Molecular Analysis, Physiological Study and Biotechnological Capabilities of Blue Pigmented Bacteria from Puerto Rico

By

Vanessa Z. Cardona Cardona

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

MASTER OF SCIENCE
In

BIOLOGY

UNIVERSITY OF PUERTO RICO
MAYAGUEZ CAMPUS

2010

Approved by:

_____	_____
Johannes Schellekens, PhD Member Graduate Committee	Date
_____	_____
Carlos J. Santos Flores, PhD Member Graduate Committee	Date
_____	_____
Carlos Ríos-Velázquez, PhD President, Graduate Committee	Date
_____	_____
Fernando Gilbes Santaella, PhD Graduate School Representative	Date
_____	_____
Nanette Diffoot Carlo, PhD Chairperson of the Department	Date

Abstract

In the microbial world, pigments are one of the most interesting characteristics. There are many bacterial pigments, but some of them, such as blue pigments are unusual. Blue-pigmented bacteria (BPB) are very uncommon; only 4 described genera share this trait. That is why the main objective of this research was to isolate and characterize BPB from Puerto Rico. Three BPB bacteria were isolated, one from Mayagüez, one from Ponce, and one from San German. The isolate from Mayagüez was lost due to a technical failure with the storage place. The other two isolates were morphologically and microscopically analyzed by performing staining and using Scanning Electron Microscopy, and by growing at optimal pH and different temperatures. It was observed that the blue pigment is only produced at 25°C, suggesting that it is sensitive to temperature. The biochemical activities were analyzed using appropriate selective and differential microbiological media and biochemical tests. Previous studies of *Vogesella indigofera* were used to compare with the results of the unknown bacteria. The 16s rDNA's of the microorganisms were amplified, sequenced and analyzed *in silico*. The result of this study showed that the unknown organism is very similar to *V. indigofera*, a microorganism used as a bioindicator for chromium-contaminated areas. The genes involved in the pigment production were amplified in both bacteria. All of the genes were compared with the one portrayed for *V. indigofera* and found to be very similar to the ones described for the mentioned bacteria. Different experiments with metals (chromium and copper) and hydrocarbons (diesel and bio-diesel) were performed to establish the biotechnological potential of the blue pigmented isolates. Of the two isolates only the one from Ponce was able to grow in one of the assays (in 0.2mM copper). Here is an in depth study of the BPB isolated from Puerto Rico.

Resumen

En el mundo microbiano los pigmentos juegan un rol muy importante. Existen varios pigmentos bacterianos, pero algunos de ellos como los pigmentos azules son muy inusuales. Las bacterias que exhiben este pigmento son sumamente extrañas y solo de han descrito 4 géneros que comparten esta única característica. Es por esta razón que el objetivo principal de esta investigación es el aislamiento y la caracterización de bacterias con pigmentación azul aisladas de Puerto Rico. Tres bacterias se aislaron; una de Mayagüez, una de Ponce y una de San Germán. La bacteria aislada en Mayagüez fue perdida luego de un percance con eléctrico en el área de almacenamiento. Las bacterias aisladas de Ponce y San Germán fueron analizadas morfológicamente y microscópicamente realizando tinciones, haciendo uso del Microscopio Electrónico de Rastreo y haciendo pruebas de crecimiento optimo en pH y temperatura. Se pudo observar que el pigmento azul solo era visible cuando la bacteria crecía a 25 °C, sugiriendo que el pigmento es sensitivo al cambio de temperatura. Las actividades bioquímicas fueron analizadas usando medio selectivo apropiado y diferentes pruebas bioquímicas. Estudios anteriores de la bacteria *Vogesella indigofera* fueron utilizados para poder comparar los resultados. El 16s ADN ribosomal de las bacterias aisladas fue amplificado, secuenciado y analizado por bioinformatica. Los resultados de estos estudios nos muestran que las bacterias aisladas son muy similares a *V. indigofera*, una bacteria que ha sido utilizada como bioindicador de áreas contaminadas con cromio hexavalente. Los genes envueltos en la producción del pigmento fueron amplificados en ambas bacterias aisladas. Todas las amplificaciones fueron comparadas con aquellas publicadas para *V. indigofera* y se encontró que son muy similares entre ellas. Diferentes experimentos con metales (cromio y cobre) e hidrocarburos (diesel y biodiesel) se realizaron para poder determinar el potencial biotecnológico de las bacterias con

pigmentación azul aisladas. Solo el aislado de Ponce pudo crecer en el ensayo de cobre (en 0.2mM cobre). A continuación un estudio de las bacterias con pigmentación azul aisladas de Puerto Rico.

Dedication

I want to dedicate this work to the two loves of my life:

To my loving husband Ismael D. Torres Arocho:

Because of you, I am here today. You stood by my side every step of the way. Thank you for your patience, for your advice and for helping me when I needed it the most. Because of you, I was able to finish all this work. Thank you for always understanding me, even when I was not rational. Thank you for putting up with the all things you had to so that I could finish this work. Thank you for always believing in me. I love you with all of my heart.

To my beloved parents Ferdinand Cardona and Iluminada Cardona:

I can not thank you enough for all of the help you have given me through all of this year. First of all, thank you for all your help and encouragement during my undergraduate and graduate studies. If it wasn't for you both I wouldn't complete my studies. Thanks for always believing in me. For all your advise, for giving me courage and all of your unconditional love. Without you I would never had grown to be the woman that I am now. I will always love you both.

Acknowledgements

First of all, I what to thank the Lord, because without Him everything is impossible. He gave me the strength finish this work.

I want to thank all of my family. My parents, for all their support and encouragement during all these years. To my two brothers who are always there for me. Thank you for all your help and support.

To Dr. Carlos Rios Velazquez. Thank you for receiving me in your lab. For all what you have taught me. For all of your support during all these years. For your guidance and for your advise. Thanks to you I can call myself a scientist.

To Dr Johannes Schellekens and Dr. Carlos Santos. Thank you for all your help and advise. Thank you for your help with the manuscript corrections

To my graduate colleges, Alexis, Gloried, Tony, Yaliz, Keyla, and Goro who have help me in one way or another.

Thanks to my very good friend Josue Malave Orengo. Thanks for all your help through all these years. Thank you for your advise, for you patience, for your input in all of this work. But more important, thank you for your friendship, you helped me through some rough times.

Also, I want to thank my great friend Kristina Soto Feliciano. Thank you for all the help that you gave to me all of these years. Thank you for your advise and thank you for your great friendship. You have been like a sister to me.

To the undergraduate students who have helped me with this investigation: Krizia Diaz, Maria de los Angeles, Jeann Marie Maldonado, Alexandra Cordero and David Arroyo.

Thanks to the laboratory technicians Jose Almodovar, for his help with the SEM, and Caroline Rivera for her help with laboratory supplies.

To Magaly Zapata, who has been more than a technician for me. I want to thank you for all the help that you have given me all of these years. Thank you for your unconditional support and for your friendship. I will never forget you.

And last, but not least, my loving husband. Thanks for EVERYTHING, without you this will not be possible. You were the reason I was able to finish this work. I love you with all my heart.

List of figures **Page**

Chapter 1

Figure1.1 Structure of the indigoidine pigment…….. 9

Figure1.2. Comparison of the indigoidine pigment and an alkylated indigoidiene… 9

Figure 1.3 Synthesis of the indigoidine molecule starting with glutamine…….. 11

Chapter 2

Figure 2.1 The Blue Pigmented Bacteria were found only in three municipalities…… 26

Figure 2.2 Macroscopic analysis of the isolated Blue Pigmented Bacteria..……. 27

Figure 2.3 Microscopy analysis of the Blue Pigmented Bacteria-Ponce and Blue Pigmented Bacteria-San German……. 28

Figure 2.4 Anaerobic Growth of the BPB isolates…… 29

Figure 2.5 16S rDNA amplification of BPB isolates in 1.0% of agarose……... 34

Figure 2.7 Phylogenetic analysis of the BPB isolates……. 35

Chapter 3

Figure 3.1 Amplification of the indigoidine genes of the Blue Pigmented Bacteria-Ponce……..……. 46

Figure 3.2 Amplification of the indigoidine genes of the Blue Pigmented Bacteria-San German…….…… 47

Figure 3.3 Sequence alignments of indigoidine genes from the Blue Pigmented Bacteria-Ponce and the Blue Pigmented Bacteria-San German……... 51

Chapter 4

Figure 4.1 Growth of *E. coli* in different concentrations of $CuSO_4$…… 61

Figure 4.2 Growth of the BPB from Ponce in $CuSO_4$……..……. 63

List of tables **Page**

Chapter 1

Table 1.1 Absorption of the indigoidine pigment in different solvents…… 8

Chapter 2

Table 2.1 Effect of temperature on the growth and pigmentation of the Blue Pigmented Bacteria isolates…….. 30

Table 2.2 Growth of the Blue Pigmented Bacteria in different salinity percentages..… 31

Table 2.3 Determination of the optimal pH for the isolates…….. 32

Table 2.4 Biochemical tests…… 33

Chapter 3

Table 3.1 Primer design for the amplification of the genes involved in the pigment production……. 42

Table 3.2 *In-silico* analysis of the indigoidine genes in Blue Pigmented Bacteria-Ponce and Blue Pigmented Bacteria-San German…..... 49

Table 3.3 *In-silico* comparison between the indigoidine genes of Blue Pigmented Bacteria-Ponce and Blue Pigmented Bacteria-San German…… 50

Chapter 4

Table 4.1 Growth of *E. coli* and the isolated Blue Pigmented Bacteria in $CuSO_4$…….. 62

Table of contents **Page**

Abstract……..ii

Resumen…….iii

Dedication……. v

Acknowledgements……vi

List of figures…….viii

List of Tables……..x

Chapter 1. Introduction and Literature Review……....1

1.1 Introduction……....2

1.2 Literature Review……...5

Chapter 2. Isolation of blue-pigmented bacteria from Puerto Rico…….14

2.1 Introduction……...15

2.2 Materials and Methods……..17

2.3. Results……..25

2.4 Discussion…….36

Chapter 3. Detection and characterization of the genes associated with the blue pigment production…….38

3.1 Introduction…….39

3.2 Materials and Methods……41

3.3. Results……45

3.4 Discussion……...51

Chapter 4. Biotechnology capabilities of the Blue Pigmented Bacteria……...53

4.1 Introduction…….54

4.2 Materials and Methods…….56

4.3. Results…….59

4.4 Discussion…….63

Chapter 5. Conclusions and Bibliography……..65

5.1 Conclusions……..66

5.2 Bibliography…….68

Appendix……..…...72

Chapter One

Introduction and Literature Review

1.1 Introduction

Pigments play an important role in physiological and molecular processes of microorganisms such as: photosynthesis, survival to oxidative damage and resistance to UV-radiation. Through the years, pigments have been used as a taxonomic tool for the identification and classification of algae, fungi, and bacteria (Kuhn et al. 1965, Szczepanowska and Lovett 1992). Pigment diversity is due to differences in their chemical structure compositions and the presence of specific chromatophores (Hui and Hulbert 1979). Pigmented microorganisms have awakened the interest of the scientific community, because of their role in taxonomic studies and their biotechnological potential in processes like fermentation and bioprocess engineering (Chatoopadhyay et al. 2008). Expression of the pigments can be affected by a number of environmental factors, including oxygen availability, nutritional condition, temperature, age of the colony, and strain variation (Starr 1958). For example, *Arthrobacter atrocyaneus* exhibits blue pigmentation when grown at 24ºC, however no pigmentation is observed at 37ºC (Kuhn and Starr 1960). Other microbes such as *Rhodospirillum rubrum, Micrococcus luteus,* and *Sarcina aurantiaca* exhibit pigmentation when grown at diverse temperatures.

The most widely distributed pigments are the carotenoids. This type of pigment also plays an important role in bacteria, for example, in photosynthetic processes, by preventing photo-damage, and conferring resistance to oxidative damage due to the production of activated forms of oxygen.

Purple and blue pigments in bacteria are less common. Violacein (purple pigment) has been found in the genera *Chromobacterium, Janthinobacter* and *Iodobacter* (Moss

2002). Indigoidine (blue pigment) has been found in bacteria that belong to the genera *Erwinia, Arthrobacter, Corynebacterium* and *Vogesella*.

Vogesella indigofera was first described in 1893 by Otto Voges (Kuhn et al. 1965) and he named it *Bacillus indigofera*. At the beginning of the 20th century, the name of the bacterium was changed to *Pseudomonas indigofera.* After a systematic study performed by Grimes et al. (1997), *P. indigofera* was placed in the genus *Vogesella*. *V. indigofera* had been isolated previously from pond sediments, natural aquatic systems, and soil. It is a member of the beta subdivision of the *Proteobacteria*, closely related to the genus *Chromobacterium*. It is an organism which might easily be overlooked during isolation of other oxidative bacteria, because the characteristic pigmentation occurs only in certain media and then only when colonies are reasonably well separated (McFadden and Howes 1960).

The blue pigment produced by this bacterium was studied by Elzari-Volcani, who established that the formation of the blue pigment was due to the production of indigoidine (Kuhn et al. 1965). The specific function of this pigment is still unknown.

In 2003, a blue pigmented bacterium (BPB) was isolated from a soil sample from Mayagüez, Puerto Rico. To our knowledge that was the first BPB isolated and described on the island. Because of the bacterium characteristic pigment, a full characterization of the organism was made. After analyzing all the results, the bacterium was placed near the genus *Vogesella*. In 2005, the bacterium was lost due to a technical failure with the storage place. Therefore, a broad and in depth search for the BPB was started. The area where the BPB was originally found was sampled with no luck. We learned that the soil from where the BPB was found came from a region near a river in Mayagüez. A search

for the bacterium was carried out in soils of nearby rivers, without success. It was not until September 2006 that a second BPB was found in Ponce, and during the summer of 2007 a third BPB was found in San German.

The main focus of this investigation was to characterize biologically, molecularly, and physiologically the BPB's. Molecular genetics was used in order to identify and amplify the genes that were associated with the production of the blue pigment. Also, to test the potential application of the pigmented bacteria, several studies were carried out to determine their capability as bioprospects.

A detailed taxonomic study was done, and here we present the description for the two pigmented bacteria, the one isolated from San German and the other one isolated from Ponce.

1.2 Literature Review

Elazari Volcani (1939) studied the pigment of *Pseudomonas indigofera* (now named *Vogesella indigofera*) and named it indigoidine (in McFadden and Howes 1960). Volcani made a chemical analysis of *Pseudomonas indigofera*; this analysis helped to compare its blue pigment with the ones produced by other bacteria. His conclusion was that there were only a few bacteria that contain this unusual pigment: *Corynebactrium insidosium, Erwinia chrysanthemi, Arthrobacter polychromagenes,* and *Arthrobacter atrocyaneus* (Kuhn et al. 1965, Starr et al. 1966).

Taxonomic Features of Blue Pigmented Bacteria (BPB)

a. *Vogesella indigofera*

What has been presumed to be the first blue pigmented bacterium was isolated in 1890 from the Spree River by Claessen, but he did not name this organism in any of his publications. In 1892, Germano mentioned the organism and incorrectly named it *Bacillus berolinensis indicus*. In 1893, Voges found a different bacterium in the water from Kiel (a city in northern Germany), and named it *Bacillus indigoferus* (Kuhn et al. 1965). In 1894, Schneider received the organism isolated by Claessen from Migula and referred to it as *Bacillus indigonaceus*.

It was not until 1900, that Migula named the bacterium isolated by Cleassen as *Pseudomonas berolinensis* and the bacterium isolated by Voges as *Pseudomonas indigofera*. But there were different opinions among the scientific community, as some scientists thought that these two bacteria were the same.

Grimes et al. (1997) performed a systematic study in which they isolated 13 blue pigmented strains. The first one was isolated in 1973 from sediments of a pond used for 20 years for the disposal of chemical wastes. The other 12 strains were isolated over the next 11 years from freshwater samples. These strains were used to demonstrate that *Pseudomonas indigofera* should be renamed *Vogesella indigofera* (Grimes et al. 1997).

b. *Corynebacterium insidiosum*

This bacterium was characterized by the formation of a blue insoluble extracellular pigment which occurs as particles in the yellow cell mass (Starr 1958). It has been reported that different conditions may affect the production of the blue pigment. Some of the conditions were: strain variation, aerobic condition, composition of the medium (pH and amino acids), crowding and aeration, temperature, and age of the colony.

Starr (1958) reported that one of the most striking properties of the blue pigment is its insolubility in the usual organic laboratory reagents. It is not soluble in water, in boiling ethanol, diethyl ether, acetone, benzene, petroleum, carbon disulfide, chloroform, and carbon tetrachloride, among others. These culture conditions are shared with other BPB. *Corynebacterium* and *Pseudomonas* pigments were identical according to the following criteria: factors affecting production by the microbe, solubility, absorption spectrum, formation and properties of derivatives (Starr 1958).

c. *Arthrobacter atrocyaneus*

This bacterium was found in the fall of 1954. It produces the blue pigment only when sugar is present in peptone medium; although heavy growth occurs without sugar, no blue pigment is formed. Another culture condition strongly affecting pigmentation is

temperature. The optimum temperature for growth was 37ºC, at which temperature no blue pigment is produced; the bacterium grows well at 24ºC with abundant blue pigmentation (Kuhn and Starr 1960). *A. atrocyaneus* exhibits no crowding effect; pigment is formed throughout confluent growth over the entire agar surface (Kuhn and Star 1960).

d. *Erwinia chrysanthemi*

E. chrysanthemi is a plant-pathogenic enterobacterium in which the main virulence determinants are pectase lyases (Reverchon 2002). It was reported that when grown in potato medium it produces more indigoidine. Also, in most cases, the blue pigment occurred transiently only in young cultures and soon disappeared, which is different when compared with *Corynebacterium insidiosum* and *Vogesella indigofera* (Starr et al. 1966).

Properties of the bacterial blue pigment Indigoidine

As mentioned, indigoidine is a characteristic blue pigment that can be found in very few bacteria. One of the first studies of the properties of the pigment was carried out on *Corynebacterium insidioum* (Starr 1958). This study presents absorption spectra of the pigment in different solvents. In pyridine the pigment has an absorption maximum at 605-606 nm. In dimethylformamide the peak was observed in 605 nm and in dimethyl sulfoxide at 617 nm (Starr 1958).

A few years later, Kuhn et al. (1965) suggested that the empirical formula of the pigment is $C_{10}H_9N_4O_4$. He also used an infra-red spectrum to determine that the structure

of the pigment is 5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenoquinone-(2,2') (figure 1.1). They also determined the absorbance of indigoidine in different solvents

Table 1.1 Absorption of the indigoidine pigment in different solvent.

Solvent	Indigoidine (nm)
Me_2SO	612
Dimethylformamide	602
N-methylpyrrolidone	605
Pyridine	603
Tetrahydrofuran	589

Starr et al. (1966) made another spectra study to the genera *Erwinia. E. maydis* presented a spectrum absorption in dimethylformamide at 599 nm, for the bacterium *E. chrysanthemi* was at 601 nm and for *E. cytolytica* at 600 to 601 nm. This is similar to the spectral results found in to *Corynebacterium insidioum*.

More recently, a new alkylated indigoidine was characterized from *Shewanella violacea* (Kobayashi et al. 2007). The bacterium exhibited a violet pigment when grown in agar media, but not in liquid media. The chemical structure of this pigment was 5,5'-didodecylamino-4,4'-dihydroxy-3,3'-diazadiphenoquinone-(2,2'), which is an alkylated indigoidine (figure 1.2) (Kobayashi et al. 2007).

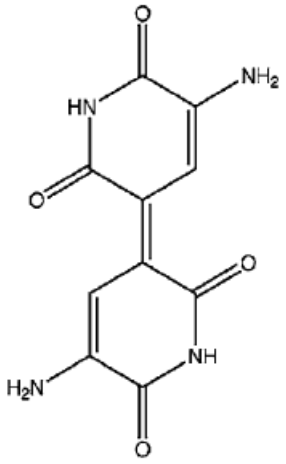


Figure1.1 Structure of indigoidine pigment. Infra-red spectrum determine that the structure of the pigment is 5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenoquinone-(2,2').

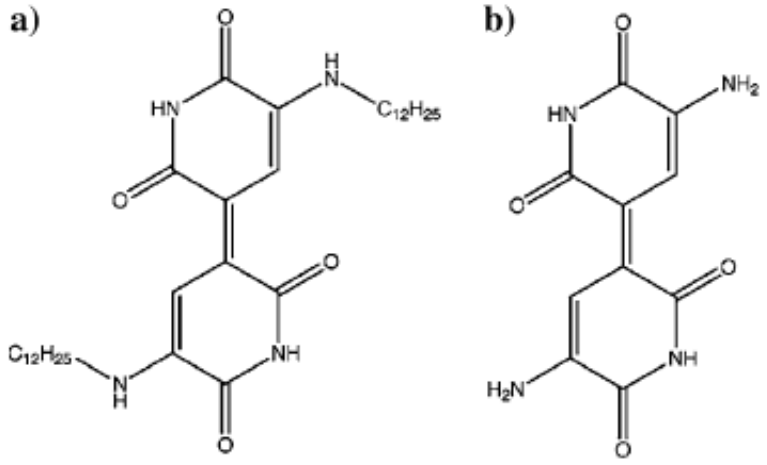


Figure 1.2. Comparison of the indigoidine pigment (a) and the alkylated indigoidine (b). The chemical structure of the pigment was 5,5'-didodecylamino-4,4'-dihydroxy-3,3'-diazadiphenoquinone-(2,2') (Kobayashi et al. 2007). The only visual difference is the alkilated portion of the structure.

A spectral study was carried to the pigment. In the solvents tetrahydrofuran (THF) or chloroform, the pigment showed a broad absorption spectrum from 500 to 700 nm. The absorption maximum of this violet pigment in THF was 616 nm, which was about 30 nm longer than that of indigoidine (587 nm) (Kobayashi et al. 2007). Both, solution and crystals of indigoidine were blue; however, the pigment of *S. violacea* was violet in a crystal or solid state. This shows that the absorption maximum of this pigment shifts down from 616 to 500–580 nm, which is in the same parameter as indigoidine (Kobayashi et al. 2007).

Blue Pigment Genetic Features in *Vogesella indigofera* and *Erwinia chrysanthemi*

Very little is known of the function and the importance of indigoidine. No recent publication exists of the pigment in *V. indigofera*, but the nucleotide sequence of genes involved in indigoidine biosynthesis in *V. indigofera* has been deposited in the European Molecular Biology Laboratory (EMBL) database. These genes are the following: *pecM, pecS, igiA, igiB, igiC, igiD,* and *igiE.* Based on sequence similarities, it was proposed that the *igiA* gene encodes a putative 4'-phosphopantetheine transferase, *igiB* encodes a putative glutamine dehydrogenase, *igiC* encodes a putative *N*-carboxymaleamide decarboxylase, *igiD* encodes a product that corresponds to the peptide synthase, and *igiE* encodes for an ATPase component of an ABC transporter probably involved in indigoidine export (Reverchon et al. 2002).

The most recent molecular study of indigoidine pigment is on the bacterium *Erwinia chrysanthemi* (Reverchon 2002). This bacterium has a regulatory locus which contains the genes *pecM* and *pecS*. It also has tree genes that are proposed to be involved

in the indigoidine biosynthesis: *indA*, *indB*, and *indC*. From the regulatory locus, *PecS* is the regulatory protein that controls the synthesis of indigoidine. The expression of all tree *ind* genes are dependant of the *pecS* gene, proposing that *PecS* is the main regulator. From the biosynthetic locus, it can be inferred the function of the tree genes. The specific function of IndA is still unclear. In contrast, IndB displays similarity to various phosphatases involved in antibiotic synthesis naphthomycin and ansatrienin in *Streptomyces collinus* and mitomycin C in *Streptomyces lavendulae.* IndC reveals significant homology with many non-ribosomal peptide synthase (NRPS). The involvement of a peptide synthetase suggests that an amino acid is the precursor of the pigment. It contains an adenylation domain showing the signature sequence for glutamine recognition and an oxidation domain similar to that found in various thiasole-forming NRPS. These data suggest that glutamine is the precursor of indigoidine. It is assumed that indigoidine results from the condensation of two glutamine molecules that have been previously cyclized by intramolecular amide bond formation and then dehydrogenated (Reverchon et al. 2002).

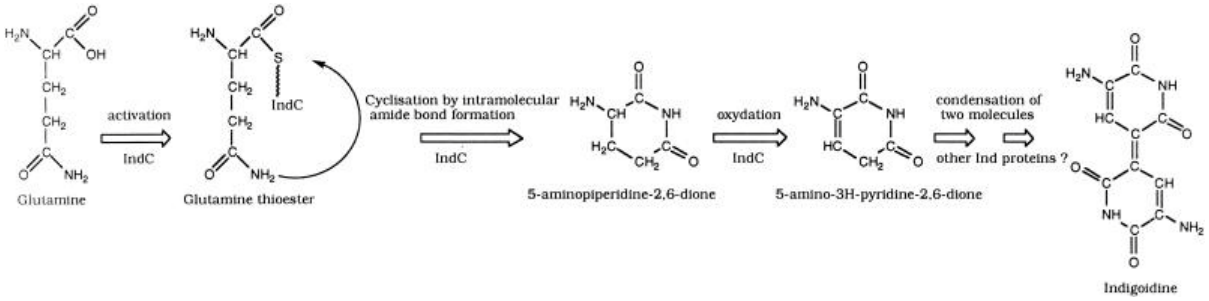


Figure 1.3 Synthesis of the indigoidine molecule starting with glutamine. It starts with the activation with IndC. Then the molecule cyclized by intramolecular amine bond formation and then oxidation by IndC again. Later the condensation of the two molecules could then generate the indigoidine (Riverchon et al. 2002).

The indigoidine loci of *Vogesella indigofera* and *Erwinia chyranthemi* are rather different. The only exceptions are the peptide synthetase genes (*indC* and *igiD*). Neither the phosphatase gene nor the *indA* homologue has been described in the *V. indigofera* indigoidine locus. This suggests that either the indigoidine biosynthesis pathways are different in these two bacteria or that the same set of genes exists in both bacteria but are organized differently (Reverchon et al. 2002). *Vogesella* contains a regulatory couple, homologous to the *pecM* and *pecS* system found in *Erwinia chrysanthemi*, suggesting that the control of indigoidine production is conserved in the two bacteria.

A study of *Streptomyces levandulae* (Takahashi et al. 2007) also mentions genes involved in the pigment production. In the study, the gene *bpsA* (which encodes for a NRPS) was cloned and transformed in *Escherichia coli*. The transformed cells produced a blue pigment, suggesting that *bpsA* is the responsible for the formation of the blue pigment. To produce the blue pigment the presence of 4'-phosphopantetheinyl transferase was necessary (Takahashi et al. 2007). It was also found that the recombinant enzyme prefers L-Gln as substrate. The chemical structure of the pigment was the same as indigoidine. BPSA has a significant similarity with the proteins IndC from *Erwinia chryanthemi* and IgiD from *Vogesella indigofera* (Takahashi et al. 2007).

Biotechnological Features in the Blue Pigmented Bacteria

Of all the BPB, the only one that has been reported to have a biotechnological potential is *V. indigofera*. As the blue color is characteristic and easily observable, the bacterium may have a potential application in the detection and monitoring of environmental pollution (Cheung and Gu 2002).

Gu and Cheung (2001) proposed that "*V. indigofera* might potentially be adopted as a metallic bio-indicator, leading to a new direction for the development and technology of bioassay detecting metal pollution". In this study the biotechnological potential of *V. indigofera* was tested using chromium as metal. In liquid culture, a general decrease in color intensity (i.e. reduced pigment production) with increments in Cr^{+6} concentrations was observed. At 300 (μg Cr^{+6}) ml^{-1} pigment production was inhibited, and bacterial growth was inhibited at 400 (μg Cr^{+6}) ml^{-1}. In solid culture the results were different. At 150 (μg Cr^{+6}) ml^{-1} pigment production was entirely inhibited and only white colonies appeared on the agar plates with. One observation was that the morphology of the bacteria varied at different concentrations of the metal. It was observed that the mean colony size of *V. indigofera* at 0 concentration of Cr^{+6} was approximately 10mm, while that at 150 (μg Cr^{+6}) ml^{-1} it was about 3mm after 10 days of incubation.

Concerning the production of the intense exopolymer, it was observed that the exopolymer production of *V. indigofera* correlated positively with the Cr^{6+} concentration in the culture media. However, the concentrations of exopolymer at each concentration of Cr^{6+} were not distinguishably different, indicating that the exopolymer production might not be induced by the Cr^{6+}. Exopolymer production could be regarded as one of the defensive mechanism performed by bacteria adhering to a surface or against stress including those imparted by metals (Gu and Cheung 2001).

Chapter Two
Isolation of blue-pigmented bacteria from Puerto Rico

2.1 Introduction

Puerto Rico is characterized for having diverse climate conditions. Although it is a small island we can find rain forests, deserts, beaches, caves, lakes and rivers. Previous reports indicate that BPB were isolated from different environments. It can be found in soil, water and in areas near water (Grimes et al. 1997), with the highest probability of finding BPB in soils near water.

There are 1,200 water bodies in Puerto Rico, only 50 of them classify as rivers and most of these flow down from the northern mountain sides to the sea. (http://www.prfrogui.com/geocities/rios.htm). The Cordillera Central divides the northern (Atlantic) and southern (Caribbean) watersheds. The northern rivers are long, rich and tranquil waters in comparison to the southern rivers, and the northern coast is wet and green. In the western region of the island, the Rio Grande de Añasco extends for about 65 km, and is part of a system of rivers that flows down from the Cordillera Central to the west, including the Rio Culebrinas, which flows through Lares and San Sebastian 45 km inland (http://www.prfrogui.com/geocities/rios.htm). In the southwest, mangroves have created a unique canal system. Puerto Rican rivers are not navigable by large vessels, but they provide electrical power and irrigation (http://welcome.topuertorico.org/geogra.shtml).

No studies of the isolation and characterization of BPB in Puerto Rico were published. In fact no recent reports on the isolation of these bacteria exist. The environment in which the BPB can be found has not been fully described. Therefore, a broad and in depth study about the BPB is an important topic to be investigated, and this study describes the isolation and characterization of BPB. The isolates described and

discussed in this section were isolated from Ponce and San German. The sampling time was from August 2003 to May 2008.

2.2 Materials and Methods

Soil analysis

A geological study was carried out for the sites where the PR-BPB was isolated, to determine the mineral composition and abundance in the collecting area.

Mineral analysis

The soil samples were sent to the Geology Department at the UPR-Mayagüez. The samples were analyzed using Siemens D500 X-Ray diffraction instrument. The instrument produces a read out where the refractive ray produces a characteristic peak at a specific angle of refraction (2Θ-) that corresponds to a plane in the crystal structure of the mineral, distinguishing the mineral species (see appendix).

Culture Media Preparation

General used media such as Nutrient Agar, Nutrient Broth, Luria Bertani (LB) Agar and LB Broth were used to grow the isolated bacteria. Nutrient Agar and Nutrient Broth were purchased from Difco ® and LB Agar and LB Broth from BD ®.

Chalk Agar Medium

Chalk Agar is a media used to enhance the pigment in pigmented bacteria. To prepare the medium, 5 g of glucose, 15 g of agar, 40 g of chalk powder, and 5 g of yeast extract were added to 1L distilled water and then boiled for 15 minutes. The mixture was stirred and boiled until it was homogeneous. The medium was sterilized by the autoclave at 15psi, 15 minutes at 121ºC. The sterilized medium was cooled until reaching approximately 50 ºC, and then poured into petri plates.

Vogesella Agar Medium

To prepare the medium, 10 g of yeast extract, 5 g of glucose, 0.5 g of sodium acetate, and 12 g of agar were measured and then added to 1L of distilled water. The mixture was stirred and boiled until it was homogeneous. The medium was sterilized by the autoclave as mentioned before above and then poured into petri plates

Sampling Areas and Sample Collection

Samples were collected from the western and south region of Puerto Rico which included the municipalities of Camuy, Isabela, Aguada, Rincon, Mayagüez, Cabo Rojo, San German, Ponce, and Utuado (Fig.1). Soil samples were collected from the shore of the nearby water bodies and then were processed.

Sample Processing

The samples were processed by soil dilution adding 1g of soil to a 10mL tube with physiologic saline solution (0.85% NaCl) at pH of 7.2. Serial dilutions were made to a final dilution of 10^{-4} and then spread on Nutrient Agar (NA) plates. The NA plates were incubated at 25ºC for 3-4 days and verified for the presence of BPB.

Macroscopic analysis

The isolates were analyzed at macroscopic level by general morphological description of the colony such as the elevation, form, and margin, using the criteria described by Harley and Prescott (1999). The colonies were also measured.

Microscopic Analysis

The bacterium was microscopically analyzed using the Olympus Optical Co., LTD CHT light microscope. To determine the dimension of the bacteria, 100 of them

were selected and measured to calculate the average size. Gram stain was performed using heat-fixed smears. To observe the shape and dimension of the bacterium, simple stain was performed. Microscopic staining was done to determine the presence of structure such as capsule and endospore (Herley 1999).

Scanning Electron Microscopy

For the Scanning Electron Microscopy (SEM) the bacterium were observed using the JEOL JSM-541 OL SEM microscope. For the analysis, the isolates were grown in Nutrient Broth for 24 hours at 25 ºC in a orbital shaker at 120 RPM. Then, approximately, 1.5 mL of the culture was transferred to a microtube and centrifuged for 1 min at 0.8 g (3,000 RPM) to obtain a cell pellet. Glutaraldehyde at 4% was added to fix the cell pellet and then was left resting for 24 hours at 4ºC. The cell pellet was then rinsed with Phosphate Buffer 1M three times. To dehydrate the pellet, different percentage of alcohol were used range from 10% to 100% in 10% intervals. Every ten minutes the pellet was transferred from alcohol to another. To completely dry the sample, a critical point drier was used. A sputter coater was used to give the samples a palladium-gold cover in order to protect the sample and to add a better conductivity to the specimen. The parameters used above were established by the Scanning Electron Microscopy Center at the University of Puerto Rico, Mayagüez Campus. The photos were taken at 15 kv.

Anaerobic Growth

The ability of the isolate to grow in the absence of oxygen was tested. Screw tubes were filled half way with Nutrient Broth, and then the isolates were inoculated in the medium. The rest of the tube was filled completely, making sure that there were no bubbles, with the rest of the media and then the tubes were sealed tightly. The tubes of

the isolate were incubated at 32ºC for one week. The controls used included: *Escherichia coli* incubated at 37ºC for one week as positive control, *Bacillus subtilis* (incubated at 25ºC for one week) as negative control, and a non inoculated tube to prove no contamination.

Growth in different percentage of NaCl

This test was done to determine the percentage of salt that inhibited the growth of the bacteria. Nutrient Broth was used as the base medium because it does not have any salt. Percentages of salt used were: 0%, 0.5%, 1.0%, 1.5% and 2.0%. Briefly, 5mL of the broth was place in tubes where the bacteria were inoculated. The samples were incubated in an orbital shaker at 32 °C for 48 hours. The bacteria used as controls were *Vogesella indigofera* 19706 and *Pseudomonas aeruginosa*.

Optimal temperature determination

To determine the optimal temperature for the pigment production, the isolates were streaked on Nutrient Agar plates. Then plates were incubated for four days at different temperatures ranging from 4 ºC to 42 ºC.

Optimal pH determination

To determine the optimal pH for the bacteria, tubes with Nutrient Broth at different pH were prepared. The pH range used was from 3.0 to 10.0 at 0.5 unit's intervals. HCl and NaOH were used to adjust the corresponding pH. Then the medium was inoculated with the isolates bacteria at logarithmic stage. The samples were incubated at 32 ºC for four days.

Biochemical Tests

In order to determine the physiology of the isolated BPB, a series of biochemical tests were performed. As controls, *Vogesella indigofera* ATCC 19706 and *Pseudomonas aeruginosa* were used. To determine the different enzymes that the bacteria may have, different tests were done. Urea broth was used to see if the bacteria can degrade urea. SIM medium were used to see if the isolates could degrade tryptophan to indole, using the enzyme tryptophanase, and to determine the production of H_2S and to see if the bacterium in motile. Starch Agar Media was used to see if the bacterium produced the enzyme amylase. The Nitrate Broth was used to see if the bacterium has the ability to reduce the nitrate to nitrite. The Nutrient Gelatin Agar was used to see if the isolate produced the exo-enzyme gelatinase. To determine the carbohydrate utilization, assays were performed with sucrose, dextrose, maltose, and mannitol broths with phenol red as indicator. The ability to use glucose, lactose, and sucrose was measured using Triple Sugar Iron Agar (TSIA) slant, the production of H_2S and gas were also observed. The utilization of citrate as only carbon source was measured using Simmons Citrate Agar slants. To detect the conversion of pyruvic acid in acetoin, the Methyl Red (MR) test was used and to see the fermentation of glucose and its transformation to pyruvic acid, we used Voges Proskauer (VP) test. All samples were incubated at 32 ºC for 48 hours. Then a post incubation treatment was added to those tests that required it. To the SIM medium, 2-3 drops of the Kovac reagent were added. To the MR medium 7 drops of Methyl Red reagent were added. To the VP medium 10 drops of α–naphthol and 5 drops of KOH 40% were added, and then results were observed after 30 minutes. To the nutrient gelatin medium the post incubation treatment was 30 minutes in the refrigerator. To the starch

agar medium 2-3 drops of iodine were added. To the nitrate medium 1 drop of sulfanilic acid and 1 drop of α-naphtilamine were added; if the result was negative, a small portion of zinc powder was added. These tests are described by Harley and Prescott (1999).

DNA extraction

The genomic DNA of the BPB isolated was extracted using the method described by Chen and Kuo (1993)**.** The samples were inoculated into Nutrient Broth for 24 hours and 1.5 µl of the sample were centrifuged at 15.7 g (13,000 RPM) to form a cell pellet. The cell lysis was performed using lysis buffer (40mM Tris-acetate pH 7.8, 20mM sodium-acetate pH 8.0, 1.0mM EDTA pH 8.0 and 1% SDS) and sodium chloride 5M, then treated with RNAse (20 µg/µl) for 30 minutes at 37ºC. An organic extraction was made using one volume of chloroform and then centrifuged at 15.7 g (13,000 RPM); this step was repeated twice. Then the sample was precipitated with absolute ethanol. The isolated genomic DNA was resuspended in 50 µl of distillated sterile water. The final concentration of the DNA was measured using a biophotometer.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed with the genomic DNA of the isolate as template in order to amplify part of the 16S rDNA gene. The amplification reaction was made using the Green Taq Master Mix by Promega and Oligo (5'-AGAGTTTGATCCTGGCTCAG-3') and Bad (5'-GGTTACCTTGTTAGGACTT-3') as universal bacterial primers (Willmotte and Wachter 1993). The following parameters were used for the reaction: initial denaturalization at 94ºC for 3 minutes; followed by 30 cycles which included denaturalization at 94ºC for 30 seconds, annealing at 52.7ºC for 30 seconds, extension at 72ºC for 1 minute. Then, a final extension at 72ºC for 10 minutes

was performed. The amplicons were purified using a PCR purification kit (Quagene Inc). The PCR product was sequenced in the facilities Macrogene USA (www.macrogenusa.com). *In silico* analysis was done by using available online databases such as Gen Bank in NCBI by the program BLAST. The complete 16S rDNA was obtained by sending the genomic DNA to the facilities of Macrogene USA. The *in-silico* analysis was done as mentioned above.

Phylogenetic Analysis

The BPB isolated 16S rDNA sequence were edited using the program Chromas Lite 2.0.0 and then aligned and edited with BioEdit 7.0.0 The phylogenetic analysis was performed using the MEGA 3.1 program. The distance model used was p-distance and the bootstrap test of phylogeny was calculated for 1000 replicates. The creation of the consensus tree was performed by the Neigbour Joining method and the final tree was drawn with the use of MEGA 3 Tree Explorer.

DNA base Composition by HPLC

The percentage of G+C of the isolates was determined by sending the samples to DSMZ Germany (http://www.dsmz.de). Briefly, 1-2g of bacteria pellet was suspended in iso-propanol / water (1:1, v/v). The samples were sent in tightly sealed screw cap tubes at ambient temperature.

Fatty acid analysis

Bacteria were grown on Nutrient Agar plates at 25ºC for 48 hours. The samples were sent to the Microbial ID, Inc. in Newark, DE (http://www.microbialid.com/). Then, analyzed by the MIDI Sherlock® Microbial Identification System.

DNA-DNA Hybridization

To determine if the isolates belong to the same genus as *Vogesella*, the samples were sent to DSMZ Germany (http://www.dsmz.de). The hybridization was done between the isolates from Ponce and San German. Also both of them were compared with *Vogesella indigofera* T. For this test, 3g of each bacteria pellet was suspended in iso-propanol / water (1:1, v/v). The samples were sent in tightly sealed screw cap tubes at ambient temperature.

2.3 Results

To blue pigmented bacteria were found. One isolated from Ponce (accession number HM236169) and one from San German (accession number HM236170). The first test that was performed was the mineral composition of the soil (see appendix). In the soil from where the BPB-P was isolated it can be observed that the strongest lines are from quartz, quartz is a common mineral in soils. Also it can be observed peaks from vermiculate. In the soils from where the BPB-SG was isolated we observed that the strongest lines are from quartz, the other peaks are very weak. These represent minerals like anorthite, calcite and vermiculite amongst others.

In both blue pigment isolates, the colonies showed a circular form, convex elevation, entire margin, and metallic deep blue color (Fig 2.2). Both isolated bacteria were gram-negative. The BPB-P forms single rods or in pairs of approximately 1- 2.5 μm, and the BPB-SG form curved rods that measured approximately 1.0-1.9 μm (Fig 2.3). Endospores or capsules were not observed in either bacterium.

The optimal temperature growth of the bacteria was also measured. The results indicate that the bacteria grow better at 32 ºC, but without pigmentation. The optimal temperature for the pigment production was 25 ºC (Table 2.1). Both bacteria were not able to grow in the absence of oxygen (Fig 2.4). The pH range that the bacteria were able to grow in was from 5.5 to 8.5 (Table 2.3). The biochemical tests performed to the BPB isolates are shown in Table 2.4. When compared with the results obtained with *V. indigofera* ATCC 19706, there are no differences in any of the test.

Amplification of the 16S rDNA was done by PCR with positive amplification of approximately 1500bp (Fig 2.5). Sequencing of the whole 16S rDNA was performed by Macrogene USA. *In-silico* analysis of both bacteria suggested that they are 99% similar

with each other, but 97% similar with *V. indigofera*. DNA-DNA hybridization suggests that the isolates from San German and Ponce are the same species as *V. indigofera*.

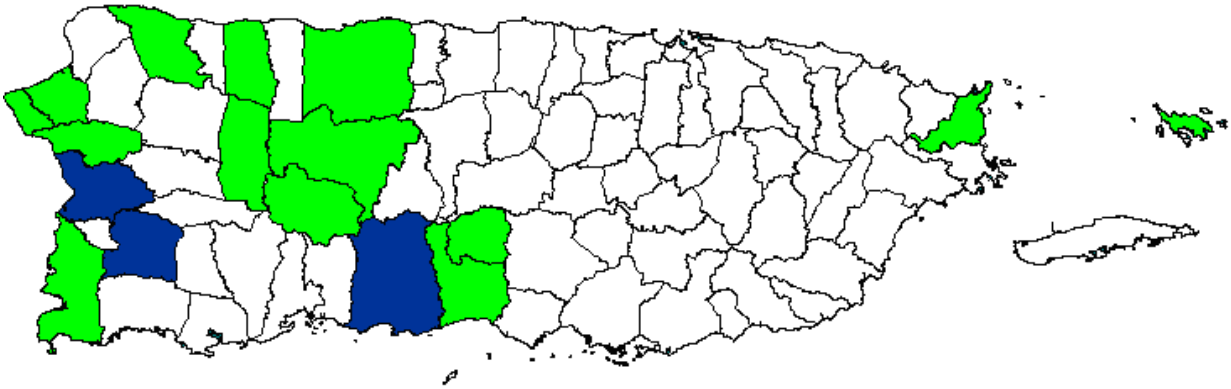


Figure 2.1. The Blue Pigmented Bacteria were found only in tree municipalities. Samples were collected randomly from different areas of Puerto Rico (colored in green and blue). BPB were found in the municipalities of Mayagüez, Ponce and San German (colored in blue).

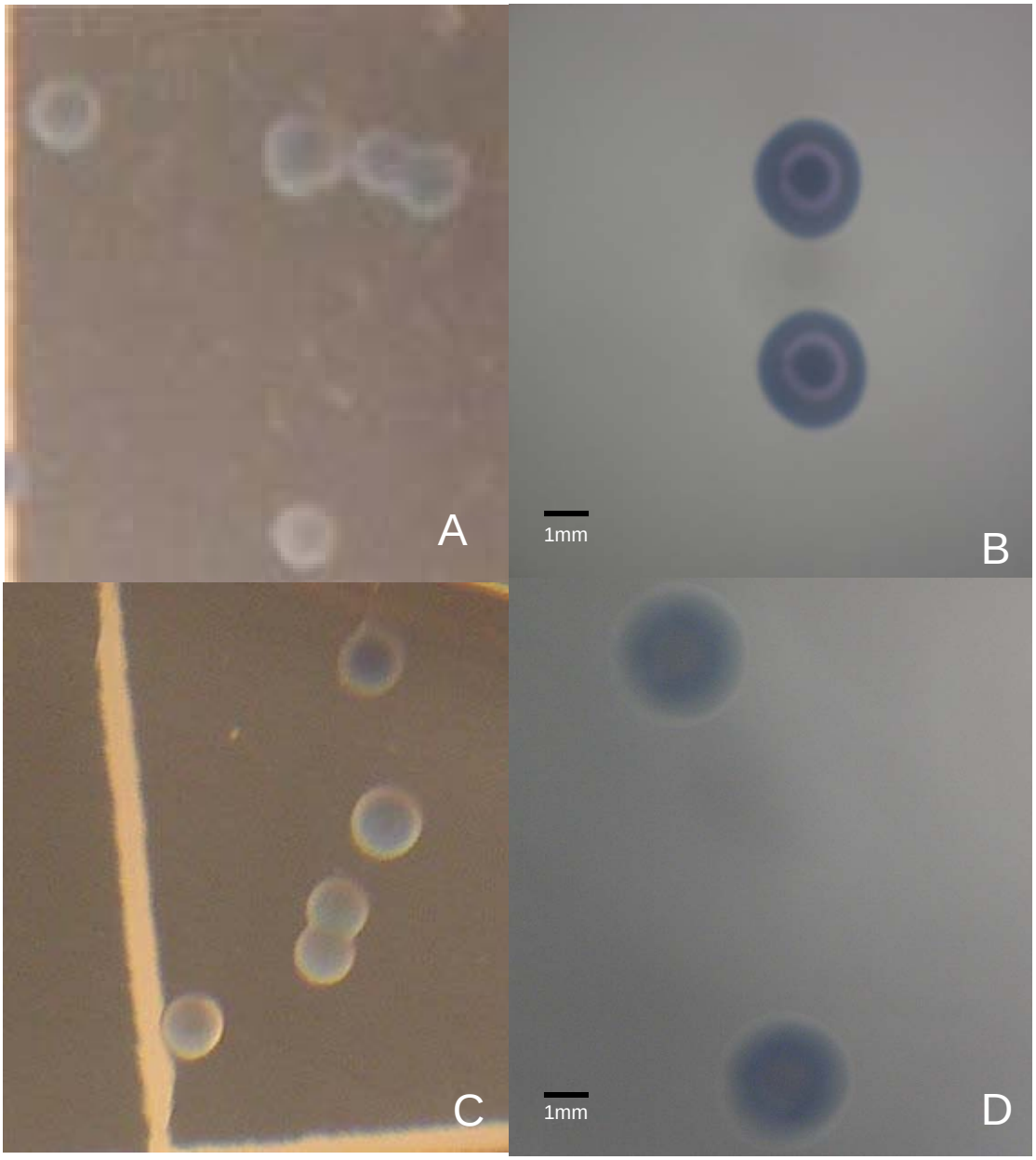


Figure 2.2 Macroscopic analysis of the Blue Pigmented Bacteria (BPB) isolated. The colonies from the BPB-Ponce (A) and the colonies from the BPB-San German (C) were round, entire, and elevated. The colonies from both Ponce (B) and San German (D) measured approximately 2mm.

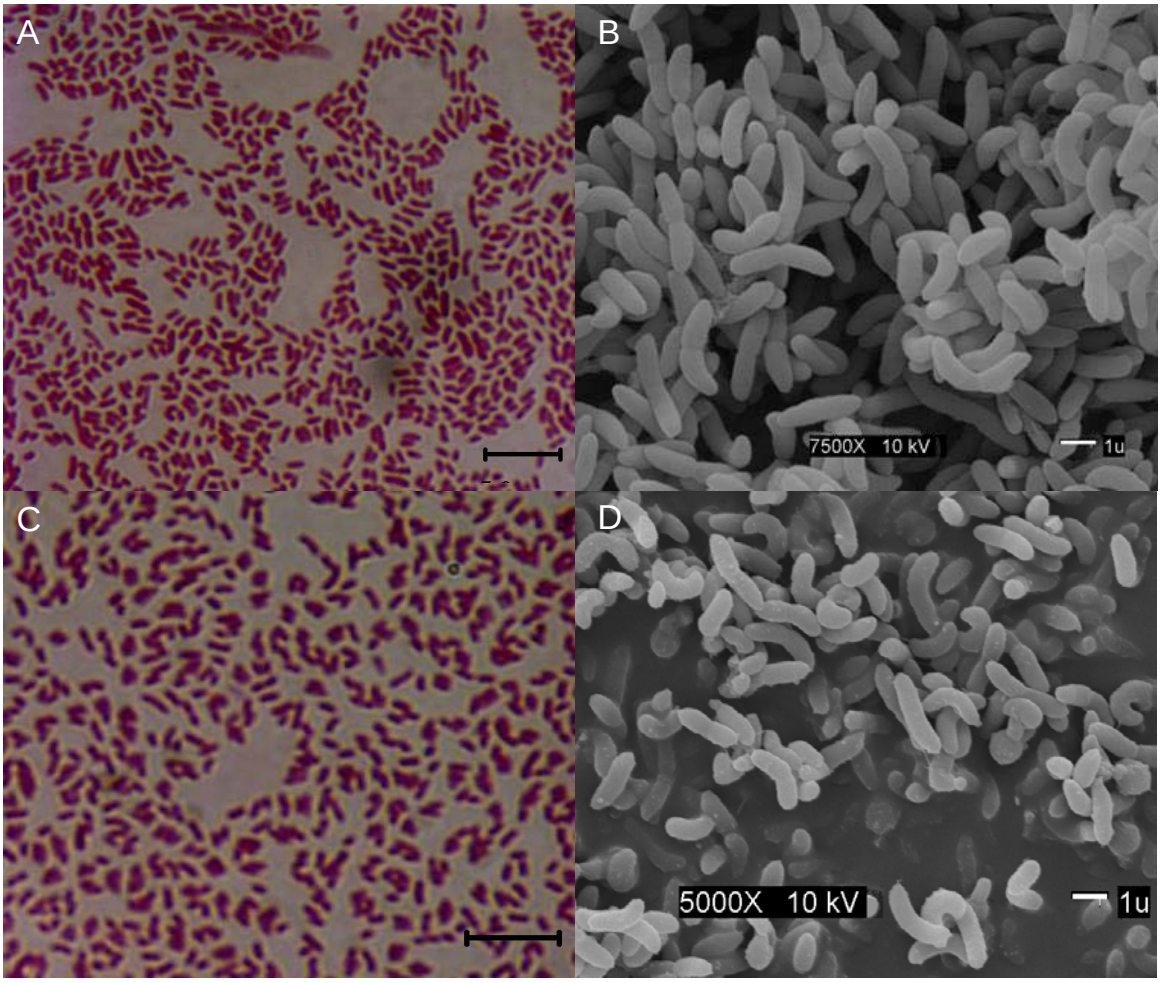


Figure 2.3. Microscopic analysis of the Blue Pigmented Bacteria-Ponce and Blue Pigmented Bacteria-San German. Gram staining of the isolated from Ponce (A) and San German (C) demonstrates that both bacteria are gram- rods (bars measure 5μm). Scanning Electron Microscopy of the isolates from Ponce (B) and San German (D), performed at the UPR-Mayagüez (Biology Department Microscopy Center).

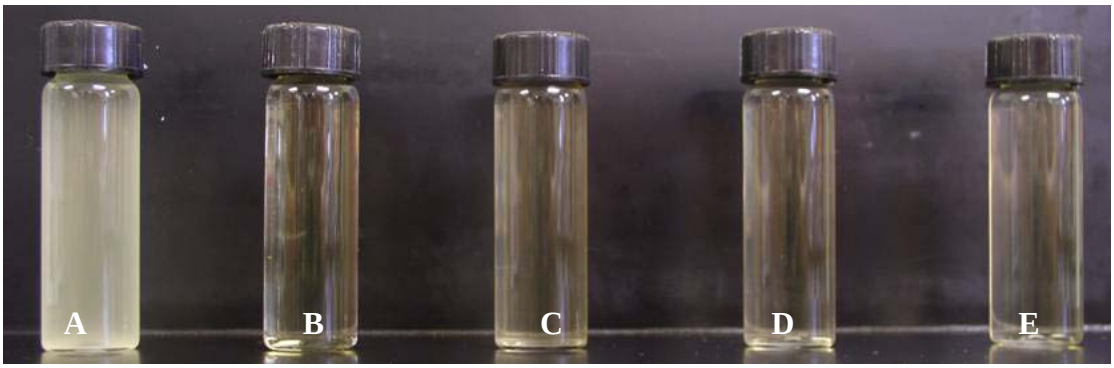


Figure 2.4. Anaerobic Growth of the BPBs isolates. The ability to grow in anaerobic condition was not detected in either the BPB-P (D) or the BPB-SG (E). The controls used in this experiment were *Escherichia coli* (A), *Bacillus subtillis* (B) and a non inoculated tube (C).

Table 2.1 Effect of the temperature on the growth and pigmentation of the BPB's

Temperature (°C)	BPB from Ponce		BPB from San German	
	Growth	Pigmentation	Growth	Pigmentation
25	++	+++	++	+++
30	+++	+	+++	+
37	++	-	++	-
40	+	-	+	-

- No growth; + Poor; ++ Some; +++ Intense

Table 2.2 Growth of the Blue Pigmented Bacteria in different salinity percentages

Salinity %	BPB from Ponce	BPB from San German
0%	**+**	**+**
0.5%	**+**	**+**
1.0%	**+**	**+**
1.5%	**+**	**+**
2.0%	**-**	**-**

+ Growth; - No growth

Table 2.3 Determination of the optimal pH for the isolates.

pH	BPB-P	BPB-SG
3.0	-	-
3.5	-	-
4.0	-	-
4.5	-	-
5.0	-	-
5.5	+	+
6.0	+	+
6.5	++	++
7.0	+++	+++
7.5	++	++
8.0	+	+
8.5	+	+
9.0	-	-
9.5	-	-
10.0	-	-

- No growth; + Poor; ++ Some; +++ Intense

Table 2.4 Biochemical tests

Biochemical test	BPB-Ponce	BPB-San German	*Vogesella indigofera* ATCC 19706	*Pseudomonas aroginosa*
Oxidase	+	+	+	+
Catalase	+	+	+	+
SIM	+/-	+/-	+/-	-/-
TSIA	K/K	K/K	K/K	K/K
SC	-	-	-	+
Urea	-	-	-	-
MSA	-	-	-	-
Nitrate	+	+	+	+
Starch	-	-	-	-
MR	-	-	-	-
VP	-	-	-	-
Gelatin	-	-	-	-

+ Positive; -Negative; K/K Alcaline

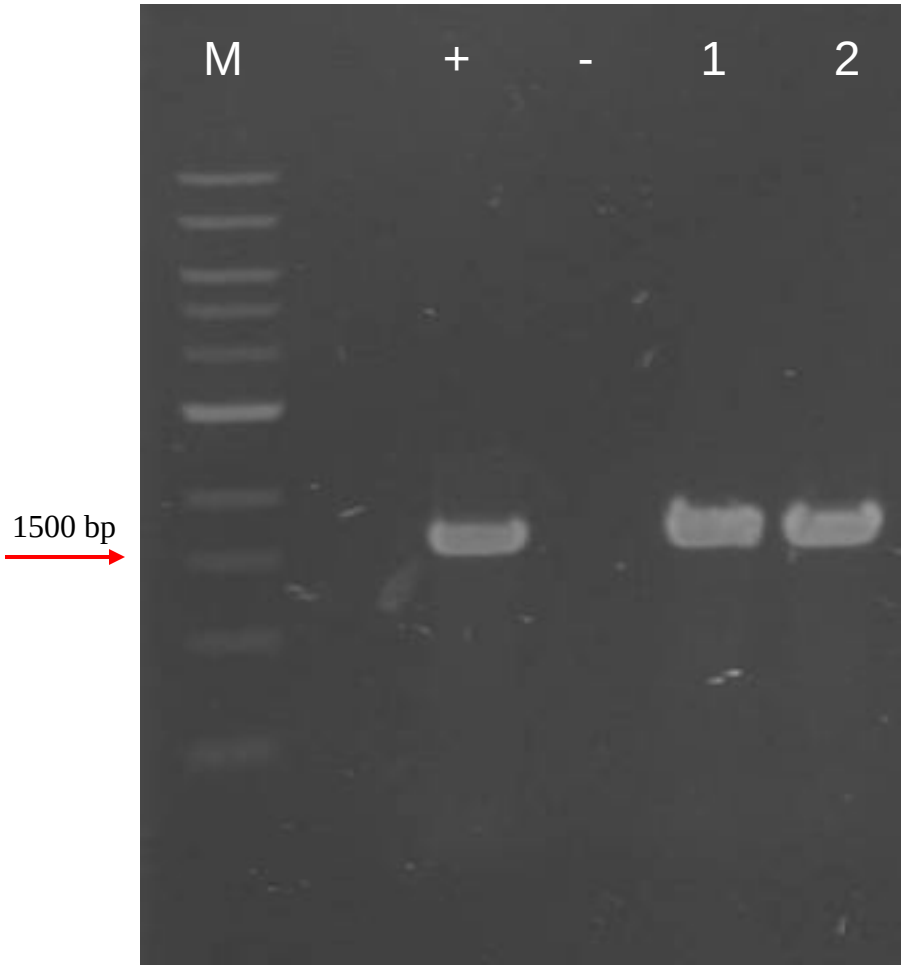


Figure 2.5 16S rDNA amplification of Blue Pigmented Bacteria isolates in 1.0% of agarose. The molecular marker (M) was 1Kb ladder. The positive control (+) was a PCR done by *E. coli* genomic DNA. The negative control (-) was the PCR mixture without DNA. The amplification of the BPB-P and BPB-SG shows an approximate size1500 bp.

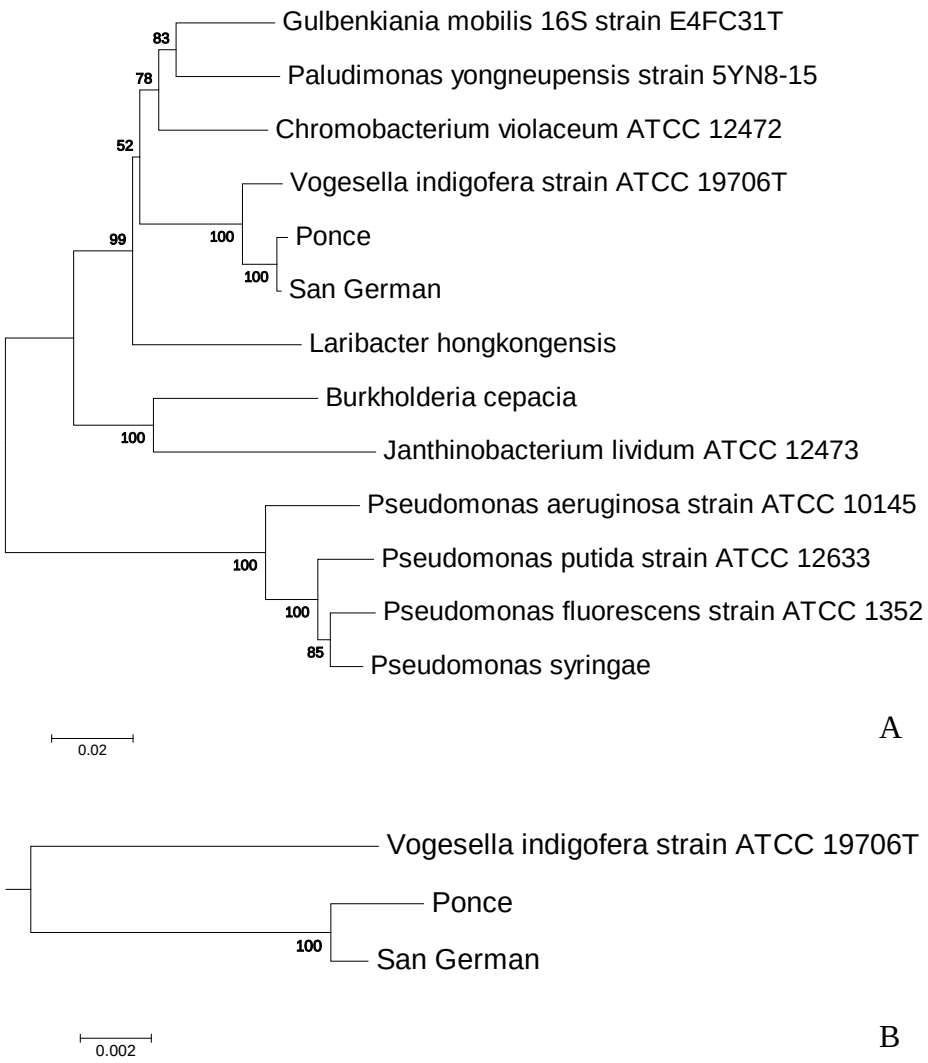


Figure 2.7 Phylogenetic analysis of the BPB. The creation of the consensus tree was made by the Neighbour-Joining method and the final tree was drawn with the use of MEGA 3 Tree Explorer. Numbers in the nodes are the bootstrap values. For the first figure (A) the bar represents 0.02 substitutions per nucleotide position. The second figure (B) is an augmentation of the fragment showing the BPB isolates with *Vogesella indigofera*. The bar represents 0.002 substitutions per nucleotide position.

2.4 Discussion

BPB are very rare so XRD analysis were carried out to see if some unusual mineral or metals were present. The minerals present in both soils are common minerals in these types of soils. No unusual minerals were present to explain the occurrence of BPB. The common feature of the soils was that they occur near water bodies, suggesting that this bacterium prefers humid environment or environments near water.

The data obtained from both BPB were compared with the published data by Grimes (1997), it can be concluded that there is no we found no microscopic, morphological, or physiologic differences between them. Both bacteria are gram negative rods. Physiologically both are aerobic, the optimal growth pH is at 7 and they can not grow in excess of 1.5% of NaCl.

The optimal temperature of both bacteria was 32 °C, but at this temperature neither of the BPB grows with the blue pigmentation. The optimal temperature to produce the pigment was measured at 25 °C. Pigments have been a very important taxonomic tool to identify bacteria species, but identification it's complicated because there are many physical conditions that can make a bacterium change its pigmentation. Some bacteria may lose the pigments when they are in some kind of stress (Kuhn and Starr 1960). In our case it seems that temperature is the stress points that can make the bacteria change its color. The experiment suggests that the bacteria may suffer from a thermal shock, causing the bacteria to lose the pigment at temperatures higher or lower than 25 °C.

When the results are compared to the portion of the 16S rDNA both bacteria have a 99% homology to each other and a 97% homology with *Vogesella indigofera*. Also the

molecular analyses of the whole 16S rDNA and the philogenetic tree suggest that the bacteria may be of the *Vogesella* genus, but they may be different species.

To decide if the bacteria are of the *indigofera* group, several analyses were carried out. One of them was to determine the percentage of G+C of each other. For this analysis, we use the findings of Grimes et al. (1997) to compare the percentages of G+C in all the *Vogesella* strains. *Vogesella indigofera* has a 65.4% of G+C. Our analysis let us know that the isolate from San German has a 64% of G+C and that the isolate from Ponce has a 71% of G+C. Comparing this with the findings of Grimes, we see that the isolate from Ponce shows a great difference. The most important test to determine weather or not they are the same species is DNA-DNA hybridization analysis. The results showed the two isolates from Puerto Rico we had 87.6% of DNA-DNA similarity. When the isolates from Ponce and San German are compared with *Vogesella indigofera* a 97.0% and 92.4% of DNA-DNA similarity is observed, respectively. Because the results were under 70%, the three bacteria should belong to the same species.

Although the two isolates have more than 70% DNA-DNA similarity, the possibility of subspecies is possible. Both bacteria presented differences in the G+C test when compared to the *Vogesella* species. Also, the isolates from Puerto Rico have some physiological difference with the *Vogesella* sp., such as the inability to produce the pigment in liquid medium. The possible existence of a sub-species required additional research.

Chapter Three
Detection and characterization of the genes associated with the blue pigment production

3.1 Introduction

Very little is known about the pigment production in BPB. To our knowledge, the only studies about the genes involved in the pigment biosynthesis are from the bacteria *V. indigofera* and *E. chrysanthemi.* From these two, only for *E. chrysanthemi* exits a more complete study of the pigment biosynthesis and the genes that are involved.

In the case of *V. indigofera*, the only information of the genes involved in the pigment production includes two sequences published in GenBank. The first sequence (accession number AF088856), provides the complete sequence for the putative indigoidine biosynthesis locus. The second sequence (accession number is AF088857), describes the complete sequence of the proposed biosynthesis regulatory locus. Based on sequence similarity, there is some proposed function for each gene. According to the explanation of each sequence in Gen Bank, the *pecM* gene (894bp) is required for the production of indigoidine and is also a putative regulator for *igiC*. The *pecS* gene (513bp) is an alleged regulative protein. The *igiA* gene (882bp) is a putative 4'-phosphopantetheine transferase and also is involved in the biosynthesis of indigoidine. The *igiB* gene (875bp) is a supposed glutamine dehydrogenase and probably involved in the biosynthesis of indigoidine. The *igiC* gene (830bp) is a putative N-carboxymaleamide decarboxylase and is evolved in the biosynthesis of indigoidine. The *igiD* gene (3866bp) is a peptide synthetase homologous and is required for the biosynthesis of indigoidine. The *igiE* gene (1226bp) encodes an ATPase component of an ABC transporter probably involved in indigoidine export.

Because the two isolates were very close to the genus *Vogesella*, the sequence that was used for the generation of specific primers to amplify all the genes were the one in GenBank for this *Vogesella*.

3.2 Material and methods

DNA extraction

The genomic DNA was extracted using the methodology described by Chen and Kuo (1993). Briefly, the samples were grown in Nutrient Broth for 24 hours and then 1.5 µl of the sample were centrifuged to form a bacterial pellet. Cell lysis was performed using lysis buffer (40mM Tris-acetate pH 7.8, 20mM sodium-acetate pH 8.0, 1.0mM EDTA pH 8.0 and 1% SDS) and sodium chloride 5M, then treated with RNAse (20 µg/µl) for 30 minutes at 37ºC. An organic extraction was made adding one volume of chloroform followed by absolute ethanol precipitation. The isolated genomic DNA was resuspended in 50 µl of distillated sterile water. The final concentration of the DNA was measured using an Eppendorf Biophotometer.

Primer design

To amplify the genes proposed to be involved in the indigoidine biosynthesis, the generation of specific primers was necessary. Due to the lack of available primers to amplify *igi* genes, we used the gene *igi* sequences described in GeneBank to design the forward and reverse primers.

Table 3.1 Primer designed for the amplification of the genes involved in the pigment production.

Primer*	Primer length	%GC	Tm	Expected product length
igi A				
F 5'-GTCGGTGGTGAAGGAAAG-3'	18bp	56%	50°C	882bp
R 5'-CAGGGTGTAGACACTGTC-3'	18bp	56%	50°C	
igi B				
F 5'-AGGTCGGGTGCCAGAAAG-3'	18bp	61%	53°C	875bp
R 5'-CACCATTGCCGTATTGGG-3'	18bp	56%	50°C	
igi C				
F 5'-TCTGGGTGGTCCGCGATTTC-3'	21bp	62%	58°C	830bp
R 5'-TGGTCATCGATGCCGCTCATG-3'	20bp	60%	56°C	
igi D1				
P1 5'-AGCCCAGATGAATACCTTGC-3'	20bp	50%	52°C	1931bp
P2 5'-TACCACCCGCTGCAGCATC-3'	19bp	63%	55°C	
igi D2				
P1 5'-GATGCTGCAGCGGGTGGTA-3'	19bp	63%	55°C	1949bp
P2 5'-AGCCGCCGTACTGGCTTC-3'	18bp	67%	55°C	
igi E				
F 5'-TCAACACACCAAGCCGGC-3'	18bp	61%	53°C	1226bp
R 5'-CAGGTCCTGCTGGAATTGC-3'	19bp	58%	53°C	

Tm= Melting Temperature; *All the primers were designed in this study

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify each gene with the genomic DNA of the isolate as template. The amplification reaction was made using the Green Taq Master Mix by Promega.

To amplify the *igiA* gene the specific primers *igi*A F and igiA R were used. The following parameters were used for the reaction: initial denaturalization at 94 ºC for 3 minutes; 30 cycles of denaturalization at 94 ºC for 30 seconds, annealing at 46.9 ºC for 30 seconds, extension at 72 ºC for 1 minute and final extension at 72ºC for 10 minutes.

To amplify the *igiB* gene the specific primers *igi*B F and igiB R were used. The following parameters were used for the reaction: initial denaturalization at 94 ºC for 3 minutes; 30 cycles of denaturalization at 94 ºC for 30 seconds, annealing at 45.0 ºC for 30 seconds, extension at 72 ºC for 1 minute and final extension at 72ºC for 10 minutes.

To amplify the *igiC* gene the specific primers igiC F and igiC R were used. The following parameters were used for the reaction: initial denaturalization at 94 ºC for 3 minutes; 30 cycles of denaturalization at 94 ºC for 30 seconds, annealing at 54.9 ºC for 30 seconds, extension at 72 ºC for 1 minute and final extension at 72ºC for 10 minutes.

The *igiD* gene measures 3,866 bp. To amplify the gene completely, two reactions were made. For the first reaction the specific primers were igiD1 P1 and igiD1 P2. The following parameters were used for the reaction: initial denaturalization at 94 ºC for 3 minutes; 30 cycles of denaturalization at 94 ºC for 30 seconds, annealing at 47.0 ºC for 30 seconds, extension at 72 ºC for 1 minute and final extension at 72 ºC for 10 minutes. For the second reaction the specific primers were IgiD2 P1 and igiD2 P2. The following parameters were used for the reaction: initial denaturalization at 94 ºC for 3 minutes; 30

cycles of denaturalization at 94 ºC for 30 seconds, annealing at 50.0 ºC for 30 seconds, extension at 72 ºC for 1 minute and final extension at 72 ºC for 10 minutes.

To amplify the *igiE* gene we used igiE F and igiE R as specific primers. The following parameters were used for the reaction: initial denaturalization at 94 ºC for 3 minutes; 30 cycles of denaturalization at 94 ºC for 30 seconds, annealing at 51.5 ºC for 30 seconds, extension at 72 ºC for 1 minute and final extension at 72ºC for 10 minutes.

Amplification was purified with a PCR purification kit (Quagene Inc). DNA sequencing of the PCR product was done in the facilities Macrogene USA (www.macrogenusa.com). *In silico* analysis was done by using available online databases such as Gen Bank in NCBI using the program BLAST.

3.3 Results

The *igiA*, *igiB*, *igiC*, *igiD* and *igiE* genes were successfully amplified (Fig 3.1 and Fig 3.2) in the bacteria isolated from Ponce and the one isolated from San German using the primers designed in the laboratory (Table 3.1). Each gene sequences were compared with the *Vogesella indigofera* sequence (Table 3.1) using Blast program. Also the indigoidine putative genes sequences of both bacteria were compared to each other (Table 3.2). In the genes of *igiA*, *igiB*, *igiC* and *igiD* no significant difference were found (Table 3.2). Only the *igiE* gene could not be compared because no significant similarity was found.

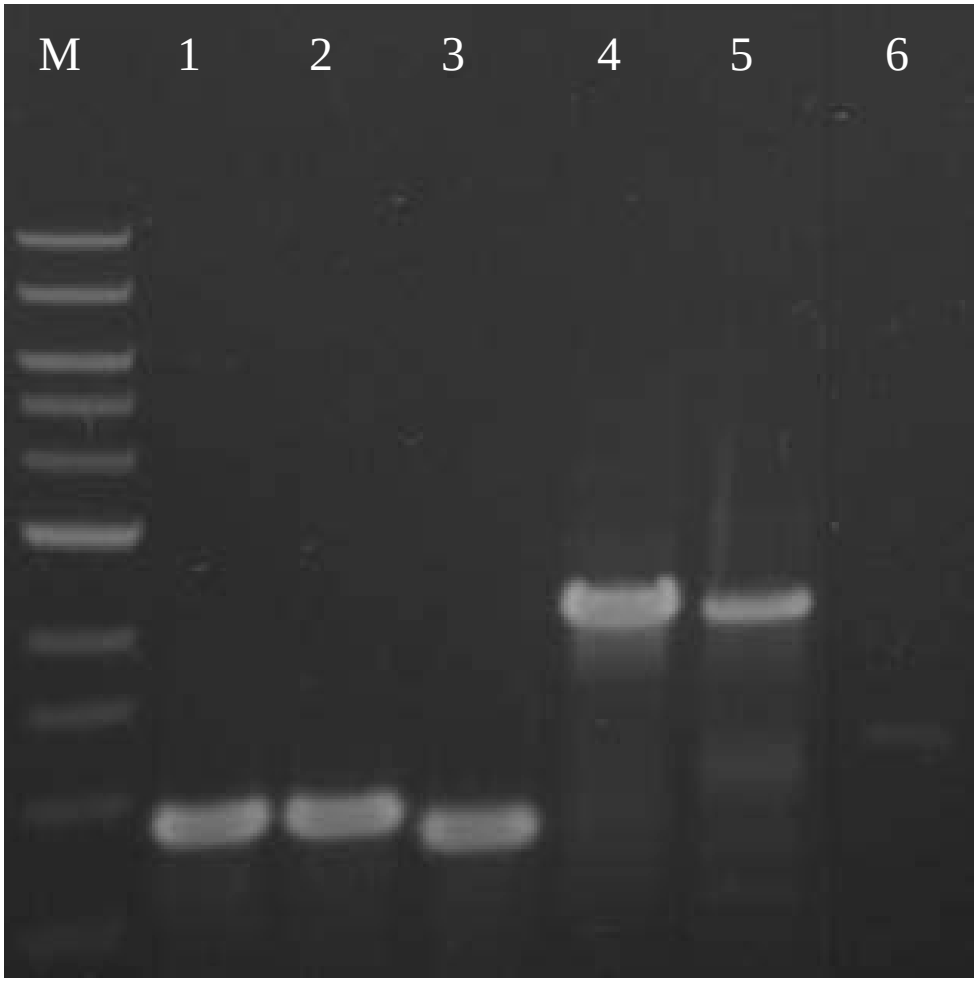


Figure 3.1 Amplification of the indigoidine genes of the Blue Pigmented Bacteria-Ponce. The samples were run by electrophoresis in a 1% agarose gel, using 1Kb (M) as molecular marker. The sample from lane 1-5 represents the amplicons from the genes *igiA, igiB, igiC igiD* (lane 4 and 5) and *igiE*, respectively.

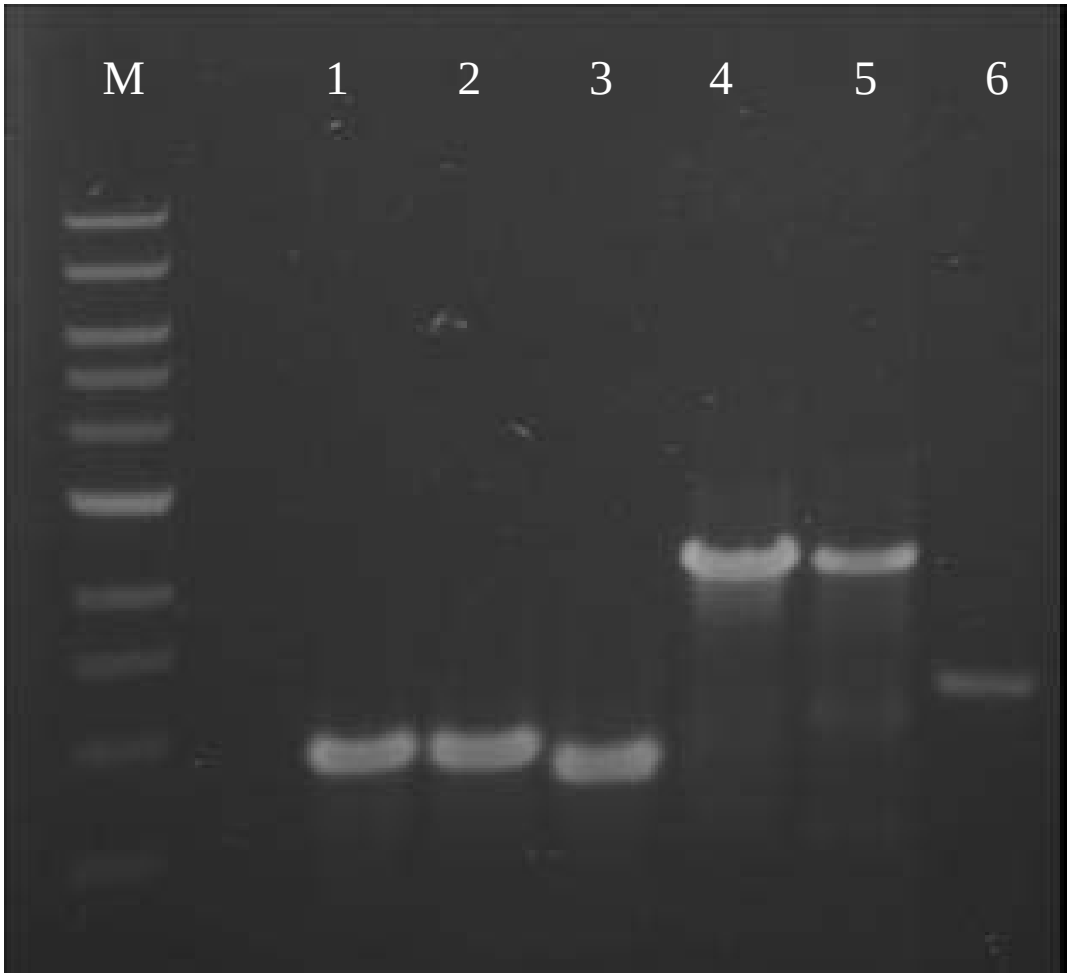


Figure 3.2 Amplification of the indigoidine genes of the Blue Pigmented Bacteria-San German. The samples were run by electrophoresis in a 1% agarose gel, using 1Kb (M) as molecular marker. The sample from lane 1-5 represents the amplicons from the genes *igiA*, *igiB*, *igiC igiD* (lane 4 and 5) and *igiE*, respectively.

Table 3.2 *In-silico* analysis of the indigoidine genes from Blue Pigmented Bacteria-Ponce and Blue Pigmented Bacteria-San German.

	Gene length in *V. indigofera*	Query length	Query coverage	Max Identity	Accession number
BPB-P					
igiA	882bp	816bp	98%	93%	HM488354
igiB	875bp	798bp	98%	95%	HM488355
igiC	830bp	743bp	98%	92%	HM488356
igiD	3866bp	2502bp	83%	93%	HM488357
igiE	1226bp	1150bp	79%	92%	HM488358
BPB-SG					
igiA	882bp	814bp	100%	93%	HM488359
igiB	875bp	797bp	98%	95%	HM488360
igiC	830bp	720bp	98%	92%	HM488361
igiD	3866bp	2375bp	99%	94%	HM488362
igiE	1226bp	917bp	92%	87%	HM488363

BPB-P= Blue pigmented bacterium from Ponce; BPB-SG= Blue Pigmented bacterium from San German.
For each one of the amplifications the E value was 0.

Table 3.3 *In-silico* comparison between the indigoidine genes from Blue Pigmented Bacteria-Ponce and Blue Pigmented Bacteria-San German.

Indigoidine genes	Total Score	Query coverage	E value	Max Identity
igiA-P vs *igi A*-SG	1445	99%	0.0	98%
igiB-P vs *igi B*-SG	1456	99%	0.0	99%
igiC-P vs *igiC*-SG	1306	96%	0.0	99%
igiD-P vs *igiD*-SG	1642	76%	0.0	99%
igiE	N/A	N/A	N/A	N/A

BPB-P= Blue pigmented bacterium from Ponce**;** BPB-SG= Blue Pigmented bacterium from San German**;** N/A= Not Applicable

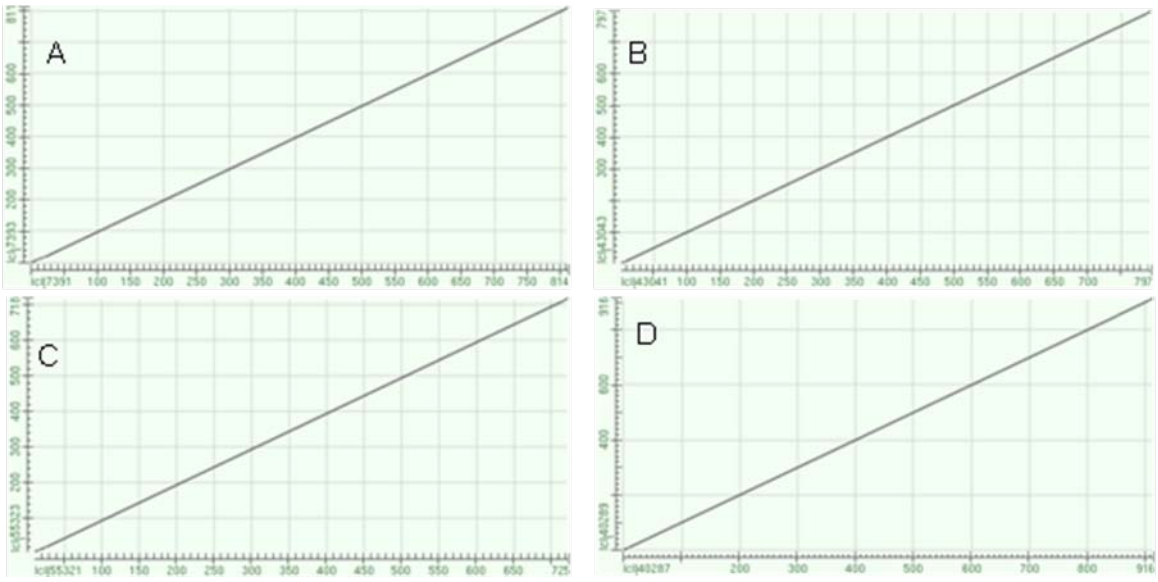


Figure 3.3 Sequence alignments of the indigoidine genes from the Blue Pigmented Bacteria-Ponce and the Blue Pigmented Bacteria-San German. The length of *igiA* (A), *igiB* (B), *igiC* (C) and *igiD* (D) are very similar between the isolated from Ponce and the isolated of San German. The *igiE* gene showed no significant similarity.

3.4 Discussion

One of the objectives of this investigation was to determine which genes were involved in the pigment production of the isolated blue pigmented bacteria. For this we used the sequence that was in the GenBank described for *Vogesella indigofera*. With this sequence we made specific primers to amplify these genes. All of the genes were positively amplified in both bacteria.

For the *igiA* gene we obtain an amplification of 816bp for the isolated from Ponce and 814 from the isolated from San German. When we compared this with the gen describe for *Vogesella indigofera* (882bp) we can see that the genes are almost the same length. Additionally, the *in-silico* analysis suggests that both isolates have a 93% of max identity when compared with the described bacteria. This percentage shows that both genes are very similar to the one described for *Vogesella indigofera*. The genes of both bacteria were also compared to each other and we obtained a maximum identity of 98%.

The *igiB* gene was also amplified in both bacteria. In this case, an amplification of 798bp and 797bp was obtained from the isolated of Ponce and for the one from San German respectively. The GenBank data base shows that *Vogesella indigofera* has a gene extension of 875bp, so both bacteria have a 95% of maximum identity with these bacteria. Between each other they have a 99% of max identity. In this case we can conclude that all of the amplified *igiB* genes are the same for the tree bacteria. The 95% can be due to the lack of sequence to compare it with.

In the case of the *igiC* gene, an amplification of 743bp and 720bp was obtained from the isolates of Ponce and for the one from San German respectively. For this gene *Vogesella indigofera* has a gene length of 830bp. When the amplicons of both bacteria are compared with the described one, we find a 92% similarity. But when the two

isolated from Puerto Rico are compared with each other they have a 99% of maximum identity. This great difference may be due to the length of the area that was amplified from the two isolated bacteria.

Because the igiD was too big, it was divided in two so it may be amplified. The obtained sequence united and compared with the one describe in *Vogesella indigofera.* Like the previous discussed genes, both sequences were very similar to each other.

The last gene that was amplified was the *igiE* gene. For this gene we obtained a 1150bp amplification for the isolate from Ponce and a 917bp amplification for the isolate from San German. The data show that the length of this gene in *V. indogofera* is of 1226. When we make an *in-silico* comparison of the genes from both isolates with the one already described we see that there is a 92% homology for the blue pigmented bacteria isolated from Ponce and an 87% homology for the one isolated from San German. This difference is due because the amplification length of the isolated from Ponce was longer that the one from San German. This is why no significant similarities were found between the genes of both isolates.

Because all of the genes that we amplified were very similar to the ones described for *Vogesella indigofera*, we can conclude that the pigment that the two isolates have is indigoidine. Genetically, not much is known about how this pigment functions in this bacterium. Actually, no work is done about the genes that produce the pigmentation. The only thing that is published are the sequence of the genes and their putative function. Both isolates share the same genes as *V.indigofera* but additional research will have to be done to determine the function of each gene in the synthesis of the pigmentation.

Chapter Four
Biotechnological capabilities of the Blue Pigmented Bacteria

4.1 Introduction

The threat of metal contamination to the public health has been a reason to remove or neutralize contaminants from the soil. In contrast to organic contaminants, such as diesel or biodiesel, heavy metals can not be biodegraded to non toxic chemical waste, but it may be bio-accumulated, bio-attenuated, or reduced direct/indirectly. Bioadsorption mechanisms involved in the process may include ion exchange, co-ordination, complexation, chelation, adsorption, micro-precipitation, diffusion through cell walls and membranes which differs depending on the species used for the origin and processing of the biomass, and solution chemistry (Igbal et al. 2005).

Every day scientists are looking for different tools or strategies to solve such contaminant problems. One of these tools may be bacterial pigmentation because of its possible use as a biotechnological tool. One of these bacterial groups are the Blue Pigmented Bacteria (BPB).

Of all the BPB, the only one that has been reported to have a biotechnological potential is *V. indigofera* that has a response to hexavalent chromium. As the blue color is characteristic and easily observable, the bacterium may have a potential application in the detection and monitoring of environmental pollution (Cheung and Gu 2002). Gu and Cheung (2001) proposed that "*V. indigofera* might potentially be adopted as a metallic bioindicator, leading to a new direction for the development and technology of bioassay detecting metal pollution". In this study the biotechnological potential of *V. indigofera* was tested using chromium. The main purpose of this objective is to determine the biotechnological capabilities of the Blue Pigmented Bacteria (BPB) isolated from Ponce and San German.

The biotechnological potential of the BPB was tested using inorganic and organic contaminants such as chromium (Cr), copper (Cu), diesel and biodiesel respectively. Two effects can be monitored with this test. First, if the growth of the bacteria is altered in the presence of the contaminant previously mentioned, and second if the bacterium pigmentation is altered or affected (decrease or increase of the intensity of the pigment) after exposure to the contaminants.

4.2 Material and Methods

Growth and preparation of the BPB

The BPB were inoculated in Nutrient Broth and incubated over night in a orbital shaker at 32°C. The Optic Density (OD) at 600nm was measured in a Eppendorph Biophotometer ®. The volume required of the inoculums at OD 600 0.025 was calculated using the OD of the overnight culture. The samples were left in a shaker at 32°C until it reached to the logarithmic phase (OD 0.4-0.6). When the samples had reached that OD, they were transferred in to a 1.5 mL micro tube. After that the cells were centrifuged at 0.54 x g (3,000 rpm) for 5 minutes. The formed pellet was washed tree times with 1 mL of a 0.85% NaCl. After each rinse the cells were centrifuged to prevent the lost of cells.

Diesel and Biodiesel media preparation and inoculation.

The use of diesel and biodiesel as the only carbon source was measured with the bacteria growth in Bushnell-Hass (BH) Difco ® broth supplemented with the corresponding hydrocarbons. The BH medium is a minimal medium with no carbon source which help us determine if the bacteria is using the carbon source that we place in the media.

To determine in which concentration of hydrocarbons the bacteria can grow different concentrations were used. Concentrations of 0.25%, 0.50%, 0.75%, 1.0% and 1.25% were prepared. Also, two control solutions were prepared: one of the BH media with no hydrocarbons and one of Nutrient Agar to determine the viability of the cells.

Each tube was labeled and inoculated with the bacterial cells that were washed as previously described. The tubes were incubated at 25 °C for 1 week. The experiment was done in triplicate.

Copper media preparation and inoculation

To determine if the isolated bacteria can grow in the presence of copper, different concentrations of the metal were prepared.

Stock Preparation $CuSO_4$ 1M

To prepare the Stock solution, 9.71g of the copper sulfate was diluted in 100ml of distilled water. The solution was sterilized by filtration.

$CuSO_4$ media preparation

Concentrations 0.0 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM- 8mM of copper sulfate ($CuSO_4$) was used and as nutritional source, 5ml of Nutrient Broth was added. With this we calculate how much of the stock we have to use to get to the desirable concentration.

The Minimal Inhibitory Concentration (MIC) of *E. coli* was also measured. This was used as our positive control. A no inoculated tube for each concentration was used to confirm the appropriate media preparation and inoculation.

The media was sterilized and then the copper solution was added. To this we added 50 µl of the bacteria isolated from Ponce and the one isolated from San German. The test was incubated at 32 ºC in a rotational shaker for 4 days. The experiment was done in triplicate.

Chromium media preparation and inoculation

For this test we used as model the experiment conducted by Gu and Cheung, 2001.

Stock Preparation of Potassium Chromate K_2CrO_4 0.5M

To prepare the stock solution, 9.71g of the potassium chromate was diluted in 100ml of distilled water. The solution was filtered to sterilize it.

K_2CrO_4 Media Preparation

The concentrations of the chromium used were 0 μg/ml, 25 μg/ml, 50 μg/ml, 75 μg/ml, 100 μg/ml, 200 μg/ml, 300 μg/ml, 400 μg/ml, 450 μg/ml and 500 μg/ml of potassium chromate, the total volume for each tube was 15 ml.

The Minimal Inhibitory Concentration in which *E. coli* can grow in the presence of chromium is 0.2 mM. This bacterium was used as our positive control. A not inoculated tube was used as a control for each concentration.

The media was sterilized and then the chromium solution was added. To inoculate the bacteria a final volume of 100 μl was added of the corresponding bacterium. All the tubes were incubated at 32 ºC in a rotational shaker for 4 days, during which it was checked every 12 hours. The experiment was prepared in triplicate.

4.3 Results

After 4 days of incubation the samples were checked for growth. In the hydrocarbon media of diesel and biodiesel no positive growth was observed in both bacteria (data not shown). Growth was observed in the positive control of the cells in Nutrient Agar, but the bacteria were not able to growth in the control of BH with no carbon source.

For the copper media experiment, the *E. coli* control gave positive results in the concentration of 0.0 μg/ml trough 5.0 μg/ml. No growth was observed in the 6.0μg/ml, 7.0 μg/ml and 8.0 μg/ml of the $CuSO_4$ solution (Figure 4.1). In the case of the isolated bacteria from San German no growth was observed in any concentration (Table 4.1), but in the isolated bacteria from Ponce, growth was observed in the 0.2 μg/ml (Table 4.1 and Figure 4.2).

In the chromium media no growth was recorded from the BPB from Ponce and San German (data not shown). The *E. coli* control gave positive results in the concentrations of 0.1mM and 0.2mM. No growth was recorded at 0.3 mM of chromium (Data not shown).

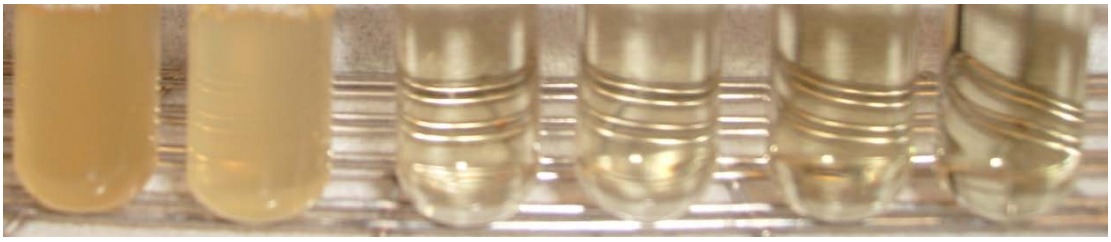
0 µg/ml 1 µg/ml 2 µg/ml 3µg/ml 4 µg/ml 5 µg/ml 6 µg/ml 7 µg/ml 8 µg/ml

Figure 4.1 Minimal Inhibitory Concentration (MIC) of *Escherichia coli* in different concentration of $CuSO_4$ was 5 µg/ml. *E. coli* gives a positive result in the concentration of 0 µg/ml through 5 µg/ml. No growth was recorded in the concentration of 6 µg/ml, 7 µg/ml and 8 µg/ml.

Table 4.1 Minimal Inhibitory Concentration (MIC) of *E. coli* and the isolated Blue Pigmented Bacteria in $CuSO_4$

$CuSO_4$ Concentration	*E. coli*	BPB-Ponce	BPB San German
0.0 µg/ml	+	+	+
0.2 µg/ml	+	+	-
0.4 µg/ml	+	-	-
0.6 µg/ml	+	-	-
0.8 µg/ml	+	-	-
1.0 µg/ml	+	-	-
2.0 µg/ml	+	-	-
3.0 µg/ml	+	-	-
4.0 µg/ml	+	-	-
5.0 µg/ml	+	-	-
6.0 µg/ml	-	-	-
7.0 µg/ml	-	-	-
8.0 µg/ml	-	-	-

BPB- Blue Pigmented Bacteria; + Positive growth; - No growth



0 μg/ml 0.2 μg/ml 0.4 μg/ml 0.6 μg/ml 0.8 μg/ml 1.0 μg/ml

Figure 4.2 Growth of the BPB from Ponce in $CuSO_4$. From the two BPB isolates the only one that shows growth in any concentration of copper was the BPB from Ponce that demonstrated growth in 0.2 μg/ml of $CuSO_4$.

4.4 Discussion

From all of the blue pigmented bacteria, the only one that had been described to have a biotechnological capability was *Vogesella indigofera*. For this reason different experiment were carried out to establish the biotechnological potential of the blue pigmented isolates.

One of the experiments was to see if the isolates were capable to grow in different percentage of diesel and biodiesel. These two compounds are very similar and have the same goal to be used as an energy source. Diesel is a compound with a combination of hydrocarbons that comes from the distillation of crude petroleum (McGrath and Harfoot 1997). Biodiesel is a compound made from plant oils and animal grease. Because this compound is less toxic it promotes a better environment for bacteria to grow (Nishio and Nakashimada 2006). In our case, neither of the bacteria was capable of growing in the presence of these hydrocarbons. Some bacteria have been reported to be capable of growing in the presence of this substance, but not all can degrade the compound (Vrdoljak et al. 2005). One of the genuses reported to be capable of growing in the presence of this compound is *Rhodobacter sphaeroides*.

The growth of the two isolates in chromium was measured because Gu and Cheung (2001) had reported that *V. indigofera* may be used as a bio-indicator of chromium contaminates sites. Nether of the isolates grew in this media, which is contradictory to the findings of Gu 2001, where he indicates that the pigment was inhibited at 300 ($\mu g\ Cr^{+6}$) ml^{-1}, and the growth was inhibited at 400 ($\mu g\ Cr^{+6}$) ml^{-1}. These results may indicate that there is some difference between *V. indigofera* and the isolates from P.R. *In silico* analysis suggests that both bacteria were 97% similar to the *V.*

indigofera, so there is physiological difference between them, and part of this difference may be this capability of growing in this metal.

Another heavy metal tested was copper. Copper is a very common metal in nature. A large number of copper occurrences are reported from the island of Puerto Rico (Cox and Briggs 1973; Bawiec et al. 2001a). The copper minerals can occur in a number of ways: as replacement of calcareous rocks adjacent to igneous intrusions (skarn), as veins and disseminations in porphyritic felsic intrusions (porphyry copper), as veins filling fractures in older rocks, or as replacements of volcanic rocks (manto-type) (Bawiec et al. 2001b). Several microorganisms, including fungi can grow in this environment. Only the bacteria isolated from Ponce showed resistance to the metal. The bacteria isolated from Ponce grew in a concentration of 0.2 μg/ml $CuSO_4$. This demonstrates that the blue pigmented bacteria may be used as a biotechnological tool for the identification of copper contaminated sites.

Chapter 5

Conclusions, Recommendations and Literature Cited

5.1 Conclusions and Recommendations

•Several samples were collected from various locations on the island of Puerto Rico. In only three samples Blue Pigmented Bacteria were found. The samples were collected from Mayagüez, Ponce and San German. The sample from Mayagüez was lost due to electrical failures in the Biology building.

- All of the isolates were obtained from soils near rivers, so future sampling is recommended of the entire island including the islands municipalities of Vieques and Culebra.

•With the tests that were performed in this work, no physiological differences were found with the two isolated bacteria and *Vogesella indigofera*.

- It is recommended to make more physiological test to find differences between the isolates and the described BPB, if they are at this level.

•Phylogenetic analysis suggests that all isolated bacteria belong to the genus *Vogesella*. But the analysis also shows evolutionary difference between the isolates from Puerto Rico and the described genus.

•G + C analysis demonstrate that both isolates have very different percentage between them and in comparison with *Vogesella* sp.

•DNA-DNA hybridization shows that the difference between them is more than 70%, which is contradictory with the result of G + C analysis.

•Molecular analysis indicates that most genes involved in the pigment production are the same compared with the one described for *Vogesella indigofera*.

- The *igi* E gene was the only one that presents significant difference between them and the *Vogesella* genus.

•Of the two isolates, the only one that presents a potential biotechnological capability is the blue pigmented bacteria isolated from Ponce.

- Further investigation will be needed to determine if these organisms are resistant to other metals or to other contaminants such as nickel or phenanthrene.
- The mechanisms that make the BPB from Ponce tolerant to copper should be further analyzed in order to determine how much it may decrease the concentration of the copper in the environment.

5.2 Bibliography and Literature Cited

1. **Ashworth, P.** 1974. Electron-Spin-Resonance studies of the structure and formation of bacterial diazodiphenoquinone pigments. Biochemical Journal. 141: 577-580.
2. **Bawiec, Walter J., Cox, Dennis P., McKelvey, Gregg E., Paidakovich, Matthew E. , Handler, Andrea, and Schellekens, Johannes H**. 2001a. Metallic and industrial mineral mines, prospects, and occurrences in Puerto Rico, as recorded in the Mineral Resource Data System (MRDS), *in* Walter J. Bawiec (compiler) Geology, geochemistry, geophysics, mineral occurrences and mineral resource assessment of the Commonwealth of Puerto Rico. U.S. Geological Survey Open File report 98-38, p. 122-126, CD-ROM.
3. **Chatoopadhyay, P., S. Chatterjee and S. K. Sen**. 2008. Biotechnological potential of natural food grade biocolorants. African Journal of Biotechnology. 7: 2972-2985.
4. **Chen, W. and T. Kuo.** 1993**.** A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. Nucleic Acids Reserve 21, 2260.
5. **Cheung, K. H. and J.D. Gu.** 2002. Bacterial color response to hexavalent chromium, Cr^{6+}. The Journal of Microbiology. 40: 234-236.
6. **Chou, J.H, Y.J. Chou, A.B. Arun, C.C. Young, C.A. Chen, J.T Wang and W.M. Chen**. 2009. *Vogesella lacus* sp. nov., isolated from a pond used for culture of soft-shell turtle. International Journal of Systematic and evolutionary Microbiology. (In press)

7. **Grimes, D. J., C. R. Woose, M.T. MacDowell, and R.T. Colwell**. 1997. Systematic Study of the Genus *Vogesella* gen. nov. and its type species, *Vegesella indigofera* comb. International Journal of Systematic Bacteriology. 47: 19-27.
8. **Grossart, H.P., M. Thorwest, I. Plitzko, T. Brinkhoff, M. Simon and A. Zeeck.** 2009. Production of a blue pigment (Glaukothalin) by *Rheinheimera marine* spp. International Journal of Microbiology. Vol. 2009, Article ID 701735. doi:10.1155/2009/701735.
9. **Gu, J. D, K. H. Cheung.** 2001. Phenotypic expression of *Vogesella indigofera* upon exposure to hexavalent chromium, Cr^{6+}. World Journal of Microbiology and Biotechnology. 17: 475-480.
10. **Harley, J. P. and L.M. Prescott.** 1999. Laboratory Exercises in Microbiology. Fourth edition. Mc Graw- Hill. Pp. 17-35, 52-54.
11. **Hiugo, W. B. and M. Turner.** 1957. A soil bacterium producing an unusual blue pigment. Journal of Bacteriology. 73: 154-157.
12. **Hui, K. M. and R. E. Hurlbert.** 1979. Modifiable Chromatophore Proteins in Photosynthetic Bacteria. Journal of Bacteriology. 138: 207-217.
13. **Iqbal A., Z. Shaheen, and A. Farah**. 2005. Heavy Metal Biosorption potential of *Aspergillus* and *Rhizopus* sp. isolated from Wastewater treated soil. Journal of Applied Science and Environment. Management. 9: 123-126.
14. **Knackmuss, H. J., G. Cosens and M. P. Starr.** 1969. The soluble blue pigment, Indochrome of *Arthrobacter polychromogenes*. European Journal of Biochemestry. 10: 90-95.

15. **Kobayashi, H., Y. Nogi and K. Horikoshi.** 2007. New violet 3,3'-bipyridyl pigment purified from deep-sea microorganism *Shewanella violacea* DSS12. Extremophiles. 11: 245-250.
16. **Kuhn, D. A. and M. P. Starr.** 1960. *Arthrobacter atrocyneus*, n. sp., and its blue pigment. Archives of Microbiology. 36: 175-181.
17. **Kuhn, R., M. P. Starr, D. A. Kuhn, H. Bauer, and H.-J. Knackmuss.** 1965. Indigoidine and other bacterial pigments related to 3-3-bipyridyl. Archives of Microbiology. 51**:**71–84.
18. **McFadden, B. A. and W. V. Howes.** 1960. *Pseudomonas indigofera*. Journal of Bacteriology. 81: 858–862.
19. **McGrath, J.E. and Harfoot, C.G.** 1997. Reductive Dehalogenation of Halocarboxylic Acids by the Phototrophic Genera *Rhodospirillum* and *Rhodopseudomonas*. Applied and Environmental Microbiology, 63: 3333-3335.
20. **Milford A. D., L. A. Achenbach, D. O. Jung and M. T. Madigan.** 2000. *Rhodobaca bogoriensis* gen. nov. and sp. nov., an alkaliphilic purple nonsulfur bacterium from African Rift Valley soda lakes. Archives of Microbiology. 117: 18-27.
21. **Moss, M. O.** 2002. Bacterial pigments. Microbiologist. 10-12
22. **Nishio N. and Y. Nakashimada.** 2007. Recent development of anaerobic digestion processes for energy recovery from wastes. Journal of Bioscience and Bioengineering. 103(2):105-112.
23. **Reverchon, S., C. Rouanet, D. Expert, and W. Nasser.** 2002. Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. Journal of Bacteriology. 3: 654-65.

24. **Sommer, E.C, W. S Silver and L. C. Vining.** 1961. Studies of pigmentation by *Pseudomonas indigofera*. Canadian Journal of Microbiology. 7: 577-585.

25. **Starr, M. P.** 1958. The blue pigment of *Corynebacterium insidiosum*. Archives of Microbiology. 30: 325-334.

26. **Starr, M. P., G. Cosens and H-J. Knackmuss.** 1966. Formation of the blue pigment indigoidine by phytopathogenic *Erwinia*. Applied Microbiology. 14: 870-872.

27. **Szczepanowska, H. and C. M. Lovett.** 1992. A study of the removal and prevention of fungal stains on paper. Journal of the American Institution for Conservation. 31: 147-160.

28. **Takahashi, H., T. Kumagai, K. Kitani, M. Mori and Y. Matoba.** 2007. Cloning and characterization of a *Streptomyces* single module type non-ribosomal peptide synthetase catalyzing a blue pigment synthesis. The Journal of Biological Chemistry. 282: 9073-9081.

29. **Vrdoljak G., Feil, W.S., Feil, H., Detter, JC., and P. Fields.** 2005. Characterization of a diesel sludge microbial consortia for bioremediation. Scanning. 27(1):8-14.

30. **Willmotte A. and R. Wachter.** 1993. Structure of the 16S Ribosomal RNA of the thermophilic cyanobacterium chlorogloeopsis HTF (*Mastigocladus laminosus* HTF) strain PCC 7518, and phylogenetic analysis. FEBS Lett. 317:96-110.

Appendix

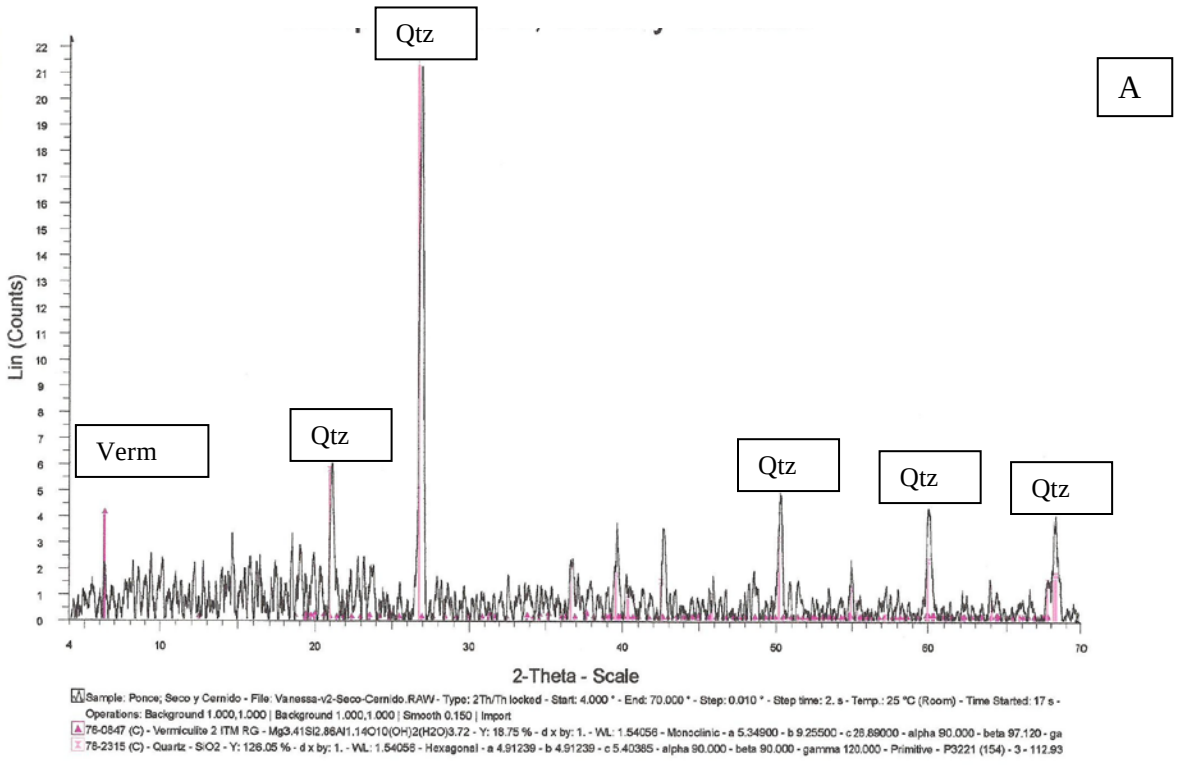


Figure A. X-ray diffraction result of the soil sample Ponce. (2 Theta linked, start at 4.000 degrees end at 70.000 degrees, step 0.010 degrees step time 2 seconds) Qtz: quartz; verm: vermiculite

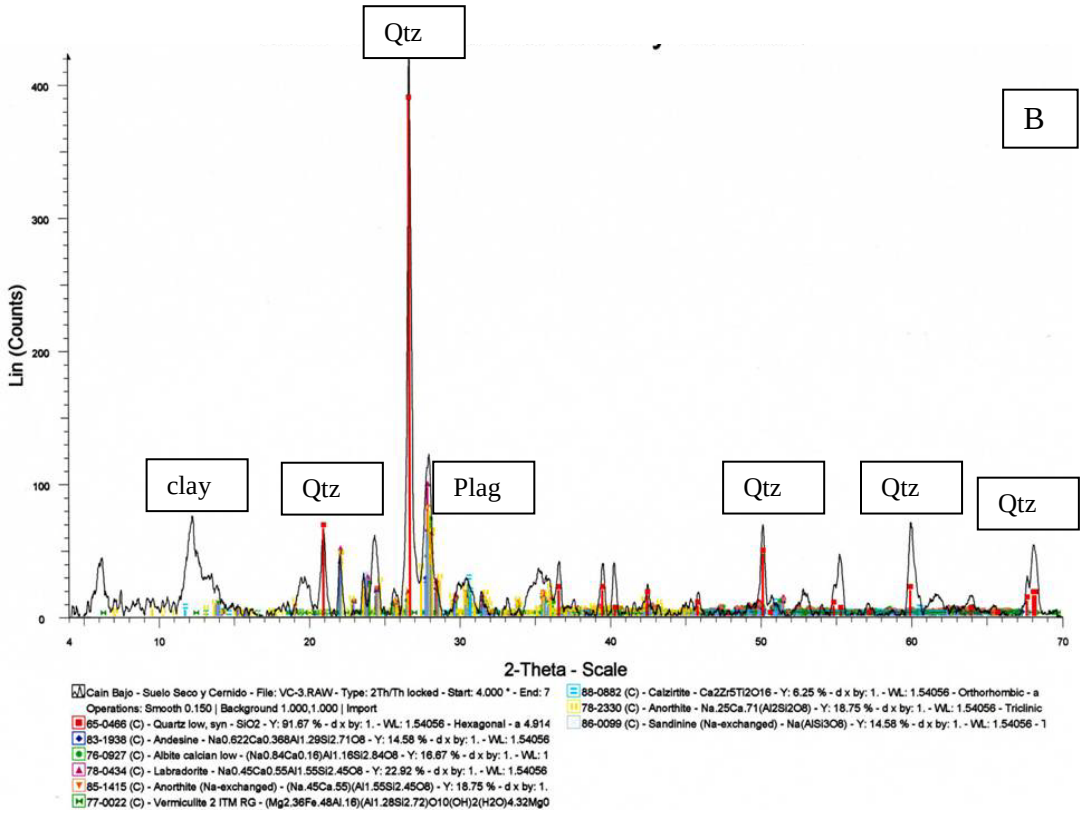


Figure B. XRD of the soil samples collected from San German (Qtz: quartz; Plag: plagioclase, nl albite to anorthite) (2 Theta linked, start at 4.000 degrees end at 70.000 degrees, step 0.010 degrees step time 2 seconds).