorganisms for this matter (Wang et al., 2016b). In our study, we found that 6 of the 16 isolates were able to produce gas under phototrophic conditions. The isolates BR0M22 (isolated from bromeliad phytotelmata), WA060, WA077, and WA056 (isolated from water reservoirs), only produced gas when acetic acid was supplied to the media as the only carbon source. Meanwhile, BR0C11 and HE0K (isolated from

bromeliad and *Heliconia* phytotelmata, respectively) produced gas when malic acid was added to the media as a carbon source. The isolate WA056 was the only bacterium that was able to produce gas when either acetic or malic acid was added to the media as the sole carbon source. When the gas produced by these isolates was exposed to the flame of a Bunsen burner, the characteristic "squeaky pop" resulting from hydrogen ignition was perceived (Elmer, 1946; Astbury et al., 2007). In terms of hydrogen production with lactic acid as the carbon source, none of our isolates were able to produce gas unlike *Rhodopseudomonas palustris*, a well-known and studied PNSB (Bianchi et al., 2010).

Since hydrogen is an environmentally friendly, renewable and highly efficient biofuel, there is a surge in the study of hydrogen producing microorganisms and optimization of the process (Katsuda et al. 2006; Basak et al., 2007; Chen et al., 2012; Hallenbeck et al., 2016). In order to optimize hydrogen production rate, different physicochemical parameters such as culture medium, illumination, carbon source, and pH and temperature ranges need to be evaluated (Wang et al., 2016). Also, like previous studies, gas chromatography can be performed to confirm hydrogen production by the isolates (Melnicki et al., 2008).

Another approach is the usage of transposon mutagenesis. For instance, *Rhodobacter sphaeroides* mutants with enhanced hydrogen production capability were obtained (Wang et al., 2018). This technique can be applied to the PNSB isolates to further explore the capabilities of these bacteria.

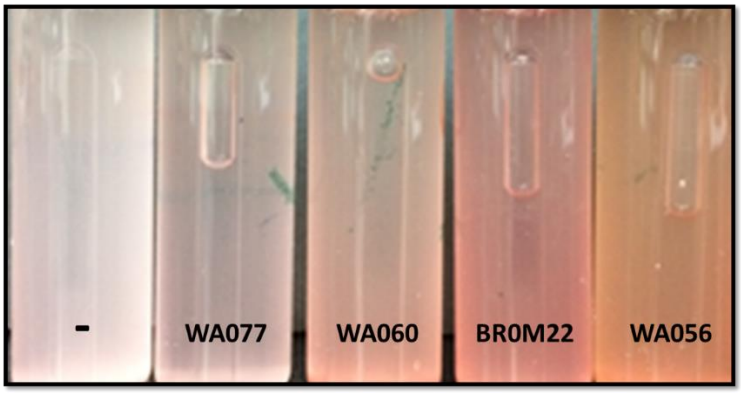


Figure 3.6: Hydrogen Production by PNSB Isolates. Gas was produced by 4 of the isolates after 1 wk of incubation under phototrophic. The – sign depicts one of the isolates unable of produced gas. The isolates WA077, WA060, and WA056 were isolated from water reservoirs and the isolate BR0M22 from bromeliad phytotelmata.

3.5.3 Antimicrobial Substances Production by PNSB

During this study, the capabilities of the isolates as producers of antimicrobial substances were addressed. Inhibition zones were observed around 14 of the 16 isolates only in the presence of *B. subtilis* as a target (Figure 3.7), suggesting specific antimicrobial substances against this bacterium. Although the isolates are producing an antimicrobial agent, it is unknown if it is bactericidal or bacteriostatic to *B. subtilis*. Also, it is important to mention that even if inhibition halos were not observed when using other targets, the capabilities of the antimicrobial substances produced by the PNSB isolates against the other targets should not be dismissed. There is a possibility that the concentration of the antimicrobial substance produced by the PNSB isolate is too low in order to affect the cell growth of other targets.

In previous studies, the production of antimicrobial substances by the PNSB *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* against *B. subtilis* was also found; however, the identity of the substance was unknown (Kaspai et al., 1977). Further identification of the antimicrobial substance produced by the PNSB isolates is strongly recommended. Also, additional analyses about the mechanisms involved in the antagonist activity by these isolates could end in the discovery of novel antimicrobials, pathways and niches for the isolation of antibiotic producers.

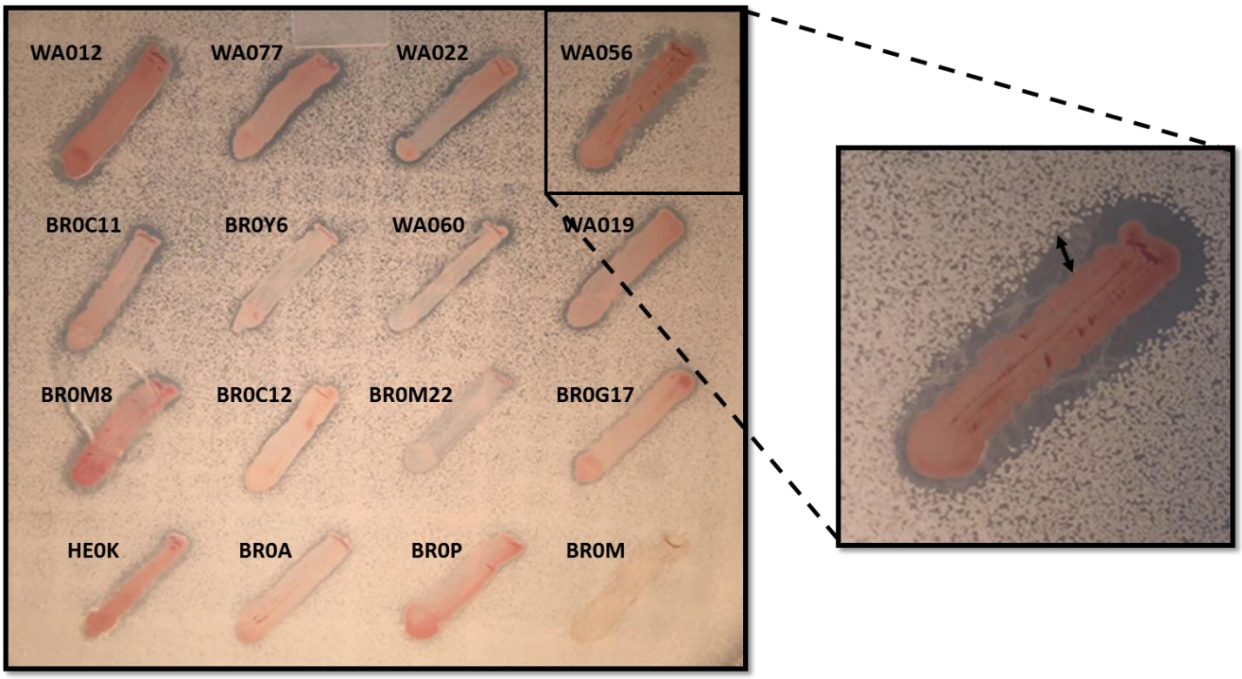


Figure 3.7: PNSB antimicrobial production against *Bacillus subtilis*. An inhibition zone (demarcated by the arrow) is visible between the PNSB isolate and the bacterium *B. subtilis* (lawn).

3.5.4 Naphthalene and Phenanthrene Degradation

Contrary to aerobic bacterial catabolism of naphthalene and phenanthrene, the pathways related to these activities under anaerobic conditions are not well understood (Chauhan et al., 2008; Seo et al., 2009; Widdel et al., 2001). For this reason in this study, we were interested in the capabilities of the PNSB isolates for naphthalene and phenanthrene degradation during anaerobic conditions.

We found that none of the isolates were able to grow using any of these polycyclic aromatics hydrocarbons (PAH'S) as the sole carbon source. In previous studies, it was found that the PNSB *Rubrivivax benzoatilyticus* was able to grow with benzoate, cyclohexanol, phenylalanine, 2-aminobenzoate, and other aromatic compounds as the sole carbon source under anaerobic

conditions (Ramana et al., 2006). For this reason, it would still be interesting to study the degradation capabilities of the PNSB isolates with other hydrocarbons.

3.6 Conclusion

While there are different studies about the biotechnological potential of PNSB, nowadays the most explored biotechnological application of these bacteria is their capability to produce hydrogen. Although there is an urgency to search for alternatives to fossil fuels there are other challenges, such as antimicrobial resistance and environmental contamination, that are also of great concern; thus, requiring solutions or alternatives as soon as possible. In addition to the Microbial and Bioprospecting Laboratory (MBB), there is no other research in Puerto Rico addressing the presence of PNSB in niches around the island nor the biotechnological potential.

Another biotechnological application that was explored during this study was the ability of the isolates to degrade the phenanthrene and naphthalene under anaerobic conditions. There is an urgency since these polycyclic aromatics hydrocarbons (PAH's) are the most difficult components to biodegrade due to its stable structure (Zhu et al., 2001). Although previous studies found that *Rps. palustris* was able to degrade phenanthrene under anaerobic conditions (Zhao et al., 2011), none of our isolates was able to degrade these contaminants. However, the capability and contribution of hydrocarbon degradation by these isolates as part of a consortium of microorganisms should not be discarded.

As confirmed by this study, PNSB are microorganisms of varied and important biotechnological applications. Also, this study explored for the first time the applications of these group of bacteria isolated from Puerto Rico. We found possible applications of PNSB in bioremediation, specifically in copper and lead removal, the biomedical field as antimicrobial producers, and as producer of hydrogen, an extensively studied and suggested as an alternative for

fossil fuel. It is important to study more extensively other applications of these isolates that were not on the scope of this study. For example, the production of enzymes of industrial interest such as amylases, proteases, and lipases that can be used at different physical and chemical conditions in industrial processes (Marti et al., 2003). Due to the pH growing range of most of our isolates (5.5 to 9.0), they should be considered for studies as providers of enzymes with possible activity at different pH ranges (Section 2.5.2). In addition, to attempt the detection of other functional activities by the isolates in this study, it is important to explore non-well studied niches for novel PNSB.

Chapter 4: General Conclusions, Recommendations and Literature Cited

4.1 Summary and Conclusions

- We confirmed that bacteria in the MMB microbial culture collection isolated previously from different aquatic environments in Puerto Rico were members of the PNSB. A total of 11 isolates from water reservoirs, 26 from Bromeliad phytotelmata and 8 from *Heliconia* phytotelmata, respectively, were confirmed as members of the PNSB.

- Besides the previous report from our laboratory about the isolation of PNSB from Bromeliads and microbial mats, we were unable to find any other report about the isolation of these organisms in Puerto Rico. Moreover, this is the first report about the isolation and partial description of PNSB isolates from *Heliconia* phytotelmata. In addition, we contribute to NCBI a total of 16 partial and 4 full 16SrDNA and 4 genome sequences.

These genome sequences can be used for the study of gene composition and regulation of these metabolically diverse bacteria.

- For the understanding of the relationship among the isolates, OrthoANI was used. This was the first time that this overall genome relatedness indices tool is used as part of the description of PNSB. By applying this technique we conclude that 3 of the isolates related to the genus *Rhodopseudomonas* are not the same species as *Rhodopseudomonas palustris*. Moreover, by the use of this technique, we conclude that reference genomes previously reported as belonging to *Rhodpseudomonas palustris* belong to different species.
- For the first time, the biotechnological potential of the PNSB from the MMB culture collection was addressed. It was found that some of the PNSB isolates had biotechnological potential applications in lead and copper bioremediation, production of hydrogen and antimicrobial substances.

4.2 Recommendations

- Due to the advance of sequencing techniques, its affordability, and the development of algorithms for species delineation, it is recommended to the International Committee on Systematics of Prokaryotes (ICSP) to evaluate the addition of full genome sequencing as a requirement for the description of novel species. Having the genomes of the described species available would facilitate species identification by the use of species delineation algorithms such as ANI and OrthoANI. In addition, the sequenced genomes can be annotated and their gene composition and function can be studied.
- Although we explored the species identities and biotechnological potential of PNSB isolates from bromeliad and *Heliconia* phytotelmata and water reservoirs, the MBB culture collection

has also PNSB isolates from microbial mats and bamboo phytotelmata whose biotechnological potentials are unknown. We highly encourage the study of these isolates and their possible applications.

- In terms of biotechnological potential, further studies of these PNSB isolates in the MBB culture collection are strongly encouraged, for additional applications in the biomedical field (i.e. enzymes of medical use such as asparaginase, lysozyme, ribonucleases, etc.), resistance to other metals (i.e. zinc, cadmium, iron, cobalt, etc.), degradation of different saturated and other polycyclic aromatic hydrocarbons (i.e. benzene, toluene, alkanes, etc.) under anaerobic and aerobic conditions, among many others that were not explored during this study. Also the capabilities of these isolates as producers of enzymes for industrial use (i.e. proteases, amylases, xylanases, ligninases, cellulases, lipases, etc.), biopolymer producers, and other activities with biotechnological applications not explored in this study yet they should be addressed.
- The PNSB isolates biotechnological potential gives insight and awareness about the environments of their origin as niches of microorganisms with further biotechnological potential. For this reason, we strongly recommend studies about the microbial community in these environments.
- Since this study provides more evidence about the ubiquity of these microorganisms and its biotechnological potential, we strongly encourage the exploration, isolation, and study of PNSB biotechnological application from other environments in Puerto Rico and around the world.

4.3 Literature Cited

Achenbach, L. A., Carey, J., & Madigan, M. T. (2001). Photosynthetic and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. *Applied and Environmental Microbiology*, 67(7), 2922–2926. https://doi.org/10.1128/AEM.67.7.2922-2926.2001

Albarracín, V. H. (2016). Ketide synthase (KS) domain prediction and analysis of iterative type II PKS gene in marine sponge-associated Actinobacteria producing biosurfactants and antimicrobial agents. *Frontiers in Microbiology*, *7*, 1–12. https://doi.org/10.3389/fmicb.2016.00063

Ansari, F. A., & Nasr, M. (2017). Phycoremediation: an eco-friendly algal technology for bioremediation and bioenergy production. *Phytoremediation Potential of Bioenergy Plants*, 431–456. https://doi.org/10.1007/978-981-10-3084-0

Astbury, G. R., & Hawksworth, S. J. (2007). Spontaneous ignition of hydrogen leaks: A review of postulated mechanisms. *International Journal of Hydrogen Energy*, 32(13), 2178–2185. https://doi.org/10.1016/j.ijhydene.2007.04.005

Barkay, T., Miller, S. M., & Summers, A. O. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews*, 27(2–3), 355–384. https://doi.org/10.1016/S0168-6445(03)00046-9

Basak, N., & Das, D. (2007). The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: The present state of the art. *World Journal of Microbiology and Biotechnology*, 23(1), 31–42. https://doi.org/10.1007/s11274-006-9190-9

Belila, A., Fazaa, I., Hassen, A., & Ghrabi, A. (2013). Anoxygenic phototrophic bacterial diversity within wastewater stabilization plant during "red water" phenomenon. *International Journal of Environmental Science and Technology*, 10(4), 837–846. https://doi.org/10.1007/s13762-012-0163-2

Bianchi, L., Mannelli, F., Viti, C., Adessi, A., & De Philippis, R. (2010). Hydrogen-producing purple non-sulfur bacteria isolated from the trophic lake Averno (Naples, Italy). *International Journal of Hydrogen Energy*, 35(22), 12216–12223. https://doi.org/10.1016/j.ijhydene.2010.08.038

Bird, L., Coleman, M., Newman, D. (2013). Iron and copper act synergistically to delay anaerobic growth of bacteria. *Applied and Environmetal Microbiology*, 79(12), 3619-3627. https://doi.org/10.1128/AEM.03944-12

Brighigna, L., Montaini, P., Favilli, F., & Trejo, A. C. (1992). Role of the nitrogen-fixing bacterial microflora in the epiphytism of *Tillandsia* (Bromeliaceae). *American Journal of Botany*, 79(7), 723–727.

Bronstein, J. L. (1986). The origin of bract liquid in a neotropical *Heliconia* species. *Biotropica*, 18(2), 111–114.

Bruins, M. R., Kapil, S., & Oehme, F. W. (2000). Microbial resistance to metals in the environment. *Ecotoxicology and Environmental Safety*, 45(3), 198–207. https://doi.org/10.1006/eesa.1999.1860

Buccolieri, A., Italiano, F., Atti, A. D., Buccolieri, G., Giotta, L., Agostiano, A., & Trotta, M. (2005). Testing the photosynthetic bacterium *Rhodobacter sphaeroides* as heavy metal removal tool. *Annali Di Chimica*, 95(May 2005).

Carmo, F. L., Santos, H. F., Peixoto, R. S., Rosado, A. S., & Araujo, F. V. (2014). Tank bromeliad water: Similar or distinct environments for research of bacterial bioactives?. *Brazilian Journal of Microbiology*, 45(1), 185–192. https://doi.org/10.1590/S1517-83822014000100024

Chauhan, A., Fazlurrahman, Oakeshott, J. G., & Jain, R. K. (2008). Bacterial metabolism of polycyclic aromatic hydrocarbons: Strategies for bioremediation. *Indian Journal of Microbiology*, 48(1), 95–113. https://doi.org/10.1007/s12088-008-0010-9

Chevenet, F., Brun, C., Bañuls, A.-L., Jacq, B., & Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics*, 7(1), 439. https://doi.org/10.1186/1471-2105-7-439

Chun, J., Lee, J., Jung, Y., Kim, M., Kim, S., Kim, B. K., & Lim, Y. (2017). EzTaxon : a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences.

International Journal of Systematics and Evolutionary Microbiology, 2259–2261. https://doi.org/10.1099/ijs.0.64915-0

Colica, G., Caparrotta, S., Bertini, G., & De Philippis, R. (2012). Gold biosorption by exopolysaccharide producing cyanobacteria and purple nonsulphur bacteria. *Journal of Applied Microbiology*, 113(6), 1380–1388. https://doi.org/10.1111/jam.12004

Courtois, S., Cappellano, C. M., Ball, M., Francou, F., Normand, P., Martinez, A. & Hopke, J. (2003). Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Applied and Environmental Microbiology*, 69(1), 49–55. https://doi.org/10.1128/AEM.69.1.49

Del Socorro, M. M., Mehid, J. B., Ladion, W. L. B., & Teves, F. G. (2013). Purple non-sulfur bacteria (PNSB) isolated from aquatic sediments and rice paddy in Iligan City, Philippines. *J Multidisciplinary Studies*, 1(1), 45–58. https://doi.org/10.7828/jmds.v1i1.394

Demin, Zhang Huifang, Yang Zhiyong, H. W. Z. & S.-J. L. (2002). *Rhodopseudomonas faecalis* sp. nov., a phototrophic bacterium isolated from an anaerobic reactor that digests chicken faeces. *International Journal of Systematic and Evolutionary Microbiology*, (52), 2055–2060. https://doi.org/10.1099/ijs.0.02259-0

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F. & Gascuel, O. (2008). Phylogeny.fr : robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*, 36, 465–469. https://doi.org/10.1093/nar/gkn180

Edgar, R. C., Drive, R. M., & Valley, M. (2004). MUSCLE : multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32(5), 1792–1797. https://doi.org/10.1093/nar/gkh340

Elmer, J. (1946). The velocity of sound in hydrogen when rotational degrees of freedom fail to be excited. *Physical Reviews*, 59(1939), 932–938.

Fouts, D. E., Brinkac, L., Beck, E., Inman, J., Sutton, G. (2012). PanOCT: automated clustering of orthologs using conserved gene neighborhood for pan-genomic analysis of bacterial strains and closely related species. *Nucleic Acids Research*, 40(22), e172. https://doi.org/10.1093/nar/gks757

Franke S, Grass G, Rensing C, Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *Journal of Bacteriology*, 185, 3804–3812.

George, M. W. (USGS). (2017). US Geological Survey, Mineral Commodity Summaries.

Girija, K. R., Sasikala, C., Ramana, C. V., Spröer, C., Takaichi, S., Thiel, V., & Imhoff, J. F. (2010). *Rhodobacter johrii* sp. nov., an endospore-producing cryptic species isolated from semi-arid tropical soils. *International Journal of Systematic and Evolutionary Microbiology*, 60(9), 2099–2107. https://doi.org/10.1099/ijs.0.011718-0

Goffredi, S. K., Kantor, A. H., & Woodside, W. T. (2011). Aquatic microbial habitats within a neotropical rainforest: Bromeliads and pH-associated trends in bacterial diversity and composition. *Microbial Ecology*, 61(3), 529–542. https://doi.org/10.1007/s00248-010-9781-8

Grégoire, D. S., & Poulain, A. J. (2016). A physiological role for Hg II during phototrophic growth. *Nature Geoscience*, 9. https://doi.org/10.1038/NGEO2629

Hales, B. J., Case, E. E., Morningstar, J. E., Dzeda, M. F., & Mauterer, L. A. (1986). Isolation of a new vanadium-containing nitrogenase from *Azotobacter vinelandii*. *Biochemistry*, 25(23), 7251–7255.

Hiraishi, A., Hoshino, Y., & Satoh, T. (1991). *Rhodoferax fermentans* gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the *Rhodocyclus-Gelatinosus*-like group. *Arch Microbiology*, 155, 330–336.

Hiraishi, A., & Okamura, K. (2017). *Rhodopseudomonas telluris* sp. nov., a phototrophic alphaproteobacterium isolated from paddy soil. *Journal of Systematic and Evolutionary Microbiology*, 1(6). https://doi.org/10.1099/ijsem.0.002120

Hirose, S., Nagashima, K. V. P., Matsuura, K., & Haruta, S. (2012). Diversity of purple phototrophic bacteria, inferred from pufM gene, within epilithic biofilm in Tama River, Japan. *Microbes Environ*, 27(3), 327–329. https://doi.org/10.1264/jsme2.ME11306

Hougardy, A., Tindall, B. J., & Klemme, J. (2000). *Rhodopseudomonas rhenobacensis* sp. nov., a new nitrate-reducing purple non-sulfur bacterium. *International Journal of Systematic and Evolutionary Microbiology*, (50), 985–992.

Hunter, C. Neil, Fevzi Daldal, M.C.T. & J.T.B. (2009). Summary for policymakers. Intergovernmental Panel on Climate Change (Ed.), Climate Change 2013 - The Physical Science Basis (Vol. 53, pp. 1–30). Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9781107415324.004

Hussey, M. A. (2016). Endospore Stain Protocol. *ASM Laboratory Protocols*, 1–11.

Idi, A., Md Nor, M. H., Abdul Wahab, M. F., & Ibrahim, Z. (2015). Photosynthetic bacteria: an eco-friendly and cheap tool for bioremediation. *Reviews in Environmental Science and Biotechnology*, 14(2), 271–285. https://doi.org/10.1007/s11157-014-9355-1

Iglesia, R. De, Pavissich, J. P., Freyhoffer, S., Andrade, S., Correa, J. A. & Gonza, B. (2010). Novel polymerase chain reaction primers for the specific detection of bacterial copper P-type ATPases gene sequences in environmental isolates and metagenomic DNA. *Letters in Applied Microbiology*, 50, 552–562. https://doi.org/10.1111/j.1472-765X.2010.02832.x

Ijoma, G. N., & Tekere, M. (2017). Potential microbial applications of co-cultures involving ligninolytic fungi in the bioremediation of recalcitrant xenobiotic compounds. *International Journal of Environmental Science and Technology*, 1–20. https://doi.org/10.1007/s13762-017-1269-3

Imhoff, J. F. (2006). The phototrophic betaproteobacteria. Prokaryotes, 593–601. https://doi.org/10.1007/0-387-30745-1

Imhoff, J. F., & Caumette, P. (2004). Recommended standards for the description of new species of anoxygenic phototrophic bacteria. *International Journal of Systematic and Evolutionary Microbiology*, 54(4), 1415–1421. https://doi.org/10.1099/ijs.0.03002-0

Janssen, P. J., van Houdt, R., Moors, H., Monsieurs, P., Morin, N., Michaux, A. & Mergeay, M. (2010). The complete genome sequence of *Cupriavidus metallidurans* strain CH34, a master survivalist in harsh and anthropogenic environments. *PLoS ONE*, 5(5). https://doi.org/10.1371/journal.pone.0010433

Jarosławiecka, A., & Piotrowska-seget, Z. (2014). Lead resistance in micro-organisms. *Microbiology*, 12–25. https://doi.org/10.1099/mic.0.070284-0

Jesús, M. De, & Sepúlveda, J. V.-. (2010). Isolation and characterization of purple non-sulfur anoxyphototropic bacteria from two microecosystems : tropical hypersaline microbial mats and bromeliads phytotelmata. *Applied Microbiology*, 109–116.

Kane, A. L., Al-Shayeb, B., Holec, P. V., Rajan, S., Le Mieux, N. E., Heinsch, S. C. & Gralnick, J. A. (2016). Toward bioremediation of methylmercury using silica encapsulated *Escherichia coli* harboring the *mer* operon. *PLoS ONE*, 11(1), 1–10. https://doi.org/10.1371/journal.pone.0147036

Kar Soon, T., Al-Zad, S., & Ransangan, J. (2014). Isolation and characterization of purple non-sulfur bacteria, *Afifella marina*, producing large Amount of carotenoids from mangrove microhabitats. *Journal of Microbiolial Biotechnology*, 24(8), 1034–1043.

Kaspai, I. I., & Klemme, J. (1977). Characterization of antibiotic activities produced by *Rhodopseudomonas sphaeroides. FEMS Microbiology Letters*, 1, 59–61.

Katsuda, T., Ooshima, H., Azuma, M., & Kato, J. (2006). New detection method for hydrogen gas for screening hydrogen-producing microorganisms using water-soluble Wilkinson's catalyst derivative. *Journal of Bioscience and Bioengineering*, 102(3), 220-226.

Kawasaki, H., Hoshino, Y., & Yamasato, K. (1993). Phylogenetic diversity of phototrophic purple non-sulfur bacteria in the Proteobacteria a group. *FEMS Microbiology Letters*, 112, 61–66.

Killick S.A., Blanchon, D.J., Large, M.F. (2014). Algal communities in phytotelmata: A comparison of native *Collospermum* and exotic bromeliads (Monocotyledonae) in New Zealand. *Journal of Plant Systematics*, 17, 303-310.

Kompantseva, E. I., Komova, A. V, Novikov, A. A., & Kostrikina, N. A. (2012). *Rhodovulum tesquicola* sp. nov., a haloalkaliphilic purple non-sulfur bacterium from brackish steppe soda lakes. *Evolutionary Journal of Systematics and Evolutionary Microbiology*, 2962–2966. https://doi.org/10.1099/ijs.0.035857-0

Kulichevskaya, I. S., Guzev, V. S., Gorlenko, V. M., Liesack, W., & Dedysh, S. N. (2006). *Rhodoblastus sphagnicola* sp. nov., a novel acidophilic purple non-sulfur bacterium from Sphagnum peat bog. *Evolutionary Journal of Systematics and Evolutionary Microbiology*, 1397–1402. https://doi.org/10.1099/ijs.0.63962-0

Kwarciak-koz, A. (2014). Phycoremediation of wastewater : heavy metal and nutrient removal processes. *International Journal of Environmental Sciences and Technology,* 25(4), 51–54. https://doi.org/10.2478/oszn-2014-0026

Lakshmi, K. V. N. S., Sasikala, C., Ramaprasad, E. V. V, & Ramana, C. V. (2013). *Rhodospirillum oryzae* sp. nov., a phototrophic bacterium isolated from rhizosphere soil of paddy. *International Journal of Systematic and Evolutionary Microbiology*, 63 (8), 3050–3055. https://doi.org/10.1099/ijs.0.049023-0

Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L. & Harwood, C. S. (2004). Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. *Nature Biotechnology*, 22(1), 55–61. https://doi.org/10.1038/nbt923

Li, X., Peng, W., Jia, Y., Lu, L., & Fan, W. (2016a). Bioremediation of lead contaminated soil with *Rhodobacter sphaeroides*. *Chemosphere*, 156, 228–235. https://doi.org/10.1016/j.chemosphere.2016.04.098

Li, X., Peng, W., Jia, Y., Lu, L., & Fan, W. (2016b). Removal of cadmium and zinc from contaminated wastewater using *Rhodobacter sphaeroides*. *Water Science and Technology*, 1–10. https://doi.org/10.2166/wst.2016.608

Liebert, C. A., Wireman, J. O. Y., Smith, T., & Summers, A. O. (1997). Phylogeny of mercury resistance (mer) operons of Gram-negative bacteria isolated from the fecal flora of primates. *Applied Environmental Microbiology*, 63(3), 1066–1076.

Liu, S. (2012). *Rhodopseudomonas faecalis* sp. nov., a phototrophic bacterium isolated from an anaerobic reactor that digests chicken faeces. *International Journal of Systematic and Environmental Microbiology*, 2055–2060. https://doi.org/10.1099/ijs.0.02259-0.The

Liu, S., Zhang, G., Li, X., & Zhang, J. (2014). Microbial production and applications of 5-aminolevulinic acid. *Applied Microbiology and Biotechnology*, 98(17), 7349–7357. https://doi.org/10.1007/s00253-014-5925-y

Luo, Z. Bin, He, J., Polle, A., & Rennenberg, H. (2016). Heavy metal accumulation and signal transduction in herbaceous and woody plants: Paving the way for enhancing phytoremediation efficiency. *Biotechnology Advances*, 34(6), 1131–1148. https://doi.org/10.1016/j.biotechadv.2016.07.003

Madigan, M. T., Jung, D. O., Woese, C. R., & Achenbach, L. A. (2000). *Rhodoferax antarcticus* sp. nov., a moderately psychrophilic purple non-sulfur bacterium isolated from an Antarctic microbial mat. *Archives of Microbiology*, 173(4), 269–277. https://doi.org/10.1007/s002030000140

Magnin, J.-P., Gondrexon, N., & Willison, J. C. (2014). Zinc biosorption by the purple non-sulphur bacterium *Rhodobacter capsulatus*. *Canadian Journal of Microbiology*, 60(12), 829–837. https://doi.org/10.1139/cjm-2014-0231

Malavé Orengo, J. (2011). A bioluminescent bacterial survey from marine ecosystems of Puerto Rico for the determination of their potential use as biosensors. MS Thesis, University of Puerto Rico at Mayaguez, Mayaguez PR.

Malaysiana, S., Awal, S., Bakau, K., Antimikrob, P. S., & Berat, B. L. (2016). Initial screening of mangrove endophytic fungi for antimicrobial compounds and heavy metal biosorption potential. *Sains Malaysiana*, 45(7), 1063–1071.

Marti, S., Mellado, E., Ventosa, A., & Sa, C. (2003). Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *Journal of Applied Microbiology*, 94, 295–300.

Mary Kensa, V. (2011). Bioremediation - An overview. *Journal of Industrial Pollution Control*, 27(2), 161–168. https://doi.org/10.1351/pac200173071163

Melnicki, M. R., Bianchi, L., De Philippis, R., & Melis, A. (2008). Hydrogen production during stationary phase in purple photosynthetic bacteria. *International Journal of Hydrogen Energy*, 33(22), 6525–6534. https://doi.org/10.1016/j.ijhydene.2008.08.041

Merugu, R., Rudra, M. P. P., Girisham, S., & Reddy, S. M. (2012). Biotechnological applications of purple non sulphur phototrophic bacteria: A minireview. I*nternational Journal of Applied Biology and Pharmaceutical Technology*, 3(1), 376–384.

Minamata Convention on Mercury. *United States Environmental Protection Agency*. Retrieved 29 October 2018.

Missagia, C.C, Alves, M.A. (2016). Florivory and floral larceny by fly larvae decrease nectar availability and hummingbird foraging visits at *Heliconia* (Heliconiaceae) flowers. *Biotropica*, 49(1), 13-17. https://doi.org/10.1111/btp.12368

Møller, A. K., Barkay, T., Hansen, M. A., Norman, A., Hansen, L. H., Sørensen, S. J. & Kroer, N. (2013). Mercuric reductase genes (*merA*) and mercury resistance plasmids in high arctic snow, freshwater and sea-ice brine. *FEMS Microbiology Ecology*, 87(1), 52-63. https://doi.org/10.1111/1574-6941.12189

Moore, E. R. B., Mihaylova, S. A., Vandamme, P., & Krichevsky, M. I. (2010). Microbial systematics and taxonomy : relevance for a microbial commons. *Research in Microbiology*, 161(6), 430–438. https://doi.org/10.1016/j.resmic.2010.05.007

Moore, W. E. C., Stackebrandt, E., Kandler, O., Colwell, R. R., Krichevsky, M. I., Truper, H. G. & Moore, L. H. (1987). Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic and Evolutionary Microbiology*, 37(4), 463–464. https://doi.org/10.1099/00207713-37-4-463

Mukkata, K. (2016). Diversity of purple non-sulfur bacteria in shrimp ponds and mercury resistant mechanisms of selected strains. MS Thesis, Prince of Songkla University, India.

Muzziotti, D., Adessi, A., Faraloni, C., Torzillo, G., & De Philippis, R. (2016). H_2 production in *Rhodopseudomonas palustris* as a way to cope with high light intensities. *Research in Microbiology*, 167(5), 350–356. https://doi.org/10.1016/j.resmic.2016.02.003

Nagashima, K. V. P., Hiraishi, A., Shimada, K., & Matsuura, K. (1997). Horizontal transfer of genes coding for the photosynthetic reaction centers of purple bacteria. *Journal of Molecular Evolution*, 45(2), 131–136. https://doi.org/10.1007/PL00006212

Nies, D. H. (1999). Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology*, 51(6), 730–750. https://doi.org/10.1007/s002530051457

Oda, Y., Samanta, S. K., Rey, F. E., Wu, L., Liu, X., Yan, T. & Harwood, C. S. (2005). Functional genomic analysis of three nitrogenase isozymes in the photosynthetic bacterium *Rhodopseudomonas palustris*. *Journal of Bacteriology*, 187(22), 7784–7794. https://doi.org/10.1128/JB.187.22.7784

Panwichian, S., Kantachote, D., Wittayaweerasak, B., & Mallavarapu, M. (2010). Isolation of purple non-sulfur bacteria for the removal of heavy metals and sodium from contaminated shrimp ponds. *Electronic Journal of Biotechnology*, 13(4). https://doi.org/10.2225/vol13-issue4-fulltext-8

Panwichian, S., Kantachote, D., Wittayaweerasak, B., & Mallavarapu, M. (2011). Removal of heavy metals by exopolymeric substances produced by resistant purple non-sulfur bacteria isolated from contaminated shrimp ponds. *Electronic Journal of Biotechnology*, 14(4), 2. https://doi.org/10.2225/vol14-issue4-fulltext-2

Pollich, M., & Klug, G. (1995). Identification and sequence analysis of genes involved in late steps of cobalamin (Vitamin B-12) *Synthesis in. Journal of Bacteriology*, 177(15), 4481–4487.

Ramana, C. V., Sasikala, C., Arunasri, K., Anil Kumar, P., Srinivas, T. N. R., Shivaji, S. & Imhoff, J. F. (2006). *Rubrivivax benzoatilyticus* sp. nov., an aromatic hydrocarbon-degrading purple betaproteobacterium. *International Journal of Systematic and Evolutionary Microbiology*, 56(9), 2157–2164. https://doi.org/10.1099/ijs.0.64209-0

Ramana, V. V., Chakravarthy, S. K., Raj, P. S., Kumar, B. V., Shobha, E., Ramaprasad, E. V. V. & Ramana, C. V. (2012). Descriptions of *Rhodopseudomonas parapalustris* sp. nov, *Rhodopseudomonas harwoodiae* sp. nov. and *Rhodopseudomonas pseudopalustris* sp. nov., and emended description of *Rhodopseudomonas palustris*. *International Journal of Systematic and Evolutionary Microbiology*, (62), 1790–1798. https://doi.org/10.1099/ijs.0.026815-0

Ramana, C. V, Sasikala, C., Arunasri, K., Kumar, P. A., Srinivas, T. N. R., Shivaji, S. & Imhoff, J. F. (2006). *Rubrivivax benzoatilyticus* sp. nov., an aromatic, hydrocarbon-degrading purple betaproteobacterium. *International Journal of Systematic and Evolutionary Microbiology* 65, 2157–2164. https://doi.org/10.1099/ijs.0.64209-0

Rensing, C., Sun, Y., Mitra, B., Rosen, B. P., Ii, Z., & Ii, C. (1998). Pb (II) -translocating P-type ATPases. *Biochemistry*, 273(49), 32614–32617.

Richardson, B. A. (1999). The bromeliad microcosm and the assessment of faunal diversity in a neotropical forest. *Biotropica*, 31(2), 321–336. https://doi.org/10.1111/j.1744-7429.1999.tb00144.x

Richardson, B. A., Rogers, C., & Richardison, M. J. (2000). Nutrients, diversity, and community structure of phytotelm systems in a lower mountain forest, Puerto Rico. *Ecological Entomology*, 25, 348–356. https://doi.org/10.1046/j.1365-2311.2000.00255.x

Roberto, P., Buosi, B., Roberta, L., Utz, P., Meira, B. R. De, Trevizan, B. & Velho, M. (1983). Rainfall influence on species composition of the ciliate community inhabiting bromeliad phytotelmata. *Zoological Studies*, 1–12.

Rosenberg, E., DeLong, E. F., Lory, S., Stackebrandt, E., & Thompson, F. (2012). The prokaryotes: Prokaryotic communities and ecophysiology. https://doi.org/10.1007/978-3-642-30123-0

Rossello-Mora, R., & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiology Ecology*, 25, 39–67.

Sakpirom, J., Kantachote, D., Nunkaew, T., & Khan, E. (2016). Characterizations of purple non-sulfur bacteria isolated from paddy fields , and identification of strains with potential for plant growth-promotion , greenhouse gas mitigation and heavy metal bioremediation. *Research in Microbiology*, 168(3), 1–10. https://doi.org/10.1016/j.resmic.2016.12.001

Sánchez-Porro, C., Martín, S., Mellado, E., & Ventosa, A. (2003). Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *Journal of Applied Microbiology*, 94(2), 295–300. https://doi.org/10.1046/j.1365-2672.2003.01834.x

Sanz-Fernández, M., Rodríguez-Serrano, M., Sevilla-Perea, A., Pena, L., Mingorance, M. D., Sandalio, L. M., & Romero-Puertas, M. C. (2017). Screening Arabidopsis mutants in genes useful for phytoremediation. *Journal of Hazardous Materials*, 335, 143–151. https://doi.org/10.1016/j.jhazmat.2017.04.021

Sasaki, K. E. N., Ikeda, S., Nishizawa, Y., & Hayashi, M. N. (1987). Production of 5-Aminolevulinic Acid by Photosynthetic Bacteria. *J. Ferment. Technol.*, 65(5), 511–515.

Sasaki, K., Watanabe, M., Suda, Y., Ishizuka, A., & Noparatnaraporn, N. (2005). Applications of photosynthetic bacteria for medical fields. *Journal of Bioscience and Bioengineering*, 100(5), 481–488. https://doi.org/10.1263/jbb.100.481

Sasikala, C., & Ramana, C. V. (1995). Biotechnological potentials of anoxygenic phototrophic bacteria. I. Production of single-cell protein, vitamins, ubiquinones, hormones, and enzymes and use in waste treatment. *Advances in Applied Microbiology*, 41, 173–226. https://doi.org/10.1016/S0065-2164(08)70310-1

Seo, J. S., Keum, Y. S., & Li, Q. X. (2009). Bacterial degradation of aromatic compounds. International *Journal of Environmental Research and Public Health*, 6(1), 278-309. https://doi.org/10.3390/ijerph6010278

Shah, J., Jain S., Shukla, A., Gupta, R., Kotnala, R. (2017). A facile non-photocatalytic technique for hydrogen gas production by hydroelectric cell. *International Journal of Hydrogen Energy*, 42(52), 30584-30590. https://doi.org/10.1016/j.ijhydene.2017.10.105

Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Systematic Biology*, 51(3), 492-508.

Silva, G., Duarte, C., Alves, G. M., Lansac-tôha, F. M., & Felipe, L. (2013). *Acta scientiarum* flagellate protist abundance in phytotelmata of *Aechmea distichantha* Lem (Bromeliaceae) in the upper Paraná river basin. *Acta Scientiarum*, 491–498. https://doi.org/10.4025/actascibiolsci.v35i4.17134

Simon, R., Priefer, U., & Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram-negative bacteria. *Biotechnology*, 1(9), 784–791. https://doi.org/10.1038/nbt1183-784

Smith, A. C., & Hussey, M. A. (2016). Gram Stain Protocols. *ASM Protocols*, 1–9.

Soto-Feliciano, K. M. (2011). The purple non-sulfur anoxyphototrophic bacteria from subtropical hypersaline microbial mats in the Cabo Rojo salterns. MS Thesis, University of Puerto Rico at Mayagüez.

Sousa, F. M. P., Morais, P. B., Rosa, C. A., & Rosa, C. A. (2014). *Hagleromyces* gen. nov., a yeast genus in the *Saccharomycetaceae* , and description of *Hagleromyces aurorensis* sp. nov., isolated from water tanks of bromeliads. *Journal of Systematic and Environmental Microbiology*, (64), 2915–2919. https://doi.org/10.1099/ijs.0.063883-0

Taghavi, S., Lesaulnier, C., Monchy, S., Wattiez, R., Mergeay, M., & Lelie, D. (2009). Lead(II) resistance in *Cupriavidus metallidurans* CH34: Interplay between plasmid and chromosomally-located functions. *International Journal of General and Molecular Microbiology*, 96(2), 171–182. https://doi.org/10.1007/s10482-008-9289-0

Tan, I., & Ramamurthi, K. (2014). Spore formation in *Bacillus subtilis*. *NIH Public Access*, 6(3), 212–225. https://doi.org/10.1126/scisignal.2001449

Tchounwou, P. B., Ayensu, W. K., Ninashvili, N., & Sutton, D. (2003). Environmental exposure to mercury and its toxicopathology implications for public health. *Environmental Toxicology*, 18(3), 149–175. https://doi.org/10.1002/tox.10116

Tumer, Z., & Moller, L. B. (2010). Menkes disease. *European Journal of Human Genetics*, 18, 511–518. https://doi.org/10.1038/ejhg.2009.187

U.S. Geological Survey (2012). Mineral commodity summaries 2016. *U.S. Geological Survey*, pp. 96-109.

Vega-Sepúlveda, J. (2009). Bacterias fototróficas anoxigénicas púrpuras no-sulfurosas en la fitotelmata de bromelias en diversos bosques de Puerto Rico. MS Thesis, University of Puerto Rico at Mayaguez, Mayaguez PR.

Venkata Ramana, V., Kalyana Chakravarthy, S., Shalem Raj, P., Vinay Kumar, B., Shobha, E., Ramaprasad, E. V. V & Ramana, C. V. (2012). Descriptions of *Rhodopseudomonas parapalustris* sp. nov., *Rhodopseudomonas harwoodiae* sp. nov. and *Rhodopseudomonas pseudopalustris* sp. nov., and emended description of *Rhodopseudomonas palustris*. *International Journal of Systematic and Evolutionary Microbiology*, 62(8), 1790–1798. https://doi.org/10.1099/ijs.0.026815-0

Vinay Kumar, B., Ramprasad, E. V. V, Sasikala, C., & Ramana, C. V. (2013a). *Rhodopseudomonas pentothenatexigens* sp. nov. and *Rhodopseudomonas thermotolerans* sp. nov., isolated from paddy soils. *International Journal of Systematic and Evolutionary Microbiology*, 63(1), 200–207. https://doi.org/10.1099/ijs.0.038620-0

Vincenzini, M., Marchini, A., Ena, A., & De Philippis, R. (1997). H_2 and poly-β-hydroxybutyrate, two alternative chemicals from purple non-sulfur bacteria. *Biotechnology Letters*, 19(8), 759–762. https://doi.org/10.1023/A:1018336209252

Wang, X., Wu, X., Hu, J., Zhang, A., Chen, D., Yang, H. & Guo, L. (2018). Isolation of a *Rhodobacter sphaeroides* mutant with enhanced hydrogen production capacity from transposon mutagenesis by NH_4^+ nitrogen resource. *International Journal of Hydrogen Energy*, 1–8. https://doi.org/10.1016/j.ijhydene.2018.01.179

Wang, Y., Zhou, P., Tong, J., & Gao, R. (2016a). Advances in the genetic modification of *Rhodobacter sphaeroides* to improve hydrogen production. *Renewable and Sustainable Energy Reviews*, 60, 1312–1318. https://doi.org/10.1016/j.rser.2016.03.001

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Biotechnology*, 173(2), 697–703.

Widdel, F., & Rabus, R. (2001). Anaerobic degradation of saturated and aromatic hydrocarbons. *Current Opinions in Biotechnology*, 12, 259–276.

Woolverton, C., Macdonald, B., & Robison, R. (2007). Colony morphology. *ASM Protocols*, 1–7.

Yukphan, P., Potacharoen, W., Tanasupawat, S., Tanticharoen, M., & Yamada, Y. (2017). *Asaia krungthepensis* sp. nov ., an acetic acid bacterium in the a-Proteobacteria. *International Journal of Systematic and Evolutionary Microbiology*, (2004), 313–316. https://doi.org/10.1099/ijs.0.02734-0

Zeng, Y. H., Chen, X. H., & Jiao, N. Z. (2007). Genetic diversity assessment of anoxygenic photosynthetic bacteria by distance-based grouping analysis of *pufM* sequences. *Letters in Applied Microbiology*, 45(6), 639–645. https://doi.org/10.1111/j.1472-765X.2007.02247.x

Zhao, L., Zhao, C., & Han, D. (2011). Anaerobic utilization of phenanthrene by *Rhodopseudomonas palustris. Biotechnology Letters,* 33, 2135–2140. https://doi.org/10.1007/s10529-011-0681-x

Zhu, X., Venosa, A. D., Suidan, M. T., & Lee, K. (2001). Guidelines for the bioremediation of marine shorelines and freshwater wetlands. US Environmental Protection Agency, Cincinnati USA.