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Bioprospecting study of cultivable prokaryotic fraction from Manaure solar saltern, La Guajira, Colombia

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Universidad de La Sabana
Doctorado en Biociencias
Chía, Colombia
2019

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Doctoral thesis

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ACKNOWLEDGMENTS

I would like to acknowledge the Universidad de La Sabana, particularly to the Dirección General de Investigación for the financial support within the project "*Bioprospección de bacterias halófilas aisladas de las salinas de Manaure, La Guajira-Colombia, como fuentes de compuestos que puedan ser usados como antibióticos* (ING 139–2013)". The collection of the samples and the study of genetic resources from Colombian biodiversity were covered under the permission granted by the Ministerio de Ambiente y Desarrollo Sostenible de Colombia to Universidad de La Sabana, through the Contrato Marco de Acceso a Recursos Genéticos y sus Productos Derivados No. 117 (Otrosí No. 2 of 05/04/2017). Additionally, I would like to thank the Dealership Big Group S.A. (Salinas Manaure) for their help during the sample collection. This research was also financial supported by COLCIENCIAS Scholarship 6172–2014 (National Doctoral Grant). I want to express my sincere thanks to Prof. Dr. Norberto Peporine Lopes from the NPPNS in the FCFRP-USP, for having me in his research lab during my research internship, to Anelize, Camila and Alan, for shared their knowledge and expertise with me. To Dr. Juan Fernando Alzate and Laura Arias, for their contribution in genomic analysis.

To my thesis advisor, Prof. Edisson Tello for his countless advises, patience, unconditional help and share his knowledge with me during these years. To Alejandro Acosta González and Luis Eduardo Diaz for their recommendations, patience and unconditional help during this research. To the agora-girls and the girls from GIBP, the most brilliant women I could ever meet during this process. Finally, I would like to thank my lovely Charlie and my mom, which made this process so much lighter with their company.

RESUMEN

El objetivo de esta investigación fue establecer el potencial bioprospectivo de la comunidad bacteriana cultivable aislada de las Salinas de Manaure en La Guajira, Colombia. Para lograrlo, se implementó una estrategia de cultivos mixtos mediante la cual se seleccionó el extracto que presentó mejor actividad citotóxica y antibacteriana y posteriormente, se caracterizaron los aislados de dicho cultivo mixto. Los resultados de la actividad biológica de los extractos de estos aislados, permitió establecer que la bacteria halófila *Vibrio diabolicus* A1SM3 fue la responsable de la actividad presentada por el cultivo mixto. A partir del fraccionamiento bioguiado del extracto de esta cepa y su posterior análisis mediante HPLC-MS/MS y RMN, se logró identificar a la isotrisindolina como el compuesto responsable de la actividad citotóxica y antibacteriana. Posteriormente, se realizaron variaciones en la fuente de carbono, nitrógeno y de salinidad en el medio de cultivo, con el fin de establecer su efecto en la producción de este compuesto. Los datos de MS/MS obtenidos de estos cultivos fueron analizados mediante redes moleculares (*molecular networking)* para determinar este efecto. Adicionalmente, se logró identificar que este microorganismo también produce polihidroxibutiratos, un biopolímero comúnmente producido y acumulado por este género de bacterias, el cual es de gran interés en la industria de los plásticos. Finalmente, mediante la secuenciación del genoma de *Vibrio diabolicus* A1SM3, se logró anotar el *cluster* de genes asociados a la biosíntesis de este biopolímero.

Palabras claves: *Vibrio diabolicus*, *molecular networking*, cultivo mixto, salinas de Manaure, polihidroxibutirato.

ABSTRACT

The objective of this research was to establish the bioprospecting potential of the cultivable bacteria isolated from the Manaure Solar Saltern in La Guajira, Colombia. To achieve this, a strategy of mixed cultures was implemented to evaluate the cytotoxic and antibacterial activity of their crude extracts. The best bioactive mixed culture was selected, and their isolates were obtained and characterized. The results from the biological assays with the crude extracts of the isolates grown individually led to establish that the halophilic bacterium *Vibrio diabolicus* A1SM3 was responsible for the biological activity of the mixed culture. From the bioguided fractionation of this extract and its analysis by HPLC-MS/MS and NMR, the isotrisindoline was identified as the compound responsible for the cytotoxic and antibacterial activity. After that, variations on the carbon and nitrogen source, and the salinity of the medium were made to determine how they affected the isotrisindoline production. The MS/MS data obtained from the crude extracts of these cultures were analyzed through molecular networking in order to establish the effect of these variations. In addition to this, it was possible to identify that this microorganism produces polyhydroxybutyrates, a biopolymer widely produced and accumulated by *Vibrio* species with great applications in the plastics industry. Finally, by sequencing the genome of *Vibrio diabolicus* A1SM3, the biosynthetic gene cluster associated with the production of this biopolymer was annotated.

Keywords: *Vibrio diabolicus*, molecular networking, mixed culture, Manaure solar saltern, polyhydroxybutyrates.

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LIST OF ABBREVIATIONS

ANI	Average nucleotide identity
AUC	Area under the curve
BGC	Biosynthetic gene clusters
CDS	Coding sequence
GNPS	Global natural product social molecular networking
HMBC	Heteronuclear multiple bond correlation
HPLC-MS/MS	High-performance liquid chromatography coupled with tandem mass spectrometry
HRMS	High resolution mass spectrometry
HSQC	Heteronuclear single quantum coherence
IMPD	Inosine 5′-monophosphate dehydrogenase
LC-MS	Liquid chromatography coupled with mass spectrometry
MALDI	matrix-assisted laser desorption
MN	Molecular networking
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS/MS	Tandem mass spectrometry
NMR	Nuclear magnetic resonance
OSMAC	One Strain, Many Compounds
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrates
TETRA	Tetranucleotide signature correlation index
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy

INTRODUCTION

Marine environments have been a significant source of bioactive metabolites that are useful for drug development (Cragg & Newman, 2013; Newman & Cragg, 2012). Despite the fact that most of the research on marine organisms has been focused on anticancer agents (Newman & Giddings, 2014), the rise in antibiotic-resistant pathogens has prompted an increase in research to identify compounds with antimicrobial activity in the marine environment. Solar salterns are typically found close to coastal areas and are commonly used to precipitate different salts, such as calcium carbonate, calcium sulfate and sodium chloride (DasSarma & DasSarma, 2012). Solar salterns are multi-pond systems in which seawater is pumped through connected ponds to increase salinity by solar evaporation, with final salinities reached that are typically ten times higher than seawater. Recently these environments are emerging as important locations in the search for new metabolites with biological activities due to their salinity gradient and the extreme conditions of UV radiation and temperature (Trigui, Masmoudi, Brochier-Armanet, Maalej, & Dukan, 2011).

Traditionally, screening for microorganisms with antimicrobial activities is dependent on the isolation of individual strains, a highly time-consuming process compared to the use of mixed cultures, which allows all of the compounds produced by the microorganisms in the mixed culture to be evaluated simultaneously (Pettit, 2009). Thus, the use of mixed cultures has been proposed as an effective strategy for the selection of a group of microorganisms with potential bioactivities compared to the traditional approach, since more samples can be processed and tested, and more culture conditions can be evaluated.

Vibrionaceae family (composed *c.a.* 128 species) is considered a Gram-negative Gammaproteobacteria, ubiquitous in marine environments and has been spotted out for its genomic flexibility. Among them, *Vibrio* species are known to be the most representative genus in the *Vibrionaceae* family (64 species) which have been widely studied for their importance as pathogens (F. Thompson, Iida, & Swings, 2004). However, some studies report the isolation of bioactive compounds from species belonging to this genus (Mansson, Gram, & Larsen, 2011).

To improve the bioprospecting research with microorganisms the combination with analytical techniques has become a necessary challenge. There are some emerging online platforms that enable analysis, interpretation, and diffusion of knowledge-based nuclear magnetic resonance (NMR) spectra and fragmentation by tandem mass spectrometry (MS/MS) data. A recent mass spectra consortium featured on the Global Natural Products Social Molecular Networking (GNPS) platform have played an important role

on analysis of large datasets of tandem MS experiments from microbial extracts allowing the visualization of clustering of MS-related data and consequently, leading to correlated biological information such as species grouping or information relative to bioactive compounds (Wang et al., 2016). On the other hand, due to the greater improvements in genome sequencing, different methods of phylogenetic analysis and species circumscription have been developed such as the calculation of the average nucleotide identity (ANI) and the tetranucleotide signature correlation index (Richter & Rosselló-Mora, 2009) but additionally, the different platforms and software to analyze and compare the information retained in the whole genome of the microorganism which opens even more the research areas.

Considering the above, the research question that leads this study was, **which is the bioprospecting potential of the prokaryotic cultivable fraction isolated from Manaure Solar saltern, La Guajira, Colombia?** and the answers to this question was developed in three main chapters:

Chapter I. Use of a mixed culture strategy to isolate halophilic bacteria with antibacterial and cytotoxic activity from the Manaure Solar Saltern in Colombia. This chapter develops the mixed culture strategy to isolate the prokaryotic fraction from brine and sediment samples from Manaure Solar Saltern obtaining 40 mixed cultures, which were evaluated to establish their potential bioactivities. Once the mixed cultures were selected by their bioactivity, the isolates were obtained, and the bioactivity was evaluated to each one of the isolates. From these results, a moderately halophilic bacterium named as A1SM3 and belonging to genus *Vibrio* was selected for further research on the metabolites responsible for the bioactivity. Considering that at the beginning of this research the aim was to study the cultivable prokaryotic fraction, some growth media reported for archaea were included, however, although a few archaea isolates were obtained from the higher salinities, the mixed culture strategy could not be developed.

Chapter II. Integrating molecular network and culture media variation to explore the production of bioactive metabolites by *Vibrio diabolicus* A1SM3. This chapter integrates molecular networking approach with experimental variation of culture media which led to the identification of the metabolite responsible for the bioactivity of the crude extract and led to explore the variations in metabolic profile by *Vibrio diabolicus* A1SM3. This was the first report on cytotoxic and antibacterial activity for crude extract for this specie, and the identification of the isotrisindoline, an indole-derivative antibiotic, previously isolated from marine organisms and responsible for the bioactivity (Bell, Carmeli, & Sar, 1994; Kobayashi et al., 1994; Veluri, Oka, Wagner-dobler, & Laatsch, 2003).

Additionally, further analysis by MS/MS on the crude extract produced by this strain let us to established the production of polyhydroxybutyrates (PHB), a biopolymer that have been widely investigating as an alternative solution to replace plastic due to their biodegradability properties (Ganapathy, Ramasamy, & Dhinakarasamy, 2018).

Chapter III. Draft genome of the PHB producer *Vibrio diabolicus* A1SM3, a moderately halophilic bacterium. The last chapter involves the analysis of whole genome sequence of *Vibrio diabolicus* A1SM3 which showed genetic potential to produce ectoine, a compound produced by halophilic bacteria to maintain turgor pressure under salt-stress condition (Müller & Köcher, 2011) and aryl polyenes, a bacterial pigments wide studied for their structural similarity with the carotenoids and its protective function against light-induced damage (Schöner et al., 2016) among others. Finally, the annotation of the biosynthetic gene cluster for the production of PHB was made and the biosynthetic pathway was reconstructed with KEGG Mapper.

After the three main chapters that answer the research question, the final chapters are focused on the general discussion about all the results, the conclusions, and perspectives conceived from the results of this research.

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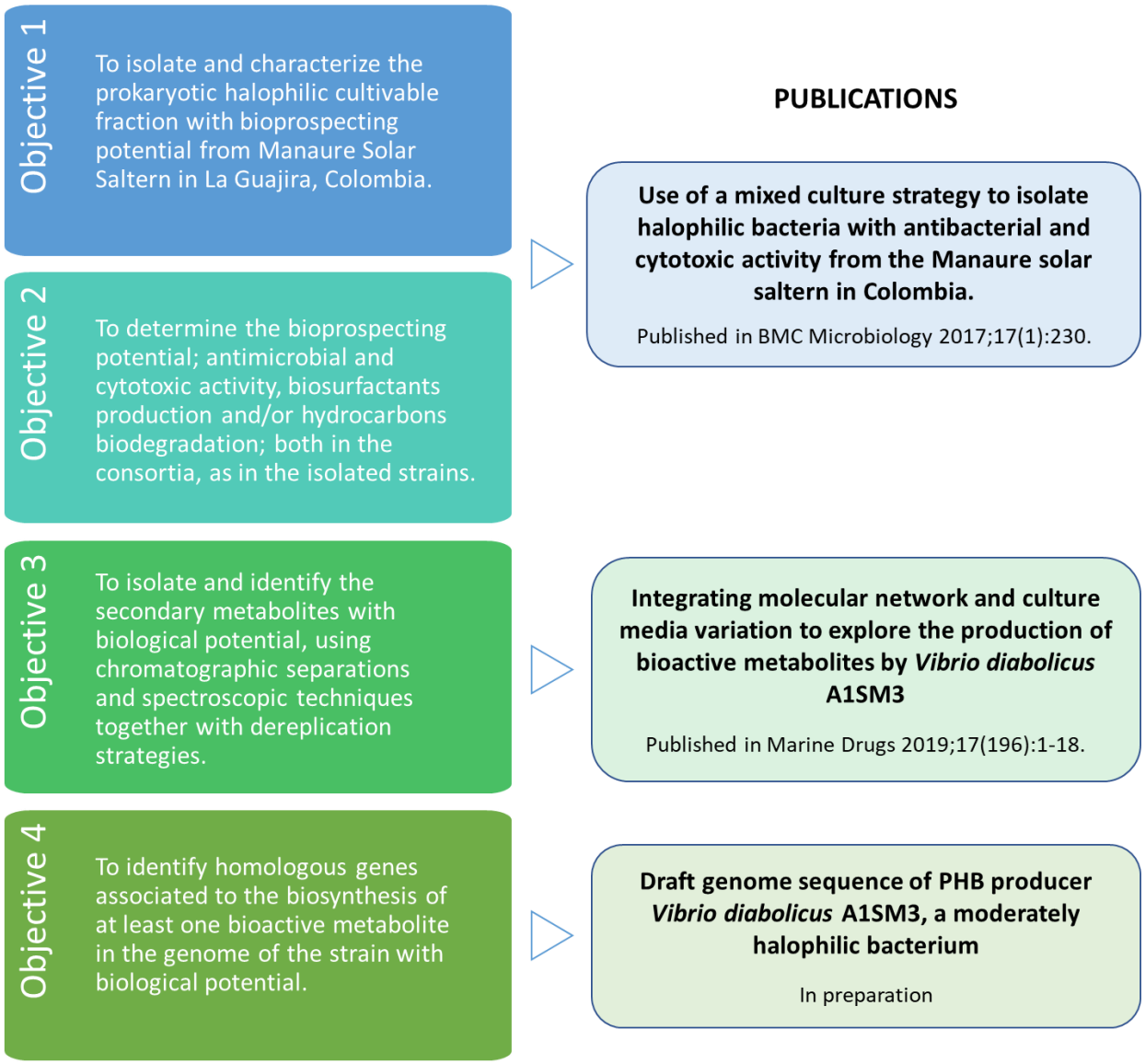
OBJECTIVES

General objective

To determine the bioprospecting potential of the prokaryotic cultivable fraction isolated from Manaure Solar Salter in La Guajira, Colombia.

Specific objectives

The specific objectives are shown in the following scheme and are related to the research articles where the results were published.



CHAPTER I: Use of a mixed culture strategy to isolate halophilic bacteria with antibacterial and cytotoxic activity from the Manaure Solar Saltern in Colombia

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Published in BMC Microbiology 2017;17(1):230. Doi: 10.1186/s12866-017-1136-x

1. ABSTRACT

Background: Water evaporation in solar salterns creates salinity gradients that promote the adaptation of microbial species to different salinities. This competitive habitat challenges the metabolic capabilities of microorganisms and promotes alterations in their production of secondary metabolites. Thus, solar salterns are a potentially important source of new natural products. In Colombia, the most important and representative solar saltern is located in Manaure (La Guajira) in the north of Colombia. The aim of this study was to develop an alternative screening strategy to select halophilic bacteria as producers of bioactive compounds from mixed microbial cultures rather than individual environmental isolates. Brine and sediment samples from different ponds (across a salinity gradient) were inoculated in seven different culture media to grow bacteria and archaea, allowing for a total of 40 different mixed cultures. An organic extract from each mixed culture was obtained and tested against multidrug-resistant pathogens, including *Klebsiella pneumoniae*, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* and *Bacillus subtilis*. In addition, the extracts were tested against two human cancer cell lines, cervical adenocarcinoma (SiHa) and lung carcinoma (A-549). **Results:** Twenty-four of the forty extracts from mixed cultures obtained from brine and sediment samples from the Manaure solar saltern showed antibacterial activity against *Bacillus subtilis*. Two extracts, referred to as A1SM3-29 and A1SM3-36, were also active against a methicillin-resistant *Staphylococcus aureus*, with the latter extract also showing slight cytotoxic activity against the assayed human lung cancer cell line. From this mixed culture, nine isolates were cultivated, and their extracts were tested against the same pathogens, resulting in the identification of a *Vibrio* sp. strain (A1SM3-36-8) with antimicrobial activity that was similar to that observed for the mixed

culture extract. The extract of this strain was subjected to a bioautography assay, and 3 different fractions exhibited antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Based on the amount obtained for each fraction, F3 was selected to isolate and identify its metabolites. The major compound was identified by NMR and HRMS as 13-*cis*-docosenamide, an amide that has been previously reported to be an antimicrobial and cytotoxic compound. **Conclusions:** Our results show the utility of our strategy in detecting bioactive molecules in initial mixed cultures by biological assays, resulting in the isolation and characterization of *Vibrio* sp. A1SM3-36-8, a halophilic strain with great antibacterial and cytotoxic potential.

2. BACKGROUND

The decrease in the discovery of new metabolites has prompted the exploration of unstudied environments as potential sources of new bioactive compounds. Because marine environments have been a significant source of bioactive metabolites that are useful for drug development (Cragg & Newman, 2013; Newman & Cragg, 2012), solar salterns are emerging as important sites in the search for new metabolites with biological activities. Despite the fact that most of the research on marine organisms has been focused on anticancer agents (Newman & Giddings, 2014), the rise in antibiotic-resistant pathogens has prompted an increase in research to identify compounds with antimicrobial activity. Such is the case for species of the genus *Salinispora* (obligate halophilic actinobacteria), among other halophilic microorganisms (Bose et al., 2015; Duncan et al., 2015; Hamedi, Mohammadipanah, & Ventosa, 2013; Wilson & Brimble, 2009).

Solar salterns are multi-pond systems in which seawater is pumped through connected ponds to increase salinity by solar evaporation, with final salinities reached that are typically ten times higher than seawater. Salterns are typically found close to coastal areas and are commonly used to precipitate different salts, such as calcium carbonate, calcium sulfate and sodium chloride (DasSarma & DasSarma, 2012). These environments have been investigated for their microbial diversity, primarily in ponds with salinities close to the saturation point (Antón, Rosselló-mora, Amann, & Anto, 2000; Hamedi et al., 2013; Oren, 2002; Ventosa, de la Haba, Sánchez-Porro, & Papke, 2015; Viver et al., 2015). Halophilic microorganisms are classified according to the optimal salt concentration required for their growth, with species ranging from slightly halophilic (grows in 1-3% salt) to moderately halophilic (grows in 3-15% salt) and extremely halophilic (grows in 15-32% salt) (Fathepure, 2014). In Colombia, the Manaure solar saltern, located in the

La Guajira region, is the most important and representative solar saltern in the country, followed by the Galerazamba solar saltern in the Atlántico region.

Traditionally, screening for microorganisms with antimicrobial activities is dependent of the isolation of individual strains, a highly time-consuming process compared to the use of mixed cultures, which allows all of the compounds produced by the microorganisms in the mixed culture to be evaluated simultaneously (Pettit, 2009). Thus, the use of mixed cultures has been proposed as an efficient strategy for the selection of microorganisms with potential bioactivities compared to the traditional approach, since more samples can be processed and tested, and more culture conditions can be evaluated. In addition, once the bioactive mixed cultures are selected, the recovery of individual strains is desirable to produce and study the metabolites associated with these activities.

Antibiotic-resistant bacteria are currently a growing global health problem. The WHO has classified carbapenem and 3rd generation cephalosporin-resistant *Klebsiella pneumoniae* as a critical priority for new antibiotics, while vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) were classified as high priority (WHO, 2017). In addition, tracheal, bronchial and lung cancers are collectively the third leading cause of cancer-related deaths among Colombian men, while breast and cervical cancers are the most common cancers in women, causing 4.087 registered deaths between 2007 and 2012 in Colombia (Pardo & Cendales, 2015), highlighting the importance of continued research on natural products. The evaluation and characterization of bioactive metabolites is considered a time-consuming practice. However, the use of common analytical techniques, such as high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) in bioassays, such as bioautography, allows bioactive metabolites to be identified more efficiently (Dewanjee, Gangopadhyay, Bhattabharya, Khanra, & Dua, 2015).

The purpose of this study was to use a mixed culture approach as a strategy to develop a screening-guided isolation method for identifying bacteria that produce bioactive compounds. Ethyl acetate extracts obtained from the mixed cultures isolated from brine and sediment samples from the Manaure Solar Saltern in La Guajira, Colombia, were used to select bioactive mixed cultures for further study. Once microbes from the mixed cultures were isolated and characterized, and their bioactivities were confirmed, a bioautography assay was used to establish which fractions had the bioactive metabolites for subsequent NMR and LC-QTOF-MS analyses. This approach led to the identification of the most abundant metabolite in a bioactive fraction that had activity against MRSA.

3. METHODS

3.1. Sampling

Brine and sediment samples were collected from 3 different ponds with salinities of 4, 9 and 15% from the Manaure Solar Saltern in the Dealership Big Group Salinas S.A. located in Manaure, La Guajira Colombia (latitude 11°46'32.0" N, longitude 72°27'27.4" W). Brine samples were collected in 500 mL sterile glass bottles. The sediment samples were collected in 50 cm long methacrylate cores. All samples were sealed and transported to the lab in a cooler at 4°C and were processed within 24 h after sampling. The salinity of each pond was determined in situ with a hand salt refractometer (Sper Scientific, China), and the pH and temperature were also measured for each pond.

3.2. Growth and enrichment of mixed cultures

Each brine sample was plated on seven different media supplemented with NaCl at a concentration corresponding to the pond from which the sample was collected (4, 9 or 15% [w/v]). For each sediment sample, the first 5 cm of the sediment core was added to 5 mL of water (0.5% salinity [w/v]) and mixed by vortexing. The supernatant was plated on the same media used for brine samples at the salinity of the pond where it was collected. The media selected for this work are presented in Table 1, each of which was supplemented with 50 mg/L of cycloheximide (OXOID, Hampshire, England) as an antifungal agent. For solid media, 15 g/L agar was added. All samples and serial dilutions (10^{-1}-10^{-4}) were plated and incubated at 30°C for three weeks. The biomass that grew in the first dilution for each media and each salinity was collected and cryopreserved at -80°C in 20% glycerol. To obtain sufficient biomass, a 200 µL aliquot of each mixed culture was inoculated in 3 mL of the respective media and incubated at 30°C for 24 h at 150 rpm. After this incubation, the entire enriched culture was transferred to fresh broth (27 mL) and incubated under the same conditions for 15 days. A total of 40 mixed cultures were obtained (Additional file 1).

Table 1. Media selected for the growth of halophilic bacteria and archaea.

Medium		Observations	References
M1	Halophile Agar I	Halophilic Bacteria	Handbook of Microbiological Media (Atlas, 2010)
M2	DSMZ 372	*Halobacterium* spp.	German Collection of Microorganisms and Cell Culture – DSMZ. Leibniz Institute.
M3	*Salinibacter ruber*	Extreme halophilic Bacteria	Antón et al., 2002 (Antón et al., 2002)
M4	*Salinibacter ruber*	Extreme halophilic Bacteria	Antón et al., 2000 (Antón et al., 2000)
M5	MCAT	Extreme halophilic Archaea	Pesenti et al., 2008 (Pesenti et al., 2008)
M6	Halophilic Actinobacteria	*Nocardiopsis* sp	Jung et al., 2000 (Jung et al., 2000)
M7	DSMZ 1018	*Haloferax* spp.	German Collection of Microorganisms and Cell Culture – DSMZ. Leibniz Institute

3.3. Extraction and characterization of crude extracts

The whole culture broth of each mixed culture and each isolate was extracted twice with ethyl acetate (1:1) and the organic fraction was subjected to rotary evaporation under vacuum (Forner, Berrué, Correa, Duncan, & Kerr, 2013). The obtained extracts were used for testing all biological activities, and uninoculated medium was extracted and used as a control.

3.4. Antibacterial activity

The antibacterial assay was conducted by direct diffusion in an agar plate. One 10 µL drop, corresponding to 150 µg of the extract (resuspended in 20% DMSO) was pipetted on an agar plate that was pre-inoculated with 100 µL of a 0.5 McFarland inoculum of each pathogenic strain and was then incubated at 37°C for 24 h (CLSI, 2012a). To ensure the diffusion of the drop through the agar plate was reproducible and comparable with standard techniques, such as a as disk diffusion assay (CLSI, 2012b), the diameter of the zone of inhibition of the drop was measured and compared with that of antimicrobial susceptibility discs (OXOID, Hampshire, England) containing the same quantity of antibiotic (30 µg), showing good results and comparable inhibition zones (Additional file 2).

The extracts were tested against multidrug resistant pathogens, including methicillin–resistant *Staphylococcus aureus* (MRSA; ATCC® BAA-44™), vancomycin-resistant *Enterococcus faecium* (VRE; ATCC® 700221™) and *Klebsiella pneumoniae* (ATCC® 700603™). *Bacillus subtilis* (ATCC ® 21556™) was used as a control strain due to its sensitivity to a broad spectrum of antibiotics. Chloramphenicol (3 mg/mL) was used as a positive control and was tested using the same procedure. A solution of 20% DMSO was used as a negative control.

3.5. Cytotoxic activity

The crude extracts were tested for cytotoxic activity against two human cancer cell lines and one non-tumor cell line by an MTT cell proliferation assay (Mosmann, 1983) using Doxorubicin as a positive control (Al-Ghamdi, 2008; Shaikh, Pawar, Aphale, & Moghe, 2012). Human cervix epithelial cells (SiHa; ATCC® HTB-35™) and a human lung cancer cell line (A-549; ATCC® CRM-CCL-185™) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Co., Darmstadt, Germany) supplemented with antibiotics (penicillin 120 IU/mL and streptomycin 100 IU/mL; Gibco/Invitrogen, Paisley, UK) and 10% fetal bovine serum (Eurobio, France). Fibroblasts (L929; ATCC® CCL-1™) were incubated in RPMI medium supplemented with 1% (v/v) L-glutamine (Sigma-Aldrich Co., Darmstadt, Germany), 10% (v/v) fetal bovine serum (Eurobio, France), 1% (v/v) penicillin and 1% (v/v) streptomycin (Gibco/Invitrogen, Paisley, UK). L929 was used as non-tumor cell line for toxicity control. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The cells were grown to approximately 1×10^5 cells per well in 96-well plates and then were incubated with different concentrations of the extracts and were diluted less than 0.5% in DMSO (10, 25, 50, 80 and 100 µg/mL) at 37°C with 5% CO_2 for 48 h. Cell viability was measured by an MTT colorimetric assay, and the 50% inhibitory concentration (IC_{50}) was calculated (Saravana Kumar et al., 2014; Sebaugh, 2011). Statistical analysis was performed using GraphPad Prism 6® and Microsoft® Excel 2016. All experiments were repeated at least three times and the results were expressed as the mean values ± standard deviation. IC_{50} values were obtained by nonlinear regression.

3.6. Enrichment, isolation and characterization of bacteria from mixed cultures

The isolation of bacteria from the bioactive mixed culture was performed by subculturing the culture onto agar plates at different dilutions (10^{-1}-10^{-4}) until an axenic culture was obtained. Isolates were cryopreserved in 1 mL of the corresponding broth containing 20% (v/v) glycerol. The isolates were deposited in the Collection of Microorganisms of Universidad de La Sabana (USAB-BIO). Single colonies were morphologically characterized according to their lifting edge, surface, texture, pigmentation and Gram

staining. For antimicrobial and cytotoxic assays, all isolates were grown in 30 mL of medium broth and incubated at 30°C for 15 days, after which the culture were extracted with ethyl acetate as described above. For *Vibrio* sp. A1SM3-36-8, the growth on TCBS (Thiosulfate Citrate Bile Salts Sucrose) agar plates (Oxoid Limited, Hampshire, England) was determined after 24 h at 30°C, and the antibiotic susceptibility profile was established using commercial antibiotic discs (Oxoid, Hampshire, England). A total of 11 antibiotics were tested, including vancomycin (5 µg), amoxicillin (10 µg), penicillin G (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), ampicillin (10 µg), tetracycline (30 µg), clindamycin (2 µg), cefoxitin (30 µg), nalidixic acid (30 µg) and rifampicin (5 µg) by disk diffusion tests (CLSI, 2012b). The susceptibility or resistance was measured by the diameter of the zones of inhibition and were classified according to Bergey's Manual of Systematics of Archaea and Bacteria for *Vibrio* species (Farmer, Brenner, Cameron, Birkhead, & Janda, 2005). To evaluate growth of isolates at different salinities, 0-20% (w/v) NaCl was supplemented in M3 broth and cultures were incubated at 30°C for 24 h. The growth was expressed as the number of colonies forming units (CFU) and the OD_{600} was measured.

3.7. Amplification and sequencing of the 16S rRNA gene

Taxonomic identification was performed by sequencing and analysis of the entire 16S rRNA gene. For amplification, isolated cells were resuspended in sterile DNAse free water. Cells suspensions were frozen using liquid nitrogen for 24 h. The 16S rRNA gene was amplified by PCR using two universal primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). PCR reactions were performed in 0.2 mL microcentrifuge tubes. Each 50 µL PCR reaction contained 2 µL of lysed cells, 1.4 µL of primers, 0.8 µL of dNTPs (25 mmol/L), 5 µL of Taq buffer (10X), 2.8 µL of $MgCl_2$ (50 mmol/L) and 0.8 µL of Taq polymerase.

The reaction mixtures were placed in a thermocycler with an initial denaturation step at 95°C for 2 minutes, followed by 30 cycles at 95°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. The PCR products were evaluated by agarose gel electrophoresis in a 1.2% (w/v) agarose gel run in 0.5X TAE buffer at 90 volts/cm for 1 h, with a HyperLadder III marker (500 bp-5 kb) used to assess the molecular weights of the products (Ballav, Kerkar, Thomas, & Augustine, 2015; Viver et al., 2015). PCR products were subjected to Sanger sequencing using four universal primers 27F, 518F (5'-CCA GCA GCC GCG GTA ATA CG-3'), 800R (5'-TAC CAG GGT ATC TAA TCC-3') and 1492R (MACROGEN, Korea).

Data obtained from sequencing were edited, corrected and assembled with CLCbio® software (https://www.qiagenbioinformatics.com/). All 16S rRNA gene sequences were compared against sequences in the SILVA (Pruesse, Peplies, & Glöckner, 2012) and NCBI databases. For tree reconstruction, the alignments were processed using the ARB software package (Ludwig et al., 2004). All sequences were aligned using the SINA tool (SILVA Incremental Aligner) (Pruesse et al., 2012) and were added to the reference dataset SILVA LTPs123_SSU (Quast et al., 2013; Yilmaz et al., 2014) using the ARB software. For the construction of *de novo* trees, the neighbor-joining algorithm with Jukes-Cantor correction were used (Viver et al., 2015). In addition, the similarity matrix was calculated using distance matrix methods with the ARB software. Sequences with identity values greater than 98.7% compared to the type strain sequences were considered to belong to the same species, and sequences with values lower than 94.5% corresponded to different genera (Yarza et al., 2014).

3.8. MALDI-TOF MS analysis

The colonies grown on agar plates were removed using a sterile stick and were spotted over the wells of a 96-well plate. Next, 1 µL of formic acid was aliquoted into each well and, after drying, 1 µL of the matrix solution (2.5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% trifluoroacetic acid and 47.5% HPLC water) was aliquoted into each well. The mixture was allowed to dry prior to analysis (Dieckmann, Graeber, Kaesler, Szewzyk, & von Döhren, 2005). Mass spectrometry measurements were performed on a Microflex spectrometer LT MALDI-TOF MS (Bruker Daltonics®) and analyzed using Bruker Flex software and a MALDI Biotyper RTC 3.0 (Bruker Daltonics®). The BTS standard (Bacterial Test Standard) *Escherichia coli* DH5α was used for instrument calibration. All spectra were analyzed in a range of 2000 to 20000 Da. The protein profiles obtained were used to calculate a correlation dendrogram using RTC Biotyper 3.0 software (Bruker Daltonics®).

3.9. Bioautography of a bioactive extract of *Vibrio sp.* A1SM3-36-8

An agar overlay assay (Dewanjee et al., 2015) was performed to detect the bioactive fractions in the crude extract of *Vibrio* sp. A1SM3-36-8 against MRSA and *B. subtilis*. The 4 x 7 cm TLC plates (ALUGRAM® SIL G/UV_{254}, Macherey-Nagel, Germany) were previously sterilized by UV radiation for 30 minutes. The crude extract was resuspended in ethyl acetate, spotted onto sterile TLC plates and developed in ethyl acetate. The developed plates were air-dried to completely remove the solvent and then were transferred into a sterile petri dish where they were covered with 10 mL of molten and seeded MH agar. One hundred µL of a 1 McFarland inoculum was used per 10 mL of MH agar for each pathogenic strain to fill the petri dish

and cover the TLC plate with a thin layer of agar. After solidification, the petri dish with the TLC plate was incubated at 37°C for 24 h. To visualize the zones of inhibition, the TLC plate was submerged into a 1 mg/mL solution of MTT reagent (Sigma-Aldrich Co., Darmstadt, Germany) for 10 s and incubated at 37°C for 2 h to assess the dehydrogenase-activity of the microorganism. The dehydrogenase converts tetrazolium salt into purple formazan, allowing the zones of inhibition to be observed as yellow, pallid halos over the purple growth of the pathogens (Choma & Grzelak, 2011). The retention factor (Rf) values at which the zones of inhibition were observed were calculated. The extract was posteriorly separated by preparative TLC plates. Fifteen mg of the crude extract was spotted onto the preparative TLC plates and developed with ethyl acetate. The fractions matching the Rfs of the bioactive spots were scratched and washed with ethyl acetate and a mixture of ethyl acetate and methanol (95:5).

3.10. Isolation and identification analysis

The fractions were analyzed by NMR experiments, including ^{1}H and ^{13}C NMR (Bruker Avance 400, 400 MHz for ^{1}H and 100 MHz for ^{13}C) using $CDCl_3$ as solvent. The fractions were analyzed with an Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight LC-QTOF-MS System (Agilent Technologies, Palo Alto, USA) with a reversed phase C18 analytical column (Agilent, Zorbax SB, 5 µm - 4.6 x 150 mm) using a gradient solvent system with A (water and 0.1% trifluoroacetic acid) and B (acetonitrile and 0.1% trifluoroacetic acid), from 20% B at zero minutes to 40% B in 4 min, to 70% B in 10 min and then to 90% B in 27 minutes. Five microliters of a 1 mg/mL solution of crude extracts were injected and analyzed at a flowrate of 0.5 mL/min in positive ion mode. The instrument was externally calibrated before the runs and the selected mass range was 100-3000 *m/z*.

4. RESULTS

4.1. Bioactivity of mixed cultures

The three ponds selected for sampling had pH values between 7.5-7.8 and a water temperature between 28-31°C. The seawater enters the multi-pond solar saltern system at the A1 pond, which had a measured salinity of 4%. The other two ponds assayed, C6 and C8, are part of the pre-concentrated ponds and had salinities of 9 and 15%, respectively (Figure 1). After inoculating the three brine (B) and sediment (S) samples (3 each) in seven different media according to the salinity of the ponds (4, 9 or 15%), a total of

40 mixed cultures were obtained (Additional file 1). The ethyl acetate extractions yielded extract amounts of between 0.01 and 0.26 mg/mL.

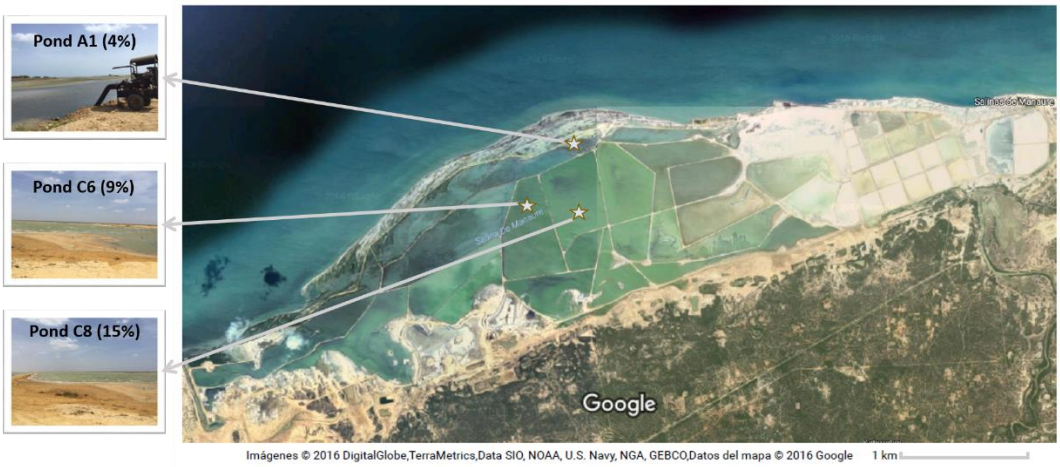

Figure 1. Solar saltern ponds located in the Big Group Salinas Manaure, La Guajira, Colombia.

The results of the antibacterial activity assays showed that 24 of the 40 extracts evaluated had activity against *B. subtilis* (Table 2). Nine extracts were obtained from pond A1 (4% salinity), nine from pond C6 (9% salinity) and 6 from the pond C8, which had the highest salinity (15%). Two extracts, A1SM1-29 and A1SM3-36, which were obtained from mixed microbial cultures of a sediment sample of pond A1 grown on different media (A1SM1-29 on medium M1 and A1SM3-36 on M3) were also active against MRSA (Table 2). The results of the cytotoxic assay showed that two mixed culture extracts displayed activity against a human lung carcinoma cell line (A549), where C8SM3-40, obtained from the sediment sample from pond C8 (15% salinity), had an IC_{50} of 64.60 ± 4.91 µg/mL, and A1SM3-36 had an IC_{50} of 44.90 ± 3.77 µg/mL. None of the extracts evaluated show any toxicity against the non-tumoral cell line L929 (IC_{50} > 100 µg/mL) at the conditions evaluated.

4.2. Isolation of microorganisms from mixed cultures and bioactivity assays

Based on the results obtained from the bioactivity assays of the mixed cultures, A1SM1-29 and A1SM3-36 were selected for the isolation of the cultivable strains with similar activities to the mixed cultures by subcultivation of the colonies that grew on agar plates at the plated dilutions until axenic cultures were obtained. A total of 8 isolates for A1SM1-29 and 26 isolates for A1SM3-36 were obtained.

The culture broth extracts from each isolate obtained from A1SM1-29 and A1SM3-36 were tested against *B. subtilis*, MRSA and the A-549 cell line under the same conditions described above. The results

showed that the A1SM3-36-8 isolate showed antibacterial activity against MRSA and *B. subtilis* (Additional file 3) and slight activity against the A-549 cell line (30% inhibition at 100 µg/mL).

4.3. Identification of isolates

The strains isolated from the A1SM1-29 mixed culture were all Gram-positive rods. The 16S rRNA gene of all strains was amplified and sequenced to construct phylogenetic trees by the neighbor-joining method using the aligned sequences. The results led to the determination that all the A1SM1-29 isolates belonged to the genus *Virgibacillus*, having as their closest relatives *V. dokdonensis* (98.8-99.7%) and *V. chiguensis* (98.8-99.6%) (Figure 2A). The isolate (A1SM1-29-8) was also a close relative of *V. dokdonensis* and *V. chiguensis* but with lower similarity (98.1 and 97.7%, respectively) than that required to classify it as the same species (98.7%).

For A1SM3-36, the protein profiles obtained by MALDI-TOF MS for each strain were used to calculate a correlation dendrogram in which 9 clades were observed. From this result, 15 of the 26 isolates were selected for amplification and sequencing of the 16S rRNA gene. The phylogenetic tree with the aligned sequences shows that one isolate (A1SM3-36-10) has *Marinobacterium mangrovicola* as the closest relative, with a similarity below the limit of circumscription (96.3%) within the same species (Yarza et al., 2014). The other isolates (A1SM3-36-1 thru A1SM3-36-15), including isolate A1SM3-36-8, which showed positive results in the bioactivity assays, belonged to the genus *Vibrio*, with *V. natriegens* (99.0-99.3%) and *V. atypicus* (99.3%) as closest relatives (Figure 2B).

Table 2. Antibacterial and cytotoxic activities of the extracts obtained from the 40 mixed cultures against the indicated multidrug resistant pathogens and human cancer cell lines.

Extract	Antibacterial Activity				Cytotoxic Activity* (IC_{50} µg/mL)		
	B. subtilis	MRSA	VRE	*K. pneumoniae*	A549	SiHa	L929
A1BM2-1	+	-	-	-	> 100	> 100	> 100
A1SM2-2	+	-	-	-	> 100	> 100	> 100
C6BM2-3	+	-	-	-	> 100	> 100	> 100
C6SM2-4	+	-	-	-	> 100	> 100	> 100
C8BM2-5	-	-	-	-	> 100	> 100	> 100
C8SM2-6	+	-	-	-	> 100	> 100	> 100
A1BM4-7	-	-	-	-	> 100	> 100	> 100
A1SM4-8	-	-	-	-	> 100	> 100	> 100
C6BM4-9	-	-	-	-	> 100	> 100	> 100
C6SM4-10	-	-	-	-	> 100	> 100	> 100
C8BM4-11	-	-	-	-	> 100	> 100	> 100
C8SM4-12	-	-	-	-	> 100	> 100	> 100
A1BM7-13	+	-	-	-	> 100	> 100	> 100
A1SM7-14	-	-	-	-	> 100	> 100	> 100
C6BM7-15	+	-	-	-	> 100	> 100	> 100
C6SM7-16	+	-	-	-	> 100	> 100	> 100
C8BM7-17	+	-	-	-	> 100	> 100	> 100
C8SM7-18	-	-	-	-	> 100	> 100	> 100
A1BM5-19	-	-	-	-	> 100	> 100	> 100
A1SM5-20	+	-	-	-	> 100	> 100	> 100
C8BM5-21	+	-	-	-	> 100	> 100	> 100
C8SM5-22	-	-	-	-	> 100	> 100	> 100
A1BM6-23	-	-	-	-	> 100	> 100	> 100
A1SM6-24	+	-	-	-	> 100	> 100	> 100
C6BM6-25	-	-	-	-	> 100	> 100	> 100
C6SM6-26	+	-	-	-	> 100	> 100	> 100
C8BM6-27	+	-	-	-	> 100	> 100	> 100
C8SM6-28	+	-	-	-	> 100	> 100	> 100
A1SM1-29	+	+	-	-	> 100	> 100	> 100
A1BM1-30	+	-	-	-	> 100	> 100	> 100
C6SM1-31	+	-	-	-	> 100	> 100	> 100
C6BM1-32	+	-	-	-	> 100	> 100	> 100
C8SM1-33	-	-	-	-	> 100	> 100	> 100
C8BM1-34	-	-	-	-	> 100	> 100	> 100
A1BM3-35	+	-	-	-	> 100	> 100	> 100
A1SM3-36	+	+	-	-	44.90 ± 3.77	> 100	> 100
C6BM3-37	+	-	-	-	> 100	> 100	> 100
C6SM3-38	+	-	-	-	> 100	> 100	> 100
C8BM3-39	+	-	-	-	> 100	> 100	> 100
C8SM3-40	-	-	-	-	64.60 ± 4.91	> 100	> 100

* > 100: IC_{50} value over 100 µg/mL are considered not cytotoxic (Mans, Rocha, & Schwartsmann, 2000).

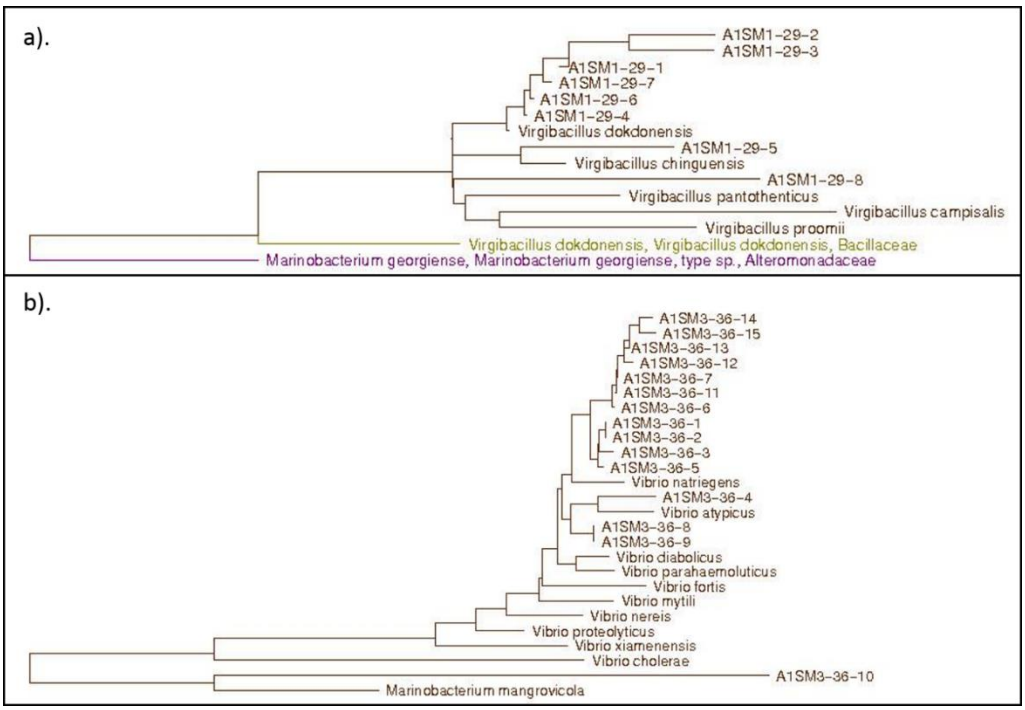

Figure 2. Phylogenetic tree reconstruction based on 16S rRNA genes of the bacterial isolates and their closest relatives. a). A1SM1-29 isolate and its closest relatives. b). A1SM3-36 isolate and its closest relatives.

4.4. Characterization of *Vibrio* sp. A1SM3-36-8

Cells were gram-negative and slightly curved rods. After 24 h of incubation, on an M3 agar plate supplemented with 4% (w/v) NaCl, circular, creamy, smooth and convex colonies were observed. To establish whether the *Vibrio* sp. A1SM3-36-8 strain was related to *Vibrio atypicus*, catalase activity was determined by the addition of a 1% (v/v) H_2O_2 solution over the colonies, and the result was compared to the previously reported activity for this species (Y. Wang, Zhang, Yu, Wang, & Austin, 2010). The isolate *Vibrio* sp. A1SM3-36-8 showed positive catalase activity that was comparable with the activity observed for *Vibrio harveyi* (ATCC® BAA-1120™), which was used as positive control, suggesting that the isolate A1SM3-36-8 was not related to *Vibrio atypicus*. The antibiotic susceptibility of this isolate was assessed to determine if the isolate had a similar antibiotic profile with other *Vibrio* species (Table 3). According to these results, the susceptibility of the isolate *Vibrio* sp. A1SM3-36-8 to tetracycline, chloramphenicol, and quinolones, such as nalidixic acid, was comparable to the susceptibilities of most environmental *Vibrio* strains, regardless of the species designation (Farmer et al., 2005; Okoh & Igbinosa, 2010).

Similarly, the resistance to beta-lactam antibiotics, such as ampicillin and penicillin G, is common for *Vibrio* species (Igbinosa, 2015). In addition, the growth of the isolate on a selective medium for the genus *Vibrio* (TCBS agar) was evaluated, and yellow, round and smooth colonies were observed after 24 h of incubation, suggesting the presence of *Vibrio* species capable of fermenting sucrose as carbohydrate source. The growth in different salinities showed that *Vibrio* sp. A1SM3-36-8 was a moderate halotolerant bacterium that could grow in salt up to a 9% (w/v) NaCl concentration.

Table 3. Antibiotic susceptibility profile of the *Vibrio* sp. A1SM3-36-8 isolate.

Antibiotic	Susceptibility*	Inhibition zone (mm)
Vancomycin	R	-
Amoxicillin	R	-
Penicillin G	R	-
Chloramphenicol	S	28
Erythromycin	I	16
Ampicillin	R	-
Tetracycline	I	19
Clindamycin	R	-
Cefoxitin	I	18
Nalidixic acid	I	20
Rifampicin	S	23

* The susceptibility ranges were classified according to Bergey's Manual of Systematics of Archaea and Bacteria (Farmer et al., 2005). Resistant (R): Inhibition zones smaller than 11 mm; Intermediate (I): Inhibition zones between 11 - 22 mm determine an intermediate susceptibility; Susceptible (S): Inhibition zones greater than 22 mm determine susceptible strains.

4.5. Isolation and identification of a bioactive fraction from *Vibrio* sp. A1SM3-36-8

From 1 L of *Vibrio* sp. A1SM3-36-8 culture broth, 27 mg of an ethyl acetate extract (2.7%) was obtained, and a bioautography assay was performed to establish at which Rf the zone of inhibition could be detected. From the bioautography assay against MRSA, three zones of inhibition were observed after the TLC plates were assessed with MTT reagent with Rf values of 0.9, 0.4 and 0.1. The bioautography assay against *B. subtilis* showed two zones of inhibition with two of the same Rfs (0.9 and 0.1) (Figure 3). A preparative TLC was performed to obtain the three primary fractions that corresponded to Rf 0.1 (F1; 2.4

mg), Rf 0.4 (F3; 6.3 mg) and Rf 0.9 (F5; 3.0 mg), which were scratched, extracted and concentrated for further analysis.

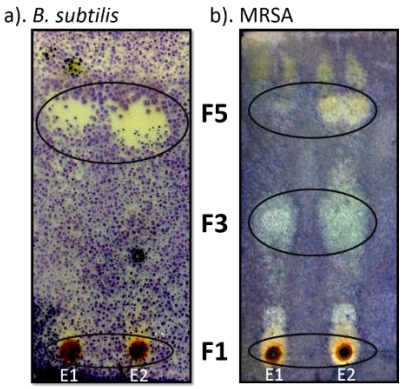

Figure 3. Bioautography assay with two extracts (E1 and E2) from the broth of different cultures of *Vibrio* sp. A1SM3-36-8 spotted on each TLC plate (a) against B. subtilis, and (b) against MRSA.

Because of the antibacterial activity exhibited by F3 against MRSA, and the amount of this fraction that was obtained, it was selected for further characterization by NMR and LC-QTOF-MS. The HRMS analysis of the F3 fraction showed the presence of an abundant ion (338.3448 *m/z*) that was consistent with a $[M+H]^+$ adduct, and an ion (360.3279 *m/z*) that was consistent with a $[M+Na]^+$ adduct, which confirmed an exact mass of 337.3358 uma, corresponding to a molecular formula of $C_{22}H_{43}NO$. The ^{13}C NMR data of F3 (Table 4) showed one signal located at δ_C 175.5 ppm, corresponding to a carbonyl carbon and non-signals for a carbon linked to an oxygen, suggesting the presence of an ester group, allowing to conclude that it could correspond to an amide group according to the nitrogen established by LC-HRMS. Two signals at δ_C 129.9 ppm, corresponding to two olefinic methines, suggested the presence of a disubstituted double bond. A group of signals between δ_C 36.0 and δ_C 22.6 ppm were assigned to 18 methylene carbons. Finally, one signal located at δ_C 14.1 ppm was assigned to the unique methyl group observed. The ^1H NMR spectrum showed one signal at δ_H 5.36 ppm (t, 2H, J = 4.7 Hz), corresponding to two identical olefinic protons, suggesting a *cis* coupling between them. One triplet methylene located at δ_H 2.22 ppm (t, 2H, J = 8 Hz) was assigned to the methylene attached to the carbonyl group. The signal at δ_H 2.01 ppm (dd, 4H, J = 12.1, 6.4 Hz) corresponded to the two methylenes adjacent to the double bond. A multiplet signal located at δ_H 1.63 ppm (m, 2H) was assigned to the methylene at beta position to the carbonyl group. A broad signal at δ_H 1.26 ppm (br s, 28H) corresponded to the methylene groups associated with an aliphatic chain. The last signal was one triplet at δ_H 0.88 (t, 3H, J = 6.7 Hz), corresponding to the methyl group at the end of the aliphatic chain (Additional file 4). These data allowed for the identification of a lineal amide with 22 carbons

and one unsaturation. Additionally, a database searches was performed in the Spectral Database for Organic Compounds (SDBS, http://sdbs.db.aist.go.jp) and in METLIN (http:// http://metlin.scripps.edu), which confirmed that the spectral information obtained for this compound corresponds to 13-*cis*-docosenamide (Figure 4).

Table 4. ^{1}H and ^{13}C NMR data for the major compound in the F3 fraction.

Position	δ_C, mult	δ_H, mult (J in Hz)
1	175.5, s	-
2	35.9, t	2.22, t (8.0)
3	25.5, t	1.63, m
4	29.2, t	
5	29.3, t	
6, 7, 8	29.5, t	
9	29.5, t	
10	29.6, t	
11	29.6, t	1.26, br s
16	29.7, t	
17,18	29.8, t	
19	29.5, t	
20	31.9, t	
21	22,7, t	
12,15	27.2, t	2.01, dd (12.1, 6.4)
13	129.9, d	5.36, t (4.7)
14	129.9, d	
22	14.1, q	0.88, t (6.7)

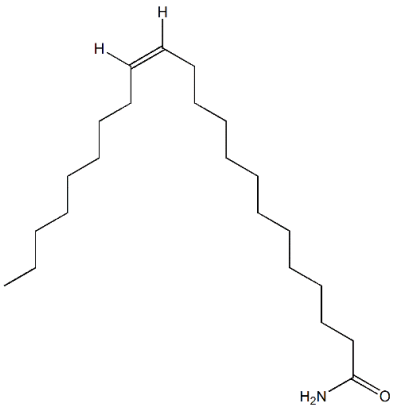

Figure 4. Molecular structure of 13-*cis*-docosenamide.

5. DISCUSSION

The strategy of using mixed cultures and subsequent isolation led to the development of a guided isolation method for the identification of bacteria producing antibacterial and cytotoxic metabolites. Two mixed cultures were selected due to their bioactivity, both of which were isolated from the same A1 pond sediment sample (4% salinity). After identifying isolates from each culture, it was evident that the medium in which the sample was inoculated influenced the growth of species from two different genera. *Virgibacillus* and *Vibrio* species were isolated using M1 and M3 media, respectively. The M1 medium had an approximately 6-fold higher amount of nutrients relative to that in the M3 medium, with yeast extract as the primary nutrient source, which has been reported to be used for the isolation of *Virgibacillus* species (Heyrman, De Vos, & Logan, 2009). Furthermore, the majority of isolated and identified strains were γ-Proteobacteria, consistent with the fact that this class is the most frequently isolated group from marine environments when using plating techniques (Joint, Mühling, & Querellou, 2010), and the genera *Virgibacillus* and *Vibrio* are largely recognized as halophilic (Heyrman et al., 2003; J. R. Thompson & Polz, 2006).

Using the strategy proposed in this study, the strain responsible for the observed activity in one of the bioactive mixed cultures (A1SM3-36) was isolated. Most of the isolates obtained from A1SM3-36 showed high similarity to *Vibrio natriegens*, a fast-growing marine bacterium associated with the sponge *Stylotella* sp. (Weinstock, Hesek, Wilson, & Gibson, 2016; Yoghiapiscessa, Batubara, & Wahyudi, 2016), and this bacterium also has been isolated from three different salt springs in the Boyacá and Cundinamarca regions of Colombia (Diaz-Cárdenas, 2011), demonstrating the affinity of *Vibrio* species for different saline environments. This species has been reported to be active against *Pseudomonas aeruginosa*, *S. aureus* and *E. coli* and toxic against *Artemia salina*, with an LC_{50} of 156.68 µg/mL (Yoghiapiscessa et al., 2016). The similarity of the isolates to *V. natriegens* may be due to their rapid growth in minimal media supplemented with glucose, similar to the M3 medium used in this work and as was previously demonstrated by Lee, Gold, and Khalil (H. H. Lee, Gold, & Khalil, 2016).

In the case of A1SM3-36, the isolation of a bioactive halophilic bacterium through the use of the mixed culture strategy was successful. This approach facilitated the later identification of isolates with bioactivities of interest, in contrast to the large number of isolates that would likely have been obtained if the conventional isolation methods had been used. For instance, in this study, seven growth media, three

salinities and two types of samples (brine and sediment) were analyzed, which could have led to a large number of isolates and a time-consuming screening process needed to establish the biological activity of each isolate. Our strategy led to the isolation and characterization of the halophilic bacterium *Vibrio* sp. A1SM3-36-8, which showed great antibacterial activity potential against the multidrug resistant pathogen MRSA and yielded good results for cytotoxic activity against the human lung cancer cell line A-549. In addition, a bioautography assay led to the identification of the bioactive Rfs, which allowed for the selective isolation and partial purification of 13-*cis*-docosenamide. This was achieved in both a practical and effective manner, considering that the amount of the F3 fraction obtained was enough for spectroscopic and spectrometric analysis, in contrast to the amounts obtained from F1 and F5, bioactive fractions from the same extract.

The amide 13-*cis*-docosenamide was previously isolated from the halophilic *Bacillus* sp. BS3, exhibiting biosurfactant activity and potential antimicrobial activity against *E. coli*, *S. aureus*, *P. aeruginosa* and *Salmonella typhi* (Donio et al., 2013). It was also identified as a common compound produced by 18 different halophilic bacterial isolates of the genera *Halobacillus*, *Sanilivibrio*, *Oceanobacillus*, *Salinicoccus* and *Thalassobacillus*, which were isolated from a South African Saltpan (Selvarajan, Sibanda, Tekere, Nyoni, & Meddows-Taylor, 2017). In a different study, a 6-*cis*-docosenamide isomer was isolated from *Asimina parviflora* fruit, and its cytotoxic activity against the cell line A-549 was observed, showing a 10-fold greater activity than that observed in this study (Ratnayake, Fang, Anderson, McLaughlin, & Evert, 1992). However, this difference could be explained because, in our study, the cytotoxic activity of the crude extract was evaluated and not the pure compound. In addition, it is also important to consider the difference in bioactivities between structural isomers.

For A1SM1-29, the obtained isolates were related to *Virgibacillus dokdonensis* and *Virgibacillus chiguensis*, both of which were previously isolated from marine environments. *V. dokdonensis* was isolated from seawater near a Korean island (Yoon, Kang, Lee, Lee, & Oh, 2005) and *V. chiguensis* was isolated from sediment from a commercial saltern in Taiwan (C.-Y. Wang, Chang, Ng, Chen, & Shyu, 2008) using the standard dilution plating method with marine and DSM 372 agar supplemented with 3% and 5% (w/v) NaCl, respectively, consistent with the salinity used in our study to culture samples taken from the A1 pond, such as A1SM1-29. *V. dokdonensis* VIT P14 has shown promise in a wide range of applications (Rajeswari, Jayaraman, & Sridharan, 2012). As far as we know, there has been no report on the biological activity or industrial applications of *V. chiguensis*. The lack of observed activity for the A1SM1-29 isolates could be explained by two different rationales. The first rationale is that the production of the bioactive metabolites

could be influenced by the interactions between the microorganisms co-existing in the culture, as was published in recent study in which the mixed culture or a co-culture was used as an excellent strategy to induce the production of new metabolites in microorganisms grown under laboratory conditions (Bertrand et al., 2014; Marmann, Aly, Lin, Wang, & Proksch, 2014). A metabolomic approach could be useful to compare the metabolic profiles of each strain when grown individually or in a mixed culture to establish which metabolites are induced by the interactions among strains. Glionitrin A, a diketopiperazine disulfide, is produced during the co-culture of a *Sphingomonas* bacterial strain and *Aspergillus fumigatus* but was not detected in the monoculture. This compound displayed an antibiotic activity against MRSA and cytotoxic activity against four human cancer cell lines, HCT-116, A-549, AGS and DU145, suggesting that microbial competition can result in the production of novel chemical structures that could be potential drug candidates (Park, Kwon, Lee, & Yang, 2009). The second rationale is related to the fact that the strain responsible for the bioactivity in A1SM1-29 could not be isolated with the subcultivation techniques used in this study, in contrast to the results obtained for the A1SM3-36 mixed culture, from which the bioactive strain *Vibrio* sp. A1SM3-36-8 was obtained. A metagenomic approach could be used to establish if all species present in the mixed cultures were isolated to develop a selective strategy for their cultivation.

6. CONCLUSION

In this study, we used a strategy in which bioactive molecules in initial mixed cultures were detected by biological assays to guide the screening of bioactive isolates of interest, which led to the isolation and characterization of a *Vibrio* sp. (strain A1SM3-36-8) with antibacterial and cytotoxic potential. However, as isolates obtained from the mixed culture A1SM1-29 did not show biological activity when grown individually, further research is needed to establish whether the production of bioactive metabolites was regulated by the interaction between the strains of this mixed culture or whether the isolation of the responsible strain is problematic. These results highlight that the strategy of using mixed cultures is an efficient alternative to identify cultures of microorganisms with pharmacological applications.

In addition, NMR and LC-QTOF-MS analyses led to identification of the major compound (13-*cis*-docosenamide) in the F3 bioactive fraction, which may be the compound responsible for the antibacterial activity against MRSA observed for the A1SM3-36 extract. The two additional fractions that displayed antibacterial activity could not be characterized due to the low extraction yield. However, this result confirmed the potential of *Vibrio* sp. A1SM3-36-8 to produce several compounds with antimicrobial activity.

One future perspective to the approach described in this study is the implementation of a wider panel of bioassays, which could allow for bioassay-guided isolation of new or known bioactive metabolites with new bioactivities.

7. DECLARATIONS

7.1. Ethics approval and consent to participate

Not applicable

7.2. Consent for publication

Not applicable

7.3. Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

7.4. Competing interests

The authors declare that they have no competing interests.

7.5. Funding

This research was supported by the Research Division of Universidad de La Sabana grant (ING 139-2013) and COLCIENCIAS Scholarship 6172-2014 (National Doctoral Grant for NCM). AAG is a recipient of an "Es Tiempo de volver 2015–2016" postdoctoral fellowship from COLCIENCIAS and the Universidad de La Sabana (Bogotá, Colombia). Besides financing the funding bodies played no role in study design, collection of data, or manuscript preparation.

7.6. Author contributions

Conceived and designed the experiments: NCM, ET, AAG and LED. Performed the experiments: NCM. Analyzed data: NCM and ET. Prepared the manuscript: NCM, ET and AAG. All authors read and approved the final manuscript

7.7. Acknowledgments

The authors thank the Dealership Big Group S.A. (Salinas Manaure) for their help during the sample collection and Claudia Parra-Giraldo at the Pontificia Universidad Javeriana for help with the MALDI-TOF MS analysis.

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9. ADDITIONAL FILES

Additional file 1

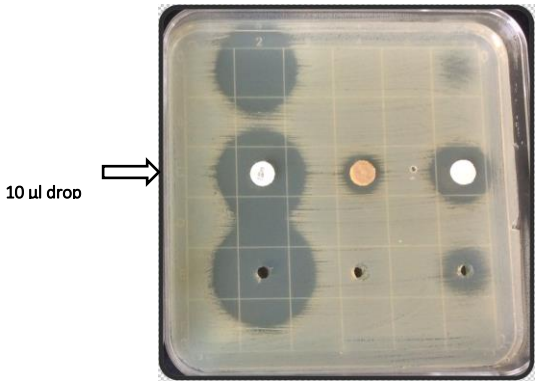
Table S1. Mixed cultures obtained from brine and sediment samples from the Manaure solar saltern and extraction yield with ethyl acetate.

Mixed Culture*	Salinity	Extraction Yield mg/mL
A1BM2-1	4%	0.15
A1SM2-2	4%	0.16
C6BM2-3	9%	0.09
C6SM2-4	9%	0.08
C8BM2-5	15%	0.13
C8SM2-6	15%	0.11
A1BM4-7	4%	0.03
A1SM4-8	4%	0.03
C6BM4-9	9%	0.03
C6SM4-10	9%	0.06
C8BM4-11	15%	0.03
C8SM4-12	15%	0.01
A1BM7-13	4%	0.09
A1SM7-14	4%	0.07
C6BM7-15	9%	0.11
C6SM7-16	9%	0.10
C8BM7-17	15%	0.11
C8SM7-18	15%	0.08
A1BM5-19	4%	0.07
A1SM5-20	4%	0.08
C8BM5-21	15%	0.09
C8SM5-22	15%	0.07
A1BM6-23	4%	0.08
A1SM6-24	4%	0.13
C6BM6-25	9%	0.06
C6SM6-26	9%	0.08
C8BM6-27	15%	0.08
C8SM6-28	15%	0.08
A1SM1-29	4%	0.26
A1BM1-30	4%	0.08
C6SM1-31	9%	0.11
C6BM1-32	9%	0.09
C8SM1-33	15%	0.11
C8BM1-34	15%	0.09
A1BM3-35	4%	0.02
A1SM3-36	4%	0.06
C6BM3-37	9%	0.02
C6SM3-38	9%	0.05
C8BM3-39	15%	0.06
C8SM3-40	15%	0.03

* The mixed cultures were named by the corresponding pond of isolation (A1 – C6 – C8); B or S for Brine or Sediment sample; the medium in which the sample was grown initially (M1 to M7, see Table 1) and a consecutive number from 1 to 40, e.g. A1BM2-1; was the mixed culture obtained from the brine sample of A1 pond and grown in M2 medium.

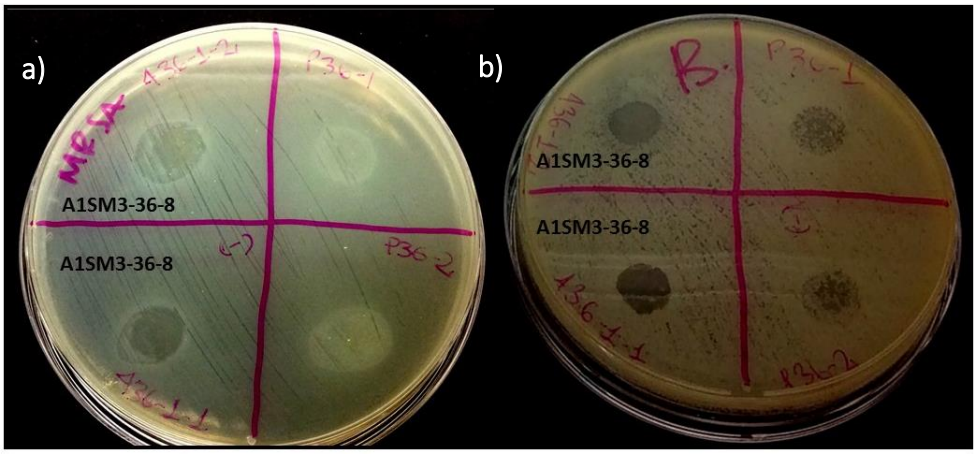
Additional file 2

Figure S1. Antibacterial assay by direct diffusion in an agar plate. Comparison of the inhibition zones of a 10 µL drop with 30 µg of chloramphenicol with the inhibition diameter of antimicrobial susceptibility discs (OXOID, Hampshire, England) on an agar plate that was pre-inoculated with a 0.5 McFarland MRSA strain inoculum and grown at 37°C for 24 h.



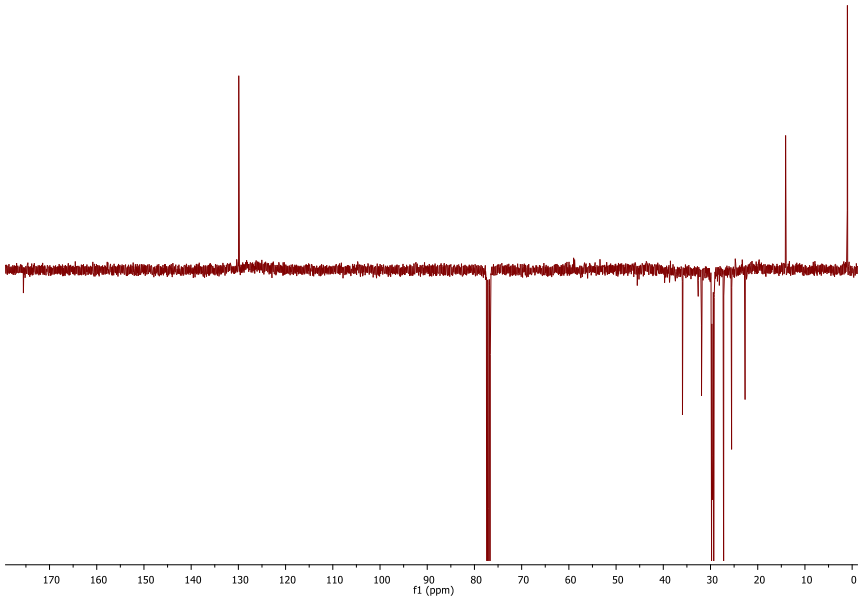
Additional file 3

Figure S2. Antibacterial activity of the A1SM3-36-8 isolate extract against. a) MRSA and b) *B. subtilis* in duplicate. The clear regions are the zones of growth inhibition caused by an aliquot containing 150 µg of the extract in 20% DMSO.

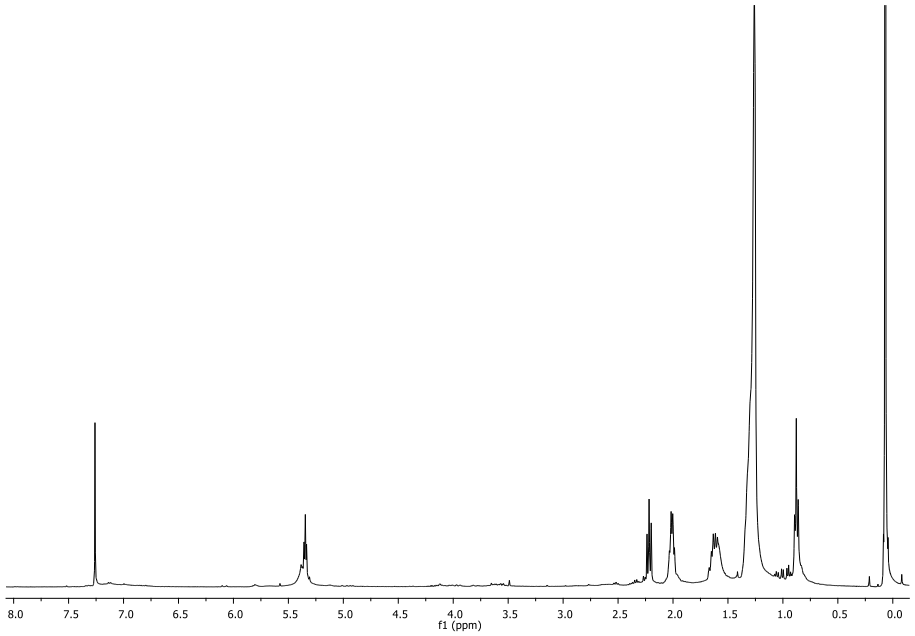


Additional file 4

Figure S3. Nuclear magnetic resonance spectra of the F3 fraction. ^{13}C (APT) NMR Spectrum of the F3 fraction (13-*cis*-docosenamide)



^{1}H NMR Spectrum of the F3 fraction (13-*cis*-docosenamide)



CHAPTER II: Integrating molecular network and culture media variation to explore the production of bioactive metabolites by *Vibrio diabolicus* A1SM3

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Published in Marine Drugs 2019;17(196):1-18. Doi: 10.3390/md17040196

1. ABSTRACT

Vibrio diabolicus A1SM3 strain was isolated from a sediment sample from Manaure Solar Saltern in La Guajira and the produced crude extracts have shown antibacterial activity against methicillin-resistant *Staphylococcus aureus* and cytotoxic activity against human lung cell line. Thus, the aim of this research was to identify the main compound responsible for the biological activity observed and to study systematically how each carbon and nitrogen sources in the growth media, and variation of the salinity, affect its production. For the characterization of the bioactive metabolites, 15 fractions obtained from *Vibrio diabolicus* A1SM3 crude extract were analyzed by HPLC-MS/MS and their activity was established. The bioactive fractions were dereplicated with Antibase and Marinlit databases, which combined with nuclear magnetic resonance (NMR) spectra and fragmentation by MS/MS, led to the identification of 2,2-di(3-indolyl)-3-indolone (isotrisindoline), an indole-derivative antibiotic, previously isolated from marine organisms. The influence of the variations of the culture media in isotrisindoline production was established by molecular network and MZmine showing that the media containing starch and peptone at 7% NaCl was the best culture media to produce it. Also, polyhydroxybutyrates (PHB) identification was established by MS/MS mainly in casamino acids media, contributing to the first report on PHB production by this strain.

Keywords: *Vibrio diabolicus*; molecular networking; 2,2-di(3-indolyl)-3-indolone; PHB; MS/MS; solar saltern

2. INTRODUCTION

Vibrionaceae family (composed *c.a.* 128 species) is considered a Gram-negative and gamma-proteobacteria, ubiquitous in marine environments and has been spotted out for its genomic flexibility and mainly for their importance as pathogens (F. Thompson et al., 2004). Until 2011, only 93 bioactive compounds have been reported to be isolated from the *Vibrionaceae* family (Mansson et al., 2011). Recently, some studies revealed the potential of *Vibrionaceae* as a source of new and bioactive natural products (Wietz, Mansson, Gotfredsen, Larsen, & Gram, 2010). Among them, *Vibrio spp*. are known to be the most representative species in the *Vibrionaceae* family (64 species) being characterized by the non-ribosomal peptides production and some hybrids synthesized by non-ribosomal peptide and polyketide synthases (Liaw et al., 2015; Mansson et al., 2011). Another family of bioactive compounds that have been widely reported is the indole alkaloids which includes the turbomycin A and B (Veluri et al., 2003), vibrindole A (Bell et al., 1994), trisindoline (Kobayashi et al., 1994) and the bis-indolylmethane derivatives, presenting anticancer activities (Li, Zhou, Zheng, Hai, & Wu, 2016; Mansson et al., 2011). On the other hand, some marine species from *Vibrio* spp. have been studied for their potential to produce polyhydroxybutyrates (PHB), a biopolymer produced and accumulated by prokaryotes, widely studied for its biotechnological potential as an alternative solution to replace plastic due to their biodegradability properties (Ganapathy et al., 2018).

Despite the biosynthetic potential of microorganisms to produce a variety of metabolites, several biological and analytical challenges still hamper their production, isolation, and identification (Aksenov, Da Silva, Knight, Lopes, & Dorrestein, 2017). The different forms of interaction between the strains and their surrounding environment (biotic and abiotic) determine the degree of metabolic diversification through a panoply of different biosynthetic pathways activation, including those related to the expression of cryptic genes—typically not expressed in normal laboratory conditions (Schroeckh et al., 2009). To address these limitations, the chemo-physiological conditions associated with different biosynthetic pathways expression have been studied by genomic strategies, metabolic engineering or system biology-based approaches such as OSMAC (One Strain, Many Compounds) and co-culture (Aghcheh & Kubicek, 2015; Bergmann et al., 2007; Bode, Bethe, Höfs, & Zeeck, 2002). In OSMAC strategy, the different metabolites production is obtained by varying the growth parameters (biotic factors: temperature, pH, time, media composition, luminosity, etc.)

using chemometric tools considering that within a sample space it is possible to correlate changes in the environment with different expressions levels of secondary metabolite biosynthesis (Bode et al., 2002).

Currently, microbial metabolomics and OSMAC studies are using advanced analytical techniques, especially LC-MS for the analysis of metabolic classes of interest (Brunetti et al., 2018). Typically, large MS/MS fragmentation data sets generated by automated analysis are evaluated by chemometric tools or web services to reduce data complexity and promote clues about trends and similarities. Among prominent approaches, molecular networking (MN) seems to be a particularly effective tool for the processing of fragmentation data from tandem mass spectrometry (MS/MS), since it allows to visualize tendencies, group and identify compounds based on spectral similarity. As structurally related compounds share similar fragmentation spectra, the molecular network tends to form closely related agglomerates (Aksenov et al., 2017).

There are some emerging online platforms that enable analysis, interpretation, and dissemination of knowledge efficiently, reliably, and quickly based on the molecular network. The recent mass spectra consortium featured on the GNPS platform have played an important role on analysis of large datasets of tandem MS experiments from microbial extracts clustering of MS-related data visualization and consequently, leading to correlated biological information such as species grouping or information relative to bioactive compounds (Wang et al., 2016).

Considering that the antibacterial and cytotoxic activity of *Vibrio diabolicus* A1SM3 was established in our group (Conde-Martínez, Acosta-González, Díaz, & Tello, 2017), we proposed here a strategy combining different growth parameters with molecular network tool for rapid and reliable clustering and characterization of bioactive compounds. For this purpose, variation in carbon and nitrogen source and salinity of the initial culture medium were evaluated to establish how these changes enhance the production of 2,2-di(3-indolyl)-3-indolone named as isotrisindoline, an antibiotic indole alkaloid isolated from *Vibrio* species (Giddings & Newman, 2015) and produced by the metagenomes genes of the sponge *Discodermia calyx* (Liu & Yang, 2017; Takeshige, Egami, Wakimoto, & Abe, 2015), and the PHB production, using a combined strategy of HPLC-MS/MS analysis and GNPS workflow.

3. RESULTS

3.1. Growth of *Vibrio diabolicus* A1SM3 in Modified Culture Media.

The initial culture medium (M3 medium) where the bioactive compounds were detected contain glucose (glu), starch (star), and sodium pyruvate (pyr) as carbon sources, and yeast extract, peptone (pep) and casamino acids (cas) as nitrogen sources (Conde-Martínez et al., 2017). From this initial composition, 12 modified culture media were proposed (Table 1) to establish how each of these nutrient sources affect the growth and the metabolic profile of *Vibrio diabolicus* A1SM3. The differences in the bacterial growth of *Vibrio diabolicus* A1SM3 in the different modified media (Supplementary Figure S1), were compared by the area under the curve (AUC) calculated for each culture at each salinity. From two-way ANOVA analysis was established a significant difference in the AUC between cultures (level: cultures; $F = 70.89$, $p = 2 \times 10^{-16}$) and the results from Tukey HSD post-hoc test showed that the growth in the media with glucose as the sole carbon source was significantly lower compared to the growth in the media with sodium pyruvate and starch, and also compared with the cultures that have only nitrogen source (Figure 1a).

Table 1. Composition of each nutrient source in the modified culture media and their coded.

Modified Culture Media	Composition of nutrient sources* (g/L)					
	Glucose	Starch	Sodium Pyruvate	Yeast Extract	Peptone	Casamino Acids
pyr_cas	0.0	0.0	1.3	0.0	0.0	1.5
pyr_pep	0.0	0.0	1.3	0.0	1.5	0.0
pyr_yeast	0.0	0.0	1.3	1.5	0.0	0.0
star_cas	0.0	1.3	0.0	0.0	0.0	1.5
star_pep	0.0	1.3	0.0	0.0	1.5	0.0
star_yeast	0.0	1.3	0.0	1.5	0.0	0.0
glu_cas	1.3	0.0	0.0	0.0	0.0	1.5
glu_pep	1.3	0.0	0.0	0.0	1.5	0.0
glu_yeast	1.3	0.0	0.0	1.5	0.0	0.0
cas	0.0	0.0	0.0	0.0	0.0	2.8
pep	0.0	0.0	0.0	0.0	2.8	0.0
yeast	0.0	0.0	0.0	2.8	0.0	0.0

* The composition is expressed as g/L. The total amount of nutrient sources was kept at 2.8 g/L, therefore, the relative proportion of carbon sources correspond to 46% (1.3 g/L) and for nitrogen sources was 54% (1.5 g/L). Glucose (glu), sodium pyruvate (pyr), starch (star), peptone (pep), casamino acids (cas) and yeast.

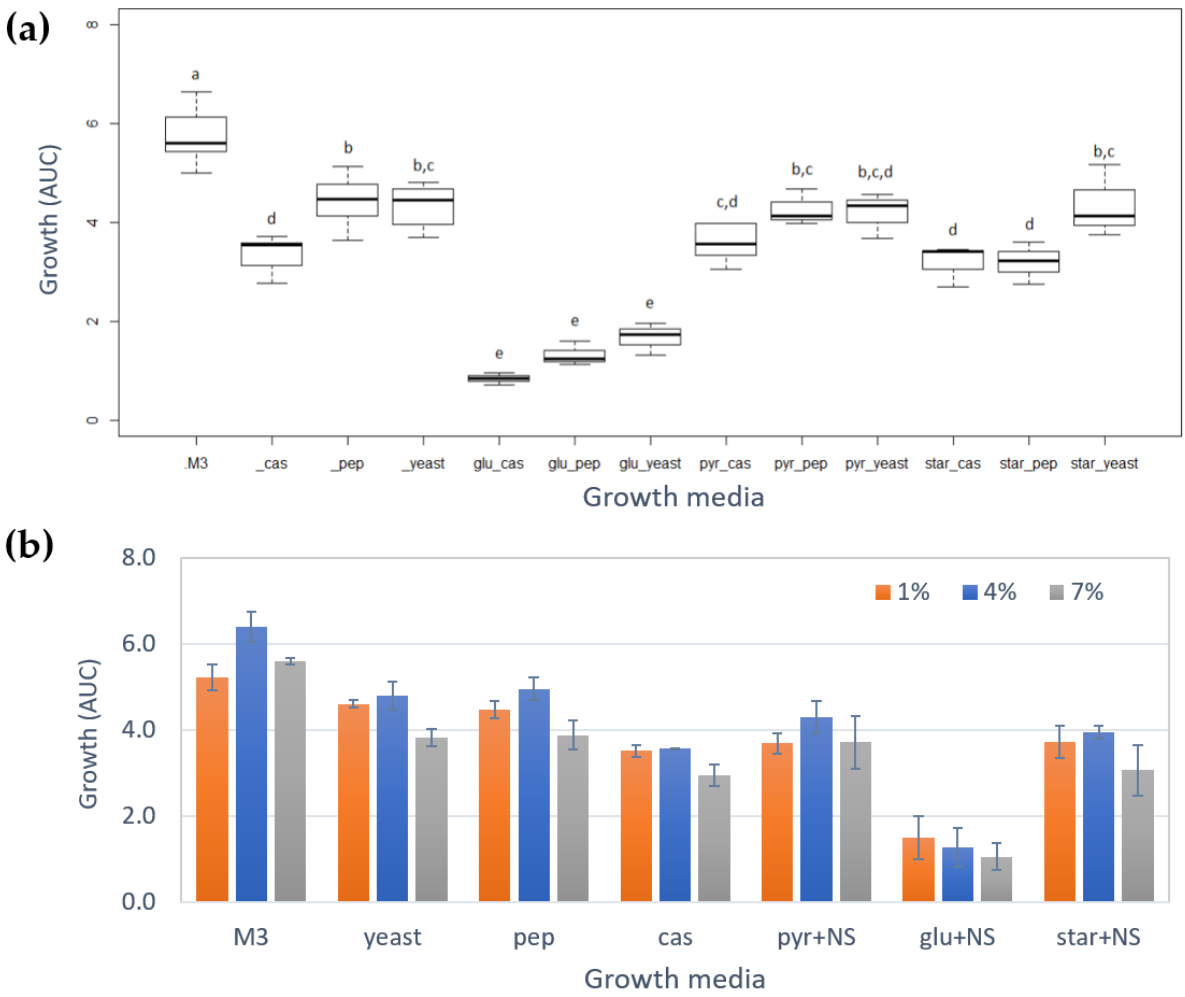

Figure 1. (**a**) Boxplot of growth of *Vibrio diabolicus* A1SM3 calculated as area under the curve (AUC) in each growth media. Glucose (glu), sodium pyruvate (pyr) and starch (star), casamino acids (cas), peptone (pep) and yeast extract. The results of AUC for each growth media are from the replicates and includes all the salinities tested. The boxes bearing different letters (a, b, c, d and e) were significantly different according to the Tukey HSD test ($P < 0.05$). (**b**) Growth of *Vibrio diabolicus* A1SM3 calculated as AUC grouped by salinity and carbon source (as a binary mixture with the nitrogen source, NS), M3 medium and growth media with only nitrogen source (without carbon source), casamino acids (cas), peptone (pep) and yeast extract.

This behavior revealed that the presence of glucose had some negative effect on *Vibrio diabolicus* A1SM3 growth under these conditions. In addition, the best medium to grow this strain was the initial M3 medium (Figure 1a).

Additionally, from two-way ANOVA considering the media and the salinity, was established that the growth of *Vibrio diabolicus* A1SM3 was significantly affected (level: salinity; $F = 19.42$, $p = 1.4 \times 10^{-6}$) by the

salinities tested (Figure 1b). In Figure 1b was evidenced the differences in the growth in the modified media by salinity. For M3 media, the higher growth was observed at 4% of NaCl.

3.2. Study of the Metabolic Profile of *Vibrio diabolicus* A1SM3 in the Modified Culture Media

The metabolic profile produced by *Vibrio diabolicus* A1SM3 in the modified culture media was investigated here by analyzing the crude extracts by HPLC-MS/MS. The data were treated in MZmine software and the peak area was used for this investigation. Figure 2a shows the ion (*m/z*) dispersion by the retention time and it is possible to observe clearly that most metabolites have less polarity characteristic. Moreover, most of the ions were up to about 800 (*m/z*), and only a few ions were observed above 1000 (*m/z*). Figure 2b shows that the lower metabolic diversity production was observed in media with yeast extract: pyr_yeast_1 (35 metabolites) and star_yeast_4 (36 metabolites), except for pyr_pep_1 (33 metabolites) which could be justified by the presence of the carbon source sodium pyruvate, which apparently is also related with few metabolites production. When considering the mixture of all the components in M3 medium, it was observed that the salinity showed an inversely proportional influence on metabolites production, the increase of salinity lead to a decrease of metabolic diversity.

On the other hand, greater metabolic diversity production was observed in the growth media containing starch plus casamino acids at 1% NaCl (295 metabolites), followed by starch plus yeast extract at 7% NaCl (230 metabolites) and M3 at 1% NaCl (222 metabolites). Therefore, comparing the same carbon source, the casamino acids was the most important parameter to induce metabolic diversity production. Interestingly, starch plus yeast extract combination at 7% NaCl, *Vibrio diabolicus* A1SM3 also produced a great number of metabolites (230), however, it was not possible to correlate this result with any variable evaluated in the culture media. In general, it seems that the nitrogen source was the most influential component in metabolites production. In Principal Component Analysis (PCA) (Figure 2c) the crude extracts obtained from star_yeast_7 and star_cas_1 were the samples with less correlation with the others (Supplementary Figure S2). Therefore, these samples were considered as outliers and were removed from the table to build a Hierarchical Clustering Analysis (HCA) (Figure 2d). In Figure 2b it is possible to evidence the clustering of the cultures in four main groups. Group 1 are formed mainly by cultures containing as nitrogen source casamino acids, as well as, group 3 formed only by growth media containing peptone and group 4 formed mainly by cultures containing yeast extract, which statistically support the hypothesis that the nitrogen sources are the most important nutrient evaluated in the culture media metabolic diversity by *Vibrio diabolicus* A1SM3. Additionally, no grouping by salinity was evidenced, meaning that, even though

the salinity influences the growth of the strain, no relationship was observed between the variations in the metabolic profile and the salinities tested (Figure 2d).

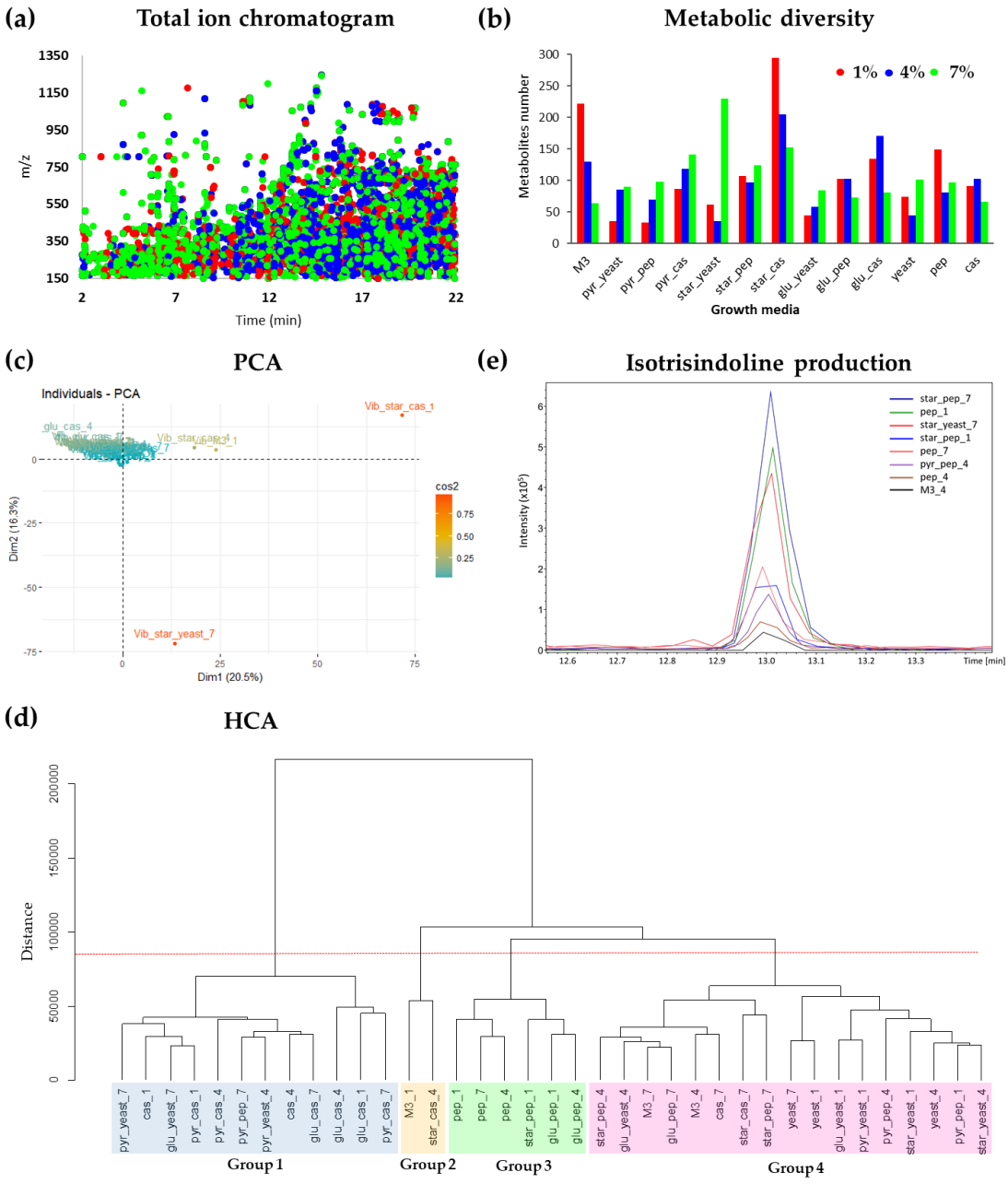

Figure 2. Metabolic profile description of the crude extracts produced by *Vibrio diabolicus* A1SM3 in modified culture media considering the HPLC-MS analyses. (**a**) Total ion chromatogram. (**b**) Metabolic diversity showing the number of metabolites produced in the mixture design. (**c**) Principal Component Analysis (PCA) showing the outlier samples star_yeast_7 and star_cas_1 (Table S1). (**d**) Hierarchical Clustering Analysis (HCA) without the outliers indicated in PCA, showing a clustering mainly by nitrogen sources. (**e**) Isotrisindoline peak (*m/z* 364) in the crude extracts of different modified culture media compared with M3 medium at 4%.

3.3. Cytotoxic Activity and Dereplication of the Fractions from *Vibrio diabolicus* A1SM3 Grown in M3 Medium

Considering that M3 medium at 4% NaCl was the initial medium to growth *Vibrio diabolicus* A1SM3 and its crude extract showed the bioactivities, 4L of culture was extracted with ethyl acetate and the crude extract was fractionated by column chromatography using as eluent solvent a gradient from n-hexana:ethyl acetate (7:3) to ethyl acetate:methanol (9:1), obtaining 15 grouped subfractions according to the TLC profile (Supplementary Table S2). The cytotoxic activity of 10 of these fractions was evaluated against two human cancer cell lines and one non-tumor cell line (control toxicity) were used. The results showed that fractions F4 and F6 were slightly cytotoxic against the human cervix epithelial cancer cell line, SiHa (ATCC® HTB – 35 ™) with IC_{50} of >100 µg/mL and 80 µg/mL, respectively. On the other hand, fraction F5 displayed a stronger activity with an IC_{50} of 28 µg/mL against the same cell line. In addition, none of these fractions showed inhibition of the non-tumor cell line (IC_{50} >100 µg/mL).

According to the analysis of the chromatographic profiles of these three cytotoxic fractions, F5 showed the most intense peak at 12.9 min (Supplementary Figure S3), which were also found in F6 with less intensity, and in F4 as a small peak. These results could be correlated with the cytotoxic assay, due to the stronger activity showed by F5 compared to F6 and F4. This peak showed *m/z* 364.1461 $[M + H]^+$ and the molecular formula was determined as $C_{24}H_{18}N_3O^+$ (calculated mass of 364.1450 Da, 3 ppm). The dereplication analysis for ion *m/z* 364.1461 using Antibase and MarinLit databases showed a match for trisindoline and isotrisindoline (2,2-di(3-indolyl)-3-indolone), an antibiotic indole-trimer alkaloids both previously isolated from associated-bacterium *Vibrio* sp. from marine sponge *Hyrtios altum* (Kobayashi et al., 1994), and from *Vibrio parahaemolyticus* (Bell et al., 1994; Veluri et al., 2003). The MS/MS spectrum (Supplementary Figure S3) showed that the fragmentation of *m/z* 364.1458 resulted in the fragment *m/z* 336 as the most intense fragment ion, corresponding to a neutral carbon monoxide loss (C=O). The ion at *m/z* 247 corresponds to a neutral loss of 117 Da, representing the cleave of an open indole moiety (C_8H_7N) which was corroborated by the ion *m/z* 247.0844 (calculated for $C_{16}H_{11}N_2O^+$; 247.0866 Da, 8.7ppm) in HR-ESI-MS spectra. Finally, the ion at *m/z* 219 corresponds to the loss of the indole moiety from fragment *m/z* 336. Considering that F5 was an impure fraction, a mono-dimensional ^1H TOCSY (600 MHz, CD_3CN) experiment at 7.18, 6.909 and 6.86 ppm signals were performed, and three spin systems were established (Figure 3, bold bond). The first one corresponding to the aromatic ring of the indole moieties from H-4′ to

H-7′. The second one corresponds to the aromatic ring of an oxy-indole moiety from H-4 to H-7 and, finally the coupling between the NH and H-2′. This information, combined with HSQC, HSQC-TOCSY and HMBC experiment (600 MHz, CD_3CN), and the reported in the literature (Bell et al., 1994; Takeshige et al., 2015) led to confirm the identification of this compound as isotrisindoline, the major component in fraction F5 and the responsible for their cytotoxic activity (Supplementary Table S3 and Figure S4). The signals on the NMR data that led to establish the difference between the isotrisindoline and the trisindoline were the corresponding to the carbons in the oxy-indole ring, mainly the signals for the quaternary carbons 3a and 7a, followed by the signals of carbons 5 and 6 (Supplementary Table S3).

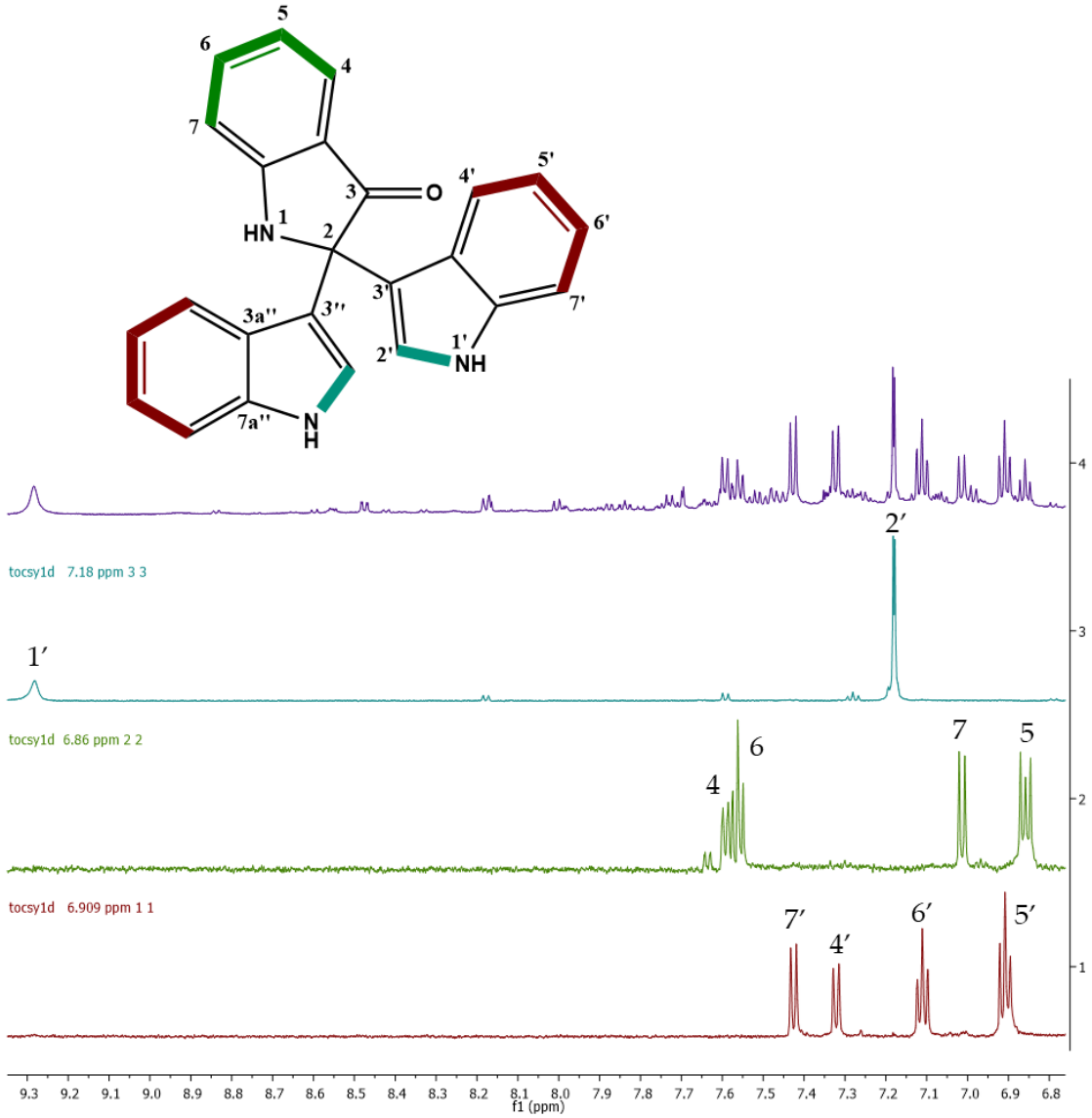

Figure 3. Mono dimensional ^{1}H-TOCSY experiment (600 MHz, CD_3CN) at 7.18, 6.909 and 6.86 ppm signals for F5 fraction. Bold bonds correspond to the spin systems established in isotrisindoline.

3.4. Molecular Networking and Isotrisindoline Production

HPLC-MS/MS data obtained from the 54 extracts from the modified culture media, 15 fractions, and 13 non-inoculated media controls, were converted to mzXML format, uploaded to GNPS web-platform and used to build a molecular network considering the online workflow. This tool clustered MS/MS spectra based on the similarity between them. After that, the data was exported and visualized into Cytoscape (Shannon et al., 2003).

A total of 705 nodes representing unique spectra (removing solvent blank nodes) were grouped in 57 clusters conformed with at least 2 nodes (Figure 4). No matches were found by GNPS library (reference compounds present in the platform), which may suggest new metabolites presence, or at least, similar compounds lacking in the GNPS library. This network was colored according to the carbon source used in the growth media and differencing the fractions (light blue) from them. This color-coded was useful to visualize the general production of metabolites under the specific conditions and which of them were also found in the fractions. In general, it was observed a correlation between the metabolites production (as nodes in the network) with the bacterial growth behavior aforementioned.

Once the ion associated with the isotrisindoline molecule (*m/z* 364.1450) was located in the molecular network, its presence in the cultures containing as sole carbon source, starch, and sodium pyruvate was established. Additionally, it was also detected in the growth media with only nitrogen source as a nutrient source; peptone and yeast extract. However, it was evidenced the lack of the M3 medium within this node. This is explained due to the low intensity and poor-quality of the MS/MS spectrum that correspond to the ion *m/z* 364.1450 in the raw data of M3 culture extracts which did not allow that GNPS algorithm to correctly grouped the MS/MS spectra from isotrisindoline in the node for this ion. Putting together this information with the peak area obtained in the aligned peak list from MZmine processing, it was possible to establish that the major peak area for isotrisindoline was in the cultures with starch plus peptone at 7% NaCl, followed by peptone at 1% and starch combined with yeast at 7% NaCl (Figure 2e). These results suggest that isotrisindoline production was higher in the cultures with starch and peptone at the three salinities tested.

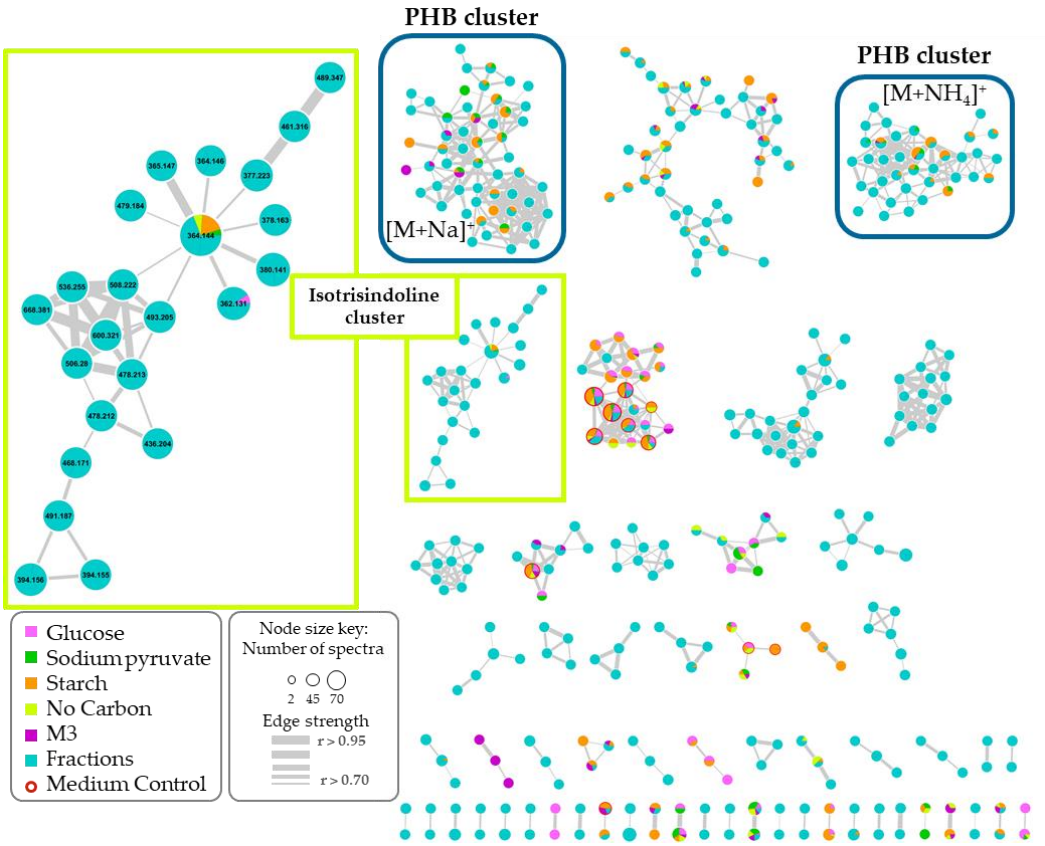

Figure 4. Molecular network for *Vibrio diabolicus* A1SM3 culture extracts in different growth media and including the fractions from the crude extract in the M3 growth medium. Each node is associated with an ion found in at least one of the growth cultures. The modified culture media were colored by carbon source as follows; glucose (pink), sodium pyruvate (dark green), starch (orange). Nodes found in the M3 medium are colored as purple and modified culture media with only nitrogen source (no carbon) are light green. The nodes found in fractions are in blue and the nodes also found in the extract of the non-inoculated medium control are circled in red. The cluster associated with isotrisindoline is marked in a light green square and PHB cluster in a blue square.

It is important to mention that the non-inoculated media were subjected to the same incubation period and extraction procedures. The search of the ion corresponding to isotrisindoline in the HPLC-MS/MS data of non-inoculated media extracts showed that this ion was not present, confirming that it is not an artifact from the medium. This proves that this compound was produced by *Vibrio diabolicus* A1SM3 and that is not generated only from media components during work-up procedures, as some authors have been described in the literature (Mansson et al., 2011; Veluri et al., 2003).

3.5. Polyhydroxybutyrates (PHB) Molecular Family

On the other hand, exploration of the vast information merged in the network and compared with literature and Antibase database, it was possible to identify two clusters associated with the

polyhydroxybutyrates (PHB) chemical family (Figure 4). These compounds are known to be the most common polyhydroxyalkanoates (PHA) produced by microorganisms under stress conditions, combined with an excess of carbon source, and accumulated as reserves of carbon and energy (Quillaguamán, Guzmán, Van-Thuoc, & Hatti-Kaul, 2010; Sasidharan, Bhat, & Chandrasekaran, 2015). Additionally, some species from *Vibrio* spp. have been described as PHB producer strains (Chien, Chen, Choi, Kung, & Wei, 2007; Numata & Doi, 2012; Sasidharan et al., 2015; Sun, Cao, Teng, & Meighen, 1994). In the network, despite to belong to the same chemical family, two clusters of nodes were associated with PHB, one cluster grouped the adducts with sodium $[M + Na]^+$ and the other with ammonium $[M + NH_4]^+$.

The identification of PHB was established by the fragmentation pattern observed in MS/MS spectra of the nodes grouped in both clusters. As an example, the ion peak at *m/z* 724.3391 corresponding to $[M + NH_4]^+$ adduct, according to peaks ions *m/z* 707.3126 as $[M+H]^+$ and *m/z* 729.2946 as $[M + Na]^+$ presence, which matches with the molecular formula of $C_{32}H_{50}O_{17}$ (calculated mass of 706.3048 Da) (Figure 5a). This pattern of adducts was observed in all nodes for both PHB clusters. The MS/MS spectra for both $[M + Na]^+$ and $[M+NH_4]^+$ adducts revealed the successive loss of 86 Da corresponding to a fragment of molecular formula $C_4H_6O_2$ which corresponds to the butyrate fragment in PHB polymer (Figure 5b).

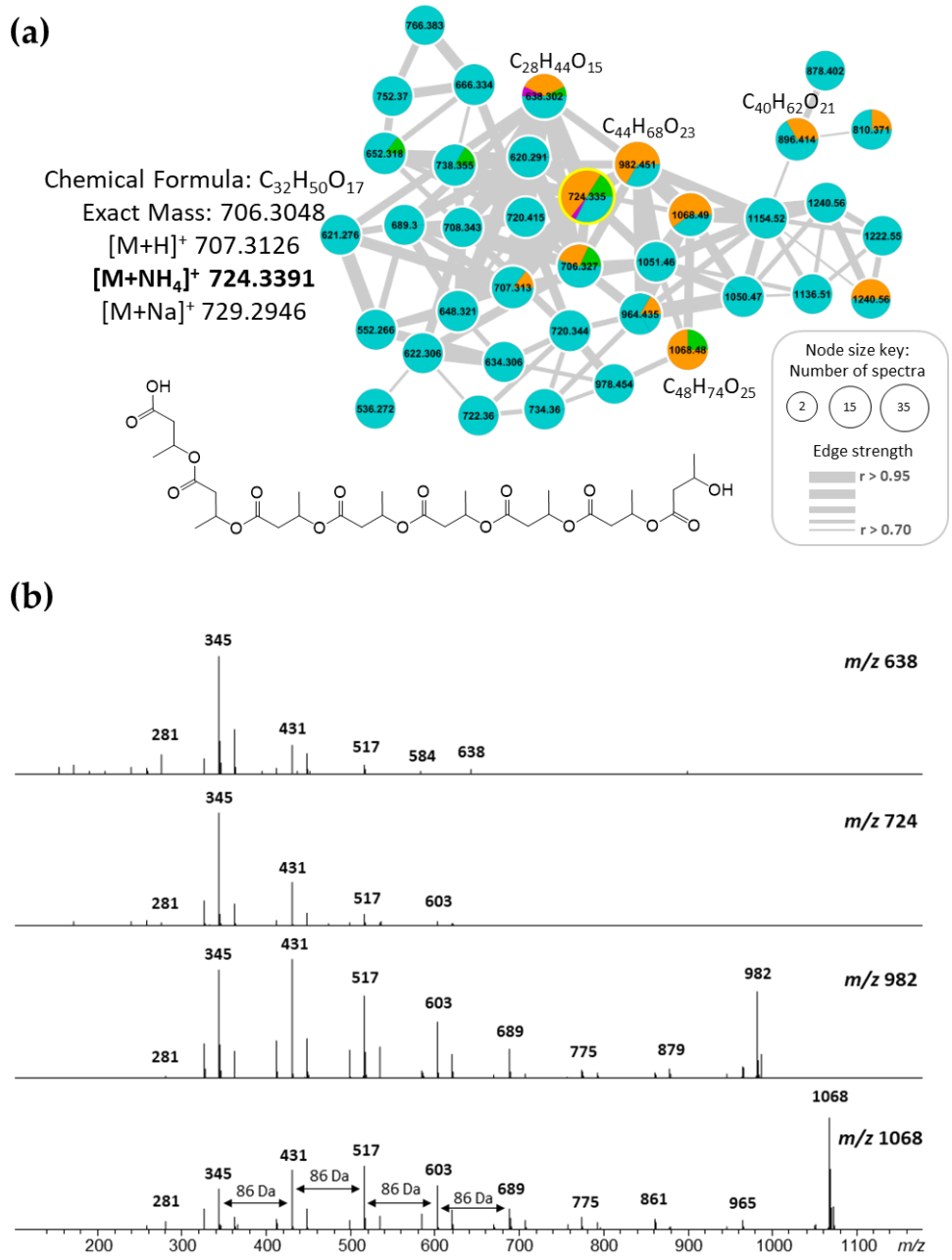

Figure 5. (a) PHB cluster of $[M + NH_4]^+$ adducts and molecular structure of PHB analog of the most representative node in the cluster. (b) MS/MS fragmentation pattern for most representative nodes in $[M + NH_4]^+$ adduct of PHB cluster.

Considering that most of the nodes observed in the first network belong to the fractions, a new network was built considering only the MS/MS data of the modified culture media to explore the variation in PHB production by *Vibrio diabolicus* A1SM3 in the different growth media (Figure 6). This network allowed to establish that the PHB production was independent of the nitrogen sources (Figure 6). However, the presence of casamino acids stimulates the production of more PHB analogs. Considering the cluster for PHB

with sodium $[M + Na]^+$ (Figure 6), 90% of the nodes were present in cultures with casamino acids, furthermore, 45% were exclusively produced in the culture media containing casamino acids. In addition, the same proportion was maintained in the cluster for $[M + NH_4]^+$ adducts.

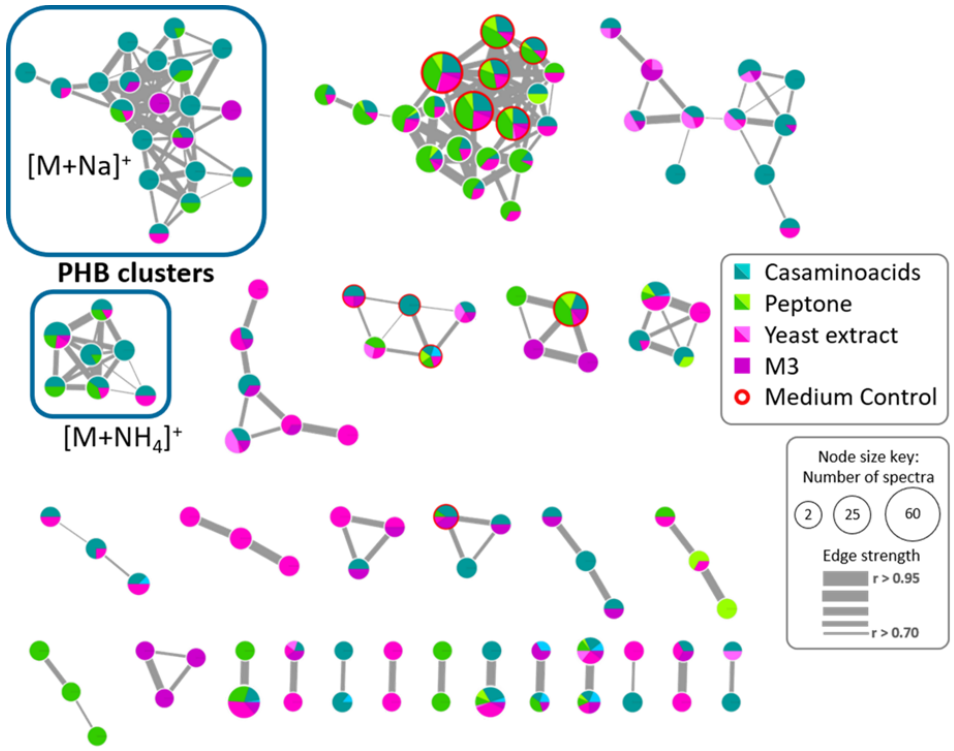

Figure 6. Molecular network for *Vibrio diabolicus* A1SM3 culture extracts in the modified culture media. The mixture design cultures by nitrogen source were colored as follows; casamino acids (blue), peptone (green), yeast extract (pink). Nodes found in the M3 medium are colored as purple and the nodes found also in the extract of the non-inoculated medium control are circled in red. The lighter tones are associated with the growth media without any of the carbon sources. The PHB related clusters are in a blue square.

Additionally, it was observed that PHB was exclusively produced in the media with sodium pyruvate and starch as the sole carbon source, this could be correlated with the negative effect in the growth of the microorganism in the culture media containing glucose as the sole carbon source (Figure 1a). In addition, it was evidenced that the carbon source presence in the growth media was necessary to PHB production because no PHB nodes were observed in the cultures with only nitrogen as a nutrient source even though the microorganism grew comparable with the other growth media. This was correlated with the biological function of the PHB family in microorganisms as a reserve of carbon and energy source.

4. DISCUSSION

Among the growth media tested at different salinities, it was possible to establish that the best growth of *Vibrio diabolicus* A1SM3 was at 4% NaCl, the same salinity of the pond from it was isolated in Manaure Solar Saltern. Additionally, it was evidenced that the nitrogen source was the most relevant parameter affecting the metabolic profile, being the casamino acids the source that enhance the number of metabolites in the culture extracts. Among the carbon sources, the starch showed to be the only that affects positively the metabolic production. Although the salinity affects significantly the growth of the strain no relationship between the salinity and the number of metabolites produced were evidenced.

The MS/MS analysis with GNPS allowed to visualize that there are many clusters from ions present only in the fractions, which means the analysis considering crude extracts could be leaving out many important ions that are in minor concentration or that does not have intense ionization. Therefore, we strongly suggest crude extracts fractionation before using in a molecular network or other metabolomic tools. Additionally, the development of MS/MS techniques and the evolution of the algorithms associated to this data are in constant improvement to enhance reproducibility and reliability under the same analysis parameters. This leads to consistent results when it comes to dealing with a biological process involving living organisms and their inherent variations related to their sensibility to environmental changes.

The strategy followed in this research led to isotrisindoline identification, a bioactive compound present in fraction F5 responsible for the cytotoxic activity against human cervix epithelial cancer cell line (SiHa) in the crude extract of *Vibrio diabolicus* A1SM3 which agrees with the cytotoxic activity previously reported for this compound (Cai et al., 2010). Additionally, antibacterial activity against *Staphylococcus aureus* reported for isotrisindoline (Bell et al., 1994) suggest that the antibacterial activity previously described against *S. aureus* methicillin-resistant (MRSA) by the crude extract of *Vibrio diabolicus* A1SM3 can be also attributed this compound. This bioactive compound was produced mainly in growth media containing peptone without distinguishing the salinity of the growth media. However, it was not possible to correlate its production with the growth of the strain. As it was evidenced in the media with the peptone only, *Vibrio diabolicus* A1SM3 showed the higher growth with one of the highest isotrisindoline production compared to the growth of the strain in starch and peptone media which showed one of the lowest growths (excluding the media with glucose) but with the major isotrisindoline production.

The indole derivatives have been commonly isolated from marine organisms, plants and bacteria (El-Sayed, Suzen, Altanlar, Ohlsen, & Hilgeroth, 2016), among them, the bis-indolylmethane moiety has a

relevant interest in pharmacological applications (Li et al., 2016) and a variety of biological activities have been described for this type of molecules as antifungal and antibacterial agents, as immunomodulators, in leukemia therapy and as anticancer agents (Li et al., 2016). So far, related to the biosynthesis of both isomers (trisindoline and isotrisindoline) it has been considered that, at least, one of the indole moieties is incorporated in post-biosynthetic reactions (Veluri et al., 2003). It has been described the role of indole as an intercellular signaling molecule that controls various bacterial phenotypes as biofilm formation and other virulence factors in Gram-positive and Gram-negative bacteria, including some species of *Vibrio* (J. H. Lee, Wood, & Lee, 2015). The indole is produced during the stationary cell growth phase and is synthesized from tryptophan by the enzyme tryptophanase encoded by the *tnaA* gene (Mueller, Beyhan, Saini, Yildiz, & Bartlett, 2009). In addition, this gene is repressed when the level of tryptophan is low (J. H. Lee & Lee, 2010), which agree with our results of isotrisindoline production in growth media with nitrogen sources rich in tryptophan, as peptone and yeast extract, compared with the non-production of this compound in the cultures grown with casamino acids. This latter nitrogen source is characterized by its low content of tryptophan due to the acid treatment during casein digestion to its production, which destroys the tryptophan (Simpson, Ducker, Diller, & Ruan, 2004). This suggests the biosynthesis of trisindoline isomers requires a source of indole moiety which is synthesized from exogenous tryptophan available in the growth media which was reported by Kwon and Weiss (2009) in the isotrisindoline production by *E. coli* during anaerobic growth (Y.-M. Kwon & Weiss, 2009). Additionally, the same authors determined that *tnaA* gene is required for the biosynthesis of isotrisindoline by the study of its production in a *tnaA* mutant (Y.-M. Kwon & Weiss, 2009).

Moreover, previous studies have been published regarding the *ipoA* gene involved in the initial oxidation of indole to indoxyl which, in equilibrium, generates 3-oxyindole that could be oxidized again to produce isatin, which has been proposed as the precursor of trisindoline and isotrisindoline by non-enzymatic reaction (N. R. Kwon et al., 2008; Yoo et al., 2008).

On the other hand, the studies conducted with the metagenome of the marine sponge *Discodermia calyx* showed that the gene corresponding to ORF 25, homologous to inosine 5′-monophosphate dehydrogenase (IMPD) was necessary for isotrisindoline production by *E. coli* (Takeshige et al., 2015). Takeshige et al. (2015) proposed the IMPD homolog as the responsible for the oxidation of the 3-oxyindole to isatin for further non-enzymatic addition of the two indole moieties to produce isotrisindoline and trisindoline. The annotation of the genome of *Vibrio diabolicus* A1SM3 recently studied in our group (data not published yet) showed the presence of *tnaA* gene responsible for the conversion of tryptophan to indole

and the IMPD homolog which putatively produce the isatin. However, the *ipoA* gene responsible for the oxidation of indole to 3-oxyindole was not annotated, this could be related with the high divergence of the strain where it was characterized (*Rhodococcus* sp. strain T104) and *Vibrio diabolicus* A1SM3.

In regard to the results obtained for PHB production in the different modified growth media, agree with previous studies where the addition of complex nitrogen sources (such as casamino acids) in the culture media, promote the PHB production by recombinant *Escherichia coli* strains and *Ralstonia eutropha* DSM 11348 (Bormann & Roth, 1999; S. Y. Lee & Chang, 1994; Yup Lee, 1998). This behavior has been explained by the amino acids and peptides composition of this source which facilitate the synthesis of proteins required for the cell functioning reducing the energy cost for the cell to synthesize them (Kalaiyezhini & Ramachandran, 2015; I. Y. Lee, Kim, Park, & Lee, 1996). Additionally, the result regarding the lower PHB production in media containing glucose was also reported by Chien et al. (2007) who studied the effect of different carbon sources on the PHB production by a marine *Vibrio* spp. finding the lower growth and production in the media containing glucose compared with glycerol, sucrose, sodium acetate and sodium succinate as carbon sources.

In conclusion, the approach implemented based on the OSMAC strategy coupled with HPLC-MS/MS and the GNPS platform guided the isotrisindoline identification, a bioactive compound with cytotoxic potential, and the PHB, which have been widely described and studied for their promising applications in biotechnology as an alternative solution to replace plastic due to their biodegradability properties (Ganapathy et al., 2018). Furthermore, the strategy employed in this work led to establishing the best nutrient sources of the culture medium to enhance these metabolites production under the conditions tested. Additionally, the articulation of these approaches represents a huge advantage in the processing and analysis of the data acquired, which allows going further in metabolites identification. Also, this study allowed determining how the variation in growth conditions, like carbon and nitrogen sources, impact the production of certain compounds. Therefore, these strategies could be also employed to study a range of environmental conditions, such as temperature, pH, co-culture, exposure to exogenous compounds, among others.

5. MATERIALS AND METHODS

5.1. *Vibrio diabolicus* A1SM3 Strain: Crude Extract Production and Fractionation

Vibrio diabolicus A1SM3 was isolated from a sediment sample recovered from a Solar Saltern in Manaure, La Guajira, Colombia. This strain was deposited in the Collection of Microorganisms of Universidad de La Sabana (USAB-BIO, Chía, Colombia) registered in the RNC Colombia. The bacteria was seed in growth medium (referred as M3 medium) with the following composition: 40 g/L NaCl, 20 g/L $MgSO_4$ $7H_2O$, 1 g/L KCl, 0.3 g/L KH_2PO_4, 0.5 g/L yeast extract, 0.5 g/L peptone (pep), 0.5 g/L casamino acids (cas), 0.5 g/L glucose (glu), 0.5 g/L starch (star) and 0.3 g/L sodium pyruvate (pyr) (Antón et al., 2002). After 15 days of incubation at 30 °C and 150 rpm, 4L of culture broth was extracted twice with ethyl acetate (1:1) and the organic fraction was concentrated in a rotary evaporator under vacuum. The extract (110 mg) was fractionated by SiO_2 column chromatography (250 mm × 15mm) using n-hexane:ethyl acetate (7:3) to ethyl acetate: methanol (9:1) gradient. A total of 80 fractions were obtained and grouped in 15 subfractions according to their TLC profiles. The grouped fractions were tested for cytotoxic activities and analyzed by HPLC-MS/MS and NMR (Bruker Ascend™ 600 MHz, Bruker Daltonics, Billerica, MA, USA).

5.2. Cytotoxic Activity

The fractions were evaluated for their cytotoxic activity against two human cancer cell lines and one non-tumor cell line by MTT cell proliferation assay (Mosmann, 1983). SiHa, human cervix epithelial cells (ATCC® HTB-35 ™) and human lung cell line A549 (ATCC® CRM-CCL-185 ™) were grown in Dulbecco's Modified Eagle's Medium—DMEM (Sigma-Aldrich Co, Darmstadt, Germany) supplemented with antibiotic agents (penicillin 120 IU/mL and streptomycin 100 IU/mL, Gibco/Invitrogen, Paisley, UK) and 10% fetal bovine serum (Eurobio, Les Ulis, France). L929, Fibroblasts (ATCC® CCL-1 ™) were incubated in RPMI medium supplemented with 1% (v/v) L-glutamine (Sigma-Aldrich Co., Darmstadt, Germany), 10% (v/v) fetal bovine serum (Eurobio, Les Ulis, France), 1% (v/v) penicillin and 1% (v/v) streptomycin (Gibco/Invitrogen, Paisley, UK). L929 was used as a non-tumor cell line for toxicity control. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Doxorubicin was used as a positive control (Al-Ghamdi, 2008). For MTT assay, 1×10^5 cells were gently placed into each well of a 96-well plate. After cell adhesion, the culture medium was changed for fresh medium containing different concentrations of the fractions (10, 25, 50, 80 and 100 µg/mL) diluted in less than 0.5% of DMSO and the cells were kept at 37 °C with 5% CO_2 for 72 h. Cell viability was measured by MTT colorimetric assay and 50% inhibitory concentration (IC_{50}) was calculated (Sebaugh, 2011). Negative controls for DMSO toxicity was also evaluated at 0.5%. Statistical analysis was

performed using GraphPad Prism 6® and Microsoft® Excel 2016. All the experiments were repeated at least three times and the results were expressed as mean values ± standard deviation. IC_{50} values were obtained by nonlinear regression.

5.3. Modified Culture Media and Metabolite Extraction of *Vibrio diabolicus* A1SM3 Cultures

The strain was cultured in different modified culture media varying the carbon and nitrogen source according to the initial growth medium composition, having into account that the total amount of the nutrient components was kept constant when the carbon and nitrogen sources of the modified media components were changed (H. Yin, Chen, Gu, & Han, 2009). A binary mixture was chosen to evaluate the effects of the three carbon sources: glucose (Merck, Darmstadt, Germany), sodium pyruvate (Fisher Scientific, Pittsburgh, PA, USA) and starch (Carlo Erba, Val de Reuil, France) combined with the three nitrogen sources: peptone (Oxoid, Basingstoke, UK), yeast extract (Oxoid, Basingstoke, UK) and casamino acids (AMRESCO Inc, VWR International, LLC; OH, USA) based on the initial composition of M3 medium. The modified media consisted of 12 combinations (Table 1) including three experiments with only one nitrogen source (peptone, casamino acids or yeast extract) as a unique nutrient source and 9 binary mixtures. Additionally, replicates of culture in M3 medium and experiments *pyr_cas*, *cas*, *yeast,* and *pep* (as named in Table 1) were done. All growth media also contained 20 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L KCl and 0.3 g/L KH_2PO_4 and NaCl according to the salinity tested (10, 40 and 70 g/L). The cultures (30 mL) were incubated for 15 days at 30 °C and 150 rpm. Samples from the cultures were taken every 24 h until day 4, after that, samples were taken in day 6, 11 and 15 to measure bacterial growth by OD_{595} and to calculate the area under the curve (AUC) for each culture. These results were analyzed using two-way ANOVA and Tukey HSD test for comparison of means in R software (R Core Team, 2013). The statistical significance was determined at p values < 0.05. After that, the whole culture broth was extracted twice with ethyl acetate (1:1) and the organic fraction was subjected to rotary evaporation under vacuum. A solution of 1 mg/mL of each extract in acetonitrile-methanol (7:3) was prepared for further HPLC-MS/MS analysis. The non-inoculated media were also extracted and used as a control.

5.4. HPLC-MS/MS Analysis

Twenty µL (1 mg/mL) of crude extracts and fractions were analyzed by HPLC-MS/MS using a Shimadzu system (Shimadzu, Tokyo, Japan), coupled with a micrOTOF-Q II mass spectrometer (Bruker Daltonics, Boston, MA, USA) equipped with an ESI source and a quadrupole-time of flight analyzer (qTOF, Billerica, MA, USA). For chromatographic analyses it was employed a Kinetex C18 column (2.6 µm, 150 × 4.6 mm)

(Phenomenex, Torrance, CA, USA) kept at 40 °C, with a flow rate of 0.8 mL/min applying a gradient solvent with phase A (water with 0.1% formic acid) and phase B (acetonitrile with 0.1% formic acid), from 10% to 40% of B in 6 min, to 70% of B in 12 min, and to 100% of B in 22 min. The MS data were acquired in positive mode using an MS range of *m/z* 50–1300. The equipment was calibrated with trifluoroacetic acid (TFA) every day, and internally during each run. The MS parameters were established as follows: nebulizer gas pressure, 4.5 Bar; dry gas flow, 9 L/min; capillary voltage, 3500 V; ion source temperature, 220 °C; spectra rate acquisition, 2 spectra/s. Auto MS/MS fragmentation was carried out for the 4 most intense ions per spectrum, and it was performed applying a gradient of collision-induced dissociation energy from 20 to 105 eV according to the parent mass. In addition, the precursor ion was released after 3 MS/MS spectra acquisition. All MS data were analyzed with Bruker Compass DataAnalysis 4.3 software (Bruker Daltonics, Boston, MA, USA).

5.5. HPLC-MS Data Processing

The converted MS data from the modified culture media and fractions were imported to MZmine 2.30 software (VTT Technical Research Center, Helsinki, Finland and Turku Center for Biotechnology, Turku, Finland) (Pluskal, Castillo, Villar-Briones, & Orešič, 2010) and processed following these steps; mass detection, chromatogram builder, chromatogram deconvolution, deisotoping, normalization, and alignment (Gorrochategui, Jaumot, Lacorte, & Tauler, 2016; Katajamaa & Orešič, 2007; Macintyre et al., 2014). The data processing parameters are shown in Supplementary Table S4. The aligned peak list was exported to CSV format with the peak areas of each feature. All features from solvent blanks were excluded. In addition, dereplication of the bioactive fractions were made using Antibase and MarinLit (http://pubs.rsc.org/marinlit/) databases.

5.6. MS/MS Data Processing for Molecular Networking

The raw data from HPLC-MS/MS was used to create a molecular network. The MS/MS data were converted to mzXML files using the MSConvert from ProteoWizard software (ProteoWizard, Palo Alto, CA, USA) (Kessner, Chambers, Burke, Agus, & Mallick, 2008) and then uploaded into the Global Natural Products Social Molecular Networking web-platform (http://gnps.ucsd.edu) (M. Wang et al., 2016). A molecular network was created using the online workflow at GNPS. The data were filtered by removing all MS/MS peaks within ± 17 Da of the precursor *m/z*. The data were then clustered with MS-Cluster with a parent mass tolerance of 0.02 Da and an MS/MS fragment ion tolerance of 0.02 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created

where edges were filtered to have a cosine score above 0.7 and more than 4 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries. The library spectra were filtered in the same way as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. For visualization and more specific analysis, the network data was exported and analyzed into Cytoscape (Version 3.6, Cytoscape consortium, San Diego, CA, USA) (Shannon et al., 2003). Two networks were constructed, the first one included the 36 extracts from modified culture media in the three salinities tested plus the 15 fractions from *Vibrio diabolicus* A1SM3, and in the second one, the 15 fractions were excluded to analyze the variation of metabolites production in the extracts from modified culture media.

5.7. NMR Analysis

The NMR experiments were carried out in a Bruker Avance III spectrometer using a 14.1 Tesla, (600.12 MHz in hydrogen frequency) magnetic field and 3 mm inverse triple cryoprobe ^{1}H, ^{13}C and ^{15}N nuclei. Each fraction was solubilized using 180 µL of CD_3CN (Sigma-Aldrich, Darmstadt, Germany). Chemical shifts to all experiments were calibrated from the solvent signal (CD_3CN) at 1.94 ppm. ^{1}H experiments were performed using the zg30 pulse sequence. Instrumental parameters were set up as follow: 1.0 s relaxation delay, spectral width from 0 to 12 ppm, 32 k points and 2.72 s of acquisition time. The post-processing was performed using a 0.1 Hz (Line Broadening—LB) exponential multiplication factor, and phase and baseline were manually corrected. ^{1}H-^{13}C HMBC experiments (hmbcetgpl3nd) were carried out using a spectral width of 0 to 10 ppm for the F2 dimension (^{1}H) and 0 to 200 ppm for the F1 dimension (^{13}C). An acquisition time of 0.0852 s, relaxation delay 2 s, and 32 scans for 128 increments was employed. Mono dimensional ^{1}H TOCSY (seldigpzs) experiments were performed at 7.18, 6.909 and 6.86 ppm. Regarding these experiments, the following conditions were used: spectral width of 0 to 10 ppm, 2.72 s acquisition time, 1.0 s relaxation delay, 32 k points, and 128 scans.

5.8. Statistical Analysis

The resulting table from the MZmine processing was used here. The matrix data were scaling by Pareto algorithm prior to multivariate analyses. Subsequent Principal Component Analysis (PCA) and Hierarchical Clustering Analysis were built using the R software version 3.3.1 employing the *factoextra* (Kassambara & Mundt, 2016) and *dendextend* (Galili, 2015) packages, respectively.

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7. SUPPLEMENTARY MATERIALS

Table S1. PCA coefficients for the variables in the 10 principal components.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Vib_cas_1	-5.7439	2.6141	-1.359	6.7812	-1.7709	1.1415	-2.6509	0.4078	-0.9579	0.0762
Vib_cas_4	-7.4338	3.4806	-5.7088	7.6803	-2.6359	1.8873	-4.016	2.2199	-2.9821	0.0491
Vib_cas_7	-2.1277	1.4339	4.2429	0.7956	-0.5252	-1.2179	0.1	-4.9625	2.2316	0.9228
Vib_glu_cas_1	-9.1492	4.9993	-15.4724	-0.3371	-0.5641	0.7097	-2.4206	2.3191	-9.0348	-5.2999
Vib_glu_cas_4	-16.3362	9.0604	-49.4769	-23.2261	3.2101	-2.7542	5.8603	-2.7288	6.4727	2.8525
Vib_glu_cas_7	-6.2318	2.6775	-3.9053	4.7924	-0.9516	1.1285	-1.6446	0.8653	-0.4835	0.074
Vib_glu_pep_1	-0.0482	-1.9648	7.4544	-6.5313	13.6193	3.9074	5.8826	3.5663	-4.702	-2.5689
Vib_glu_pep_4	-0.0967	-2.3693	8.3629	-7.4576	16.8054	4.2325	7.1721	4.6115	-6.7993	-3.7672
Vib_glu_pep_7	-2.4776	0.3828	4.1105	0.7916	4.7136	1.2269	2.2345	0.5311	1.3871	0.9845
Vib_glu_yeast_1	-4.1292	0.8673	2.4539	5.0455	0.079	0.8885	0.5926	-0.8054	4.7922	2.3944
Vib_glu_yeast_4	-4.5341	0.9934	1.9617	5.5787	0.0059	1.391	0.2028	-0.8117	3.9445	2.8791
Vib_glu_yeast_7	-6.5489	2.5228	-2.3945	7.7646	-1.6162	1.9219	-2.6529	0.8998	-0.8012	0.5174
Vib_m3_1	23.8515	3.4415	1.8441	-3.4679	-17.2023	-13.6931	17.8214	-1.0341	-14.4761	13.261
Vib_m3_4	-0.0204	0.9628	7.5389	-4.7089	-2.4039	-2.0916	3.4026	-26.9919	-4.0187	-14.1368
Vib_m3_7	-1.5938	0.5965	5.013	-0.9591	-0.6981	-0.7107	0.7526	-3.9618	1.4835	0.3603
Vib_pep_1	1.1507	-1.2055	13.7931	-25.8704	-22.9004	18.9448	-6.4934	7.0166	2.7295	-3.148
Vib_pep_4	-2.6642	0.2775	5.041	-0.4723	-1.2563	1.2514	1.2844	-2.5183	2.4502	2.8168
Vib_pep_7	-1.4818	-0.0023	8.783	-8.8311	-4.8633	5.1803	0.0727	-1.5399	1.9107	1.1325
Vib_pyr_cas_1	-5.2435	2.1632	-1.3923	4.6853	-0.9651	0.917	-1.1686	-0.2244	0.9491	0.5626
Vib_pyr_cas_4	-2.6505	2.1356	1.8922	1.0914	-0.7912	-4.1273	-4.7348	-1.0861	0.5952	-0.6383
Vib_pyr_cas_7	-8.7068	4.7577	-10.9204	6.517	-4.0523	1.667	-7.2537	4.4256	-15.4672	-7.4771
Vib_pyr_pep_1	-3.9401	1.1186	2.6773	4.3703	0.2793	0.2796	0.9265	-1.8585	4.664	2.7019
Vib_pyr_pep_4	-2.4355	0.5724	4.9539	-0.121	1.8569	0.6015	1.7456	-2.767	2.0944	1.1442
Vib_pyr_pep_7	-6.8106	2.8891	-3.4867	7.5333	-2.1013	1.6304	-3.2254	1.4319	-1.5971	0.4501
Vib_pyr_yeast_1	-4.2034	1.1283	2.4188	5.1892	-0.4351	0.9546	0.3254	-1.2439	4.7406	2.8567
Vib_pyr_yeast_4	-6.8943	2.9436	-2.9082	9.2583	-2.9674	2.3029	-4.0843	1.9878	-1.8597	0.9968
Vib_pyr_yeast_7	-7.6281	3.2204	-4.8226	9.2368	-3.2592	2.5801	-4.3932	2.8474	-3.4651	0.0987
Vib_star_cas_1	71.6993	16.9427	-13.7742	3.395	6.9206	11.1208	-9.005	-2.8848	1.6183	0.8458
Vib_star_cas_4	18.4288	4.4041	0.2312	1.9052	-5.6719	-17.2395	10.0685	13.9373	8.4546	-18.0979
Vib_star_cas_7	2.7474	0.4688	10.6052	-15.3313	4.586	-25.7735	-25.3736	0.3303	-0.427	3.7919
Vib_star_pep_1	-0.9789	-0.5348	7.2167	-5.2065	7.5776	1.9502	3.7497	1.4927	-1.6606	1.0593
Vib_star_pep_4	-1.1381	-1.4219	7.1503	-4.6828	10.8054	2.617	4.2909	3.4583	-1.4746	1.0777
Vib_star_pep_7	-0.7362	-2.4509	9.2728	-9.5224	10.6578	1.338	2.5567	4.5433	-3.9074	3.3517
Vib_star_yeast_1	-3.1458	0.0928	3.1313	3.222	2.2931	0.4976	1.289	-0.2513	3.0196	2.3933
Vib_star_yeast_4	-3.9444	1.108	2.39	4.2505	0.4195	0.2902	0.6084	-1.5014	4.2583	2.303
Vib_star_yeast_7	13.3619	-71.8045	-12.7836	2.3279	-1.3605	0.2324	-2.0474	-0.5535	0.0018	-0.4538
Vib_yeast_1	-0.8445	0.9684	2.3266	4.2955	-1.3992	-1.5087	3.4075	-0.3076	5.3416	1.9336
Vib_yeast_4	-4.3529	1.3267	1.8368	5.0585	-0.0583	0.6105	0.2376	-0.9515	3.8299	2.0238
Vib_yeast_7	3.0318	1.203	1.7027	5.1596	-3.3798	-4.2848	6.5802	2.0926	7.1449	-0.3238

Table S2. Fractionation of crude extract and cytotoxic activity from the fractions of *Vibrio diabolicus* A1SM3 grown in M3 medium.

Fraction	Weight (mg)	Cytotoxic activity IC_{50} (µg/mL)		
		SiHa	A549	L929
F1	12.4	>100	>100	>100
F2	3.5	>100	>100	>100
F3	27.0	>100	>100	>100
F4	2.6	>100	>100	>100
F5	3.7	28	>100	>100
F6	3.3	80	>100	>100
F7	1.7	NT*	NT*	NT*
F8	1.4	NT*	NT*	NT*
F9	9.0	>100	>100	>100
F10	1.1	NT*	NT*	NT*
F11	5.3	>100	>100	>100
F12	4.2	>100	>100	>100
F13	9.2	>100	>100	>100
F14	5.2	>100	>100	>100
F15	3.3	>100	>100	>100

* NT. Not tested

Table S3. NMR data of isotrisindoline (2,2-di(3-indolyl)-3-indolone) measured in CD_3CN (^1H: 600 MHz; ^{13}C: 150 MHz) compared with the NMR data reported in literature and the NMR data of trisindoline.

Position C-H	^1H NMR and ^{13}C NMR for isotrisindoline in CD_3CN acquired in this work				^1H and ^{13}C NMR for isotrisindoline in $CDCl_3$ *		^1H and ^{13}C NMR for isotrisindoline in $CDCl_3$ **		^1H and ^{13}C NMR for trisindoline in CD_3OD ***	
	δ_H, Mult	δ_C, HSQC	Mult	HMBC (δ_H to δ_C)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)
5	6.83, t	119.7	CH	C7, C7a	6.89	119.4	6.87	118.7	6.97	124.5
5'	6.88, t	120.1	CH	C4', C3a'	7.19	119.9	6.93	118.7	6.81	120.3
4	7.57, d	125.9	CH	C3a	7.73	125.5	7.69	124.8	7.27	127.3
6'	7.08, t	122.9	CH	C7', C7a'	7.09	122.3	7.11	121.2	7.03	122.9
2'	7.15, d	124.7	CH	3', 3a', 7a'	6.94	124.1	7.13	124.0	6.90	126.4
7'	7.40, d	112.6	CH	C4', C5', C6'	7.62	111.4	7.37	111.2	7.32	113.1
4'	7.29, d	121.6	CH	C5', C3a'	7.36	120.4	7.40	119.9	7.27	123.2
6	7.54, t	138.4	CH	C7	7.51	137.6	7.52	137.5	7.25	129.8
7	6.99, d	113.4	CH	C2, C6	6.91	112.9	6.94	112.3	7.05	111.8
3'	-	115.0	C			115.1		113.7		116.5
3a	-	119.5	C			120.8		119.0		137.5
3a'	-	126.7	C			125.7		125.3		128.1
7a'	-	138.0	C			137.0		136.9		139.6
7a		161.3				160.3		160.5		143.1
2	-	-	C			68.2		68.2		183.4
3	-	-	C=O			201.5		201.8		55.7

* NMR data obtained from Takeshige, Y.; Egami, Y.; Wakimoto, T.; Abe, I. Production of indole antibiotics induced by exogenous gene derived from sponge metagenomes. Mol. BioSyst. 2015, 11, 1290–1294.

** NMR data obtained from Bell, R.; Carmeli, S.; Sar, N. Vibrindole A, a Metabolite of the Marine Bacterium, *Vibrio parahaemolyticus*, Isolated from the Toxic Mucus of the Boxfish *Ostracion cubicus*. J. Nat. Prod. 1994, 57, 1587–1590.

*** NMR data obtained from Yoo, M.; Choi, S.-U.; Choi, K.Y.; Yon, G.H.; Chae, J.-C.; Kim, D.; Zylstra, G.J.; Kim, E. Trisindoline synthesis and anticancer activity. Biochem. Biophys. Res. Commun. 2008, 376, 96–99.

Table S4. MZmine 2.30 processing parameters

Step	Parameter	Value
Baseline corrector	*m/z* bin width	1.0000
	Smoothing	2
	Asymmetry	0.001
Mass detection	Mass detector	Centroid
	Noise level	1.0E4
Chromatogram builder	Min time span (min)	0.07
	Min height	1.0E4
	m/z tolerance	0.005
Chromatogram deconvolution	Algorithm	Baseline cut-off
	Min peak height	1.0E4
	Peak duration range (min)	0.04 - 3.00
	Baseline level	1.1E4
Deisotoping	*m/z* tolerance	0.005
	Retention time tolerance (min)	0.3
	Maximum charge	1
	Representative isotope	Most intense
Normalization	Linear normalizer	Total raw signal peak area
Alignment	algorithm	Join aligner
	m/z tolerance	0.005
	Weight for *m/z*	20
	Retention time tolerance	0.5
	Weight for RT	10

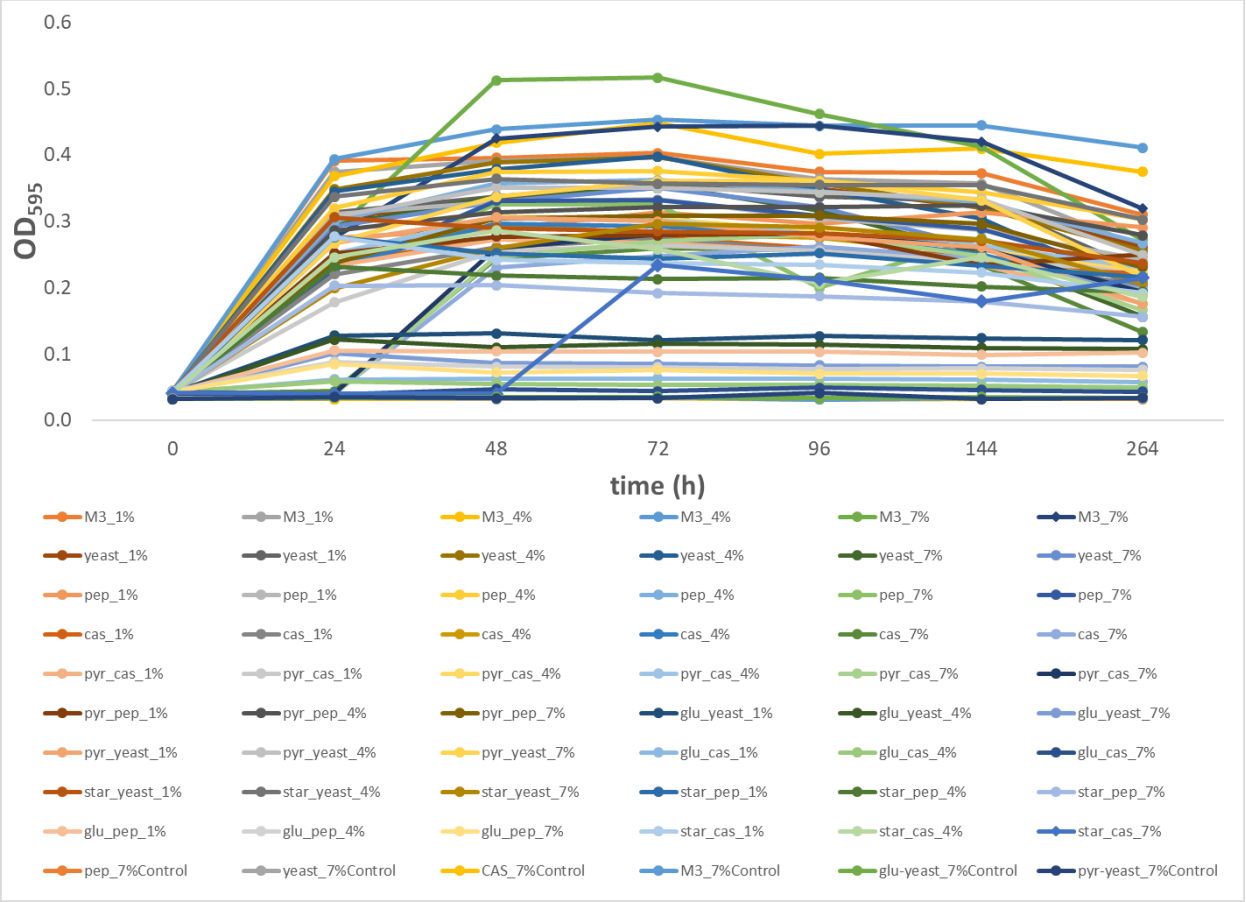


Figure S1. Bacterial growth of *Vibrio diabolicus* A1SM3 in the modified growth media

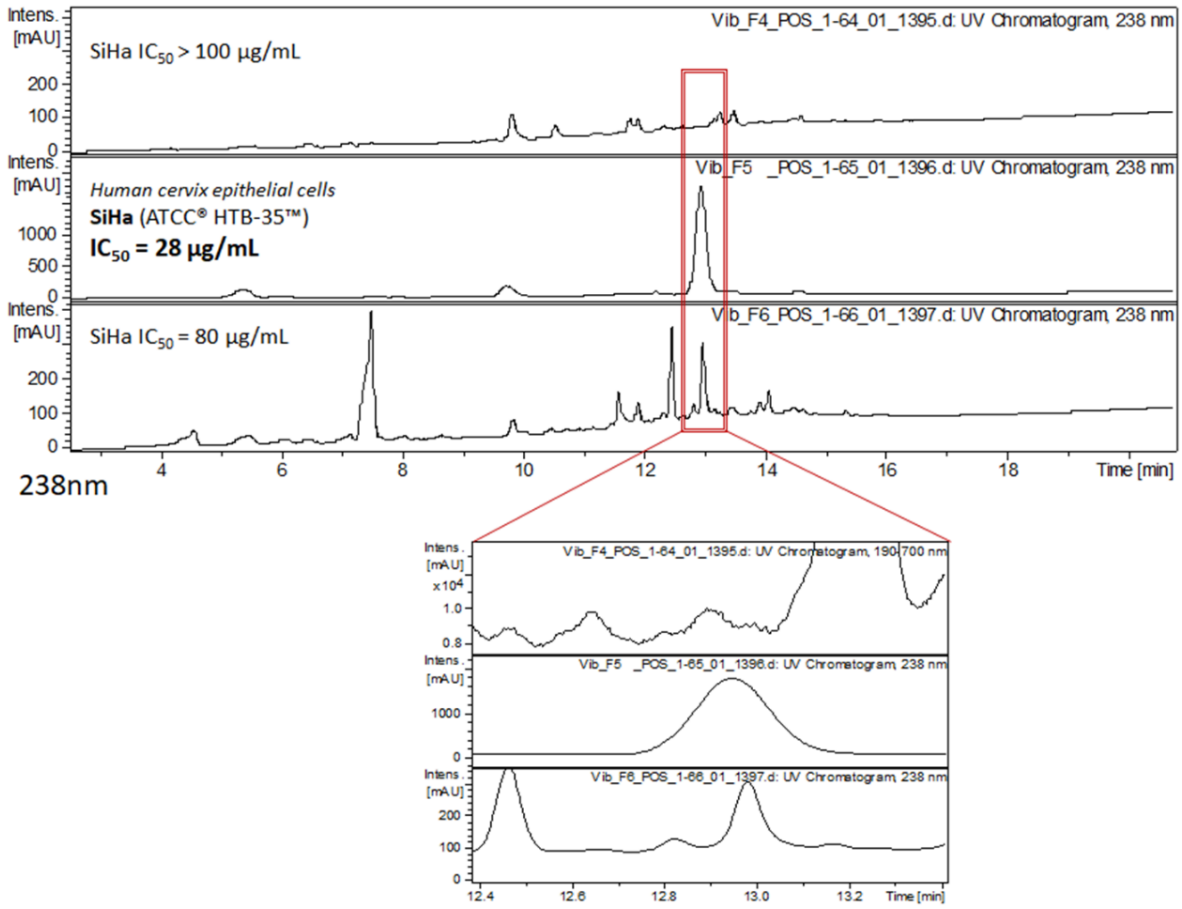


Figure S2. Chromatograms of the cytotoxic fractions of *Vibrio diabolicus* A1SM3 with IC_{50} values.

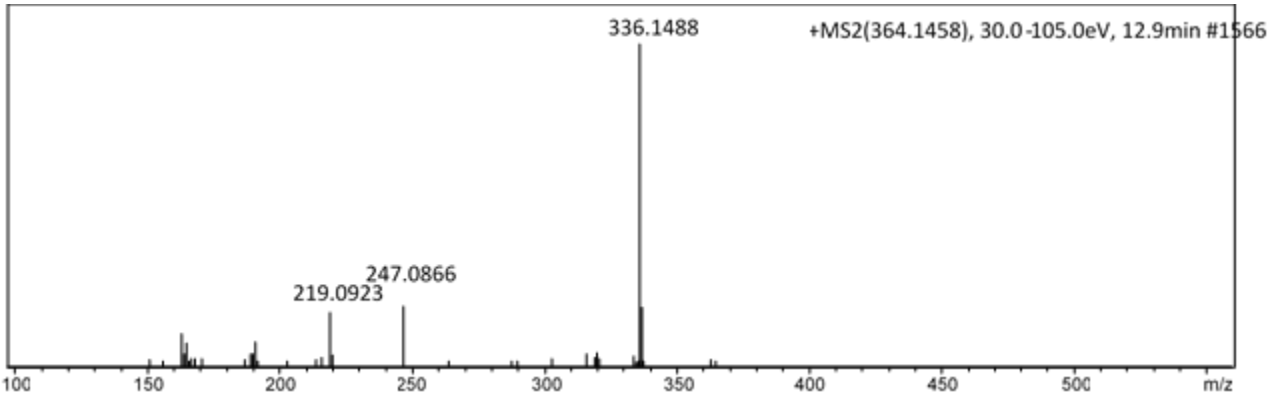
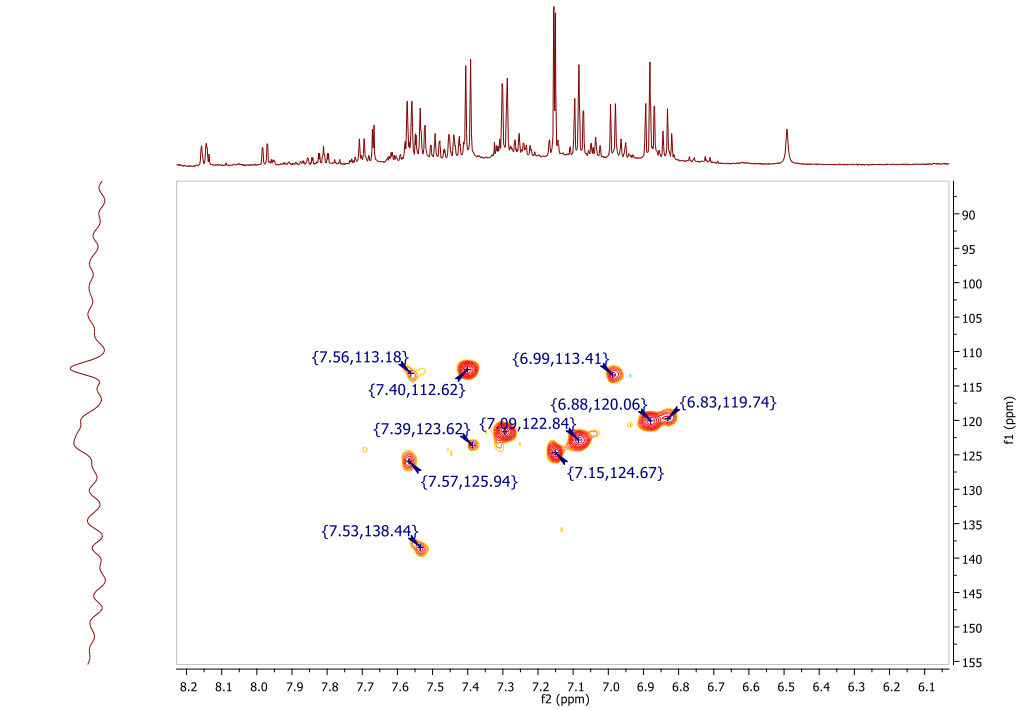


Figure S3. MS/MS spectrum for isotrisindoline in fraction F5.



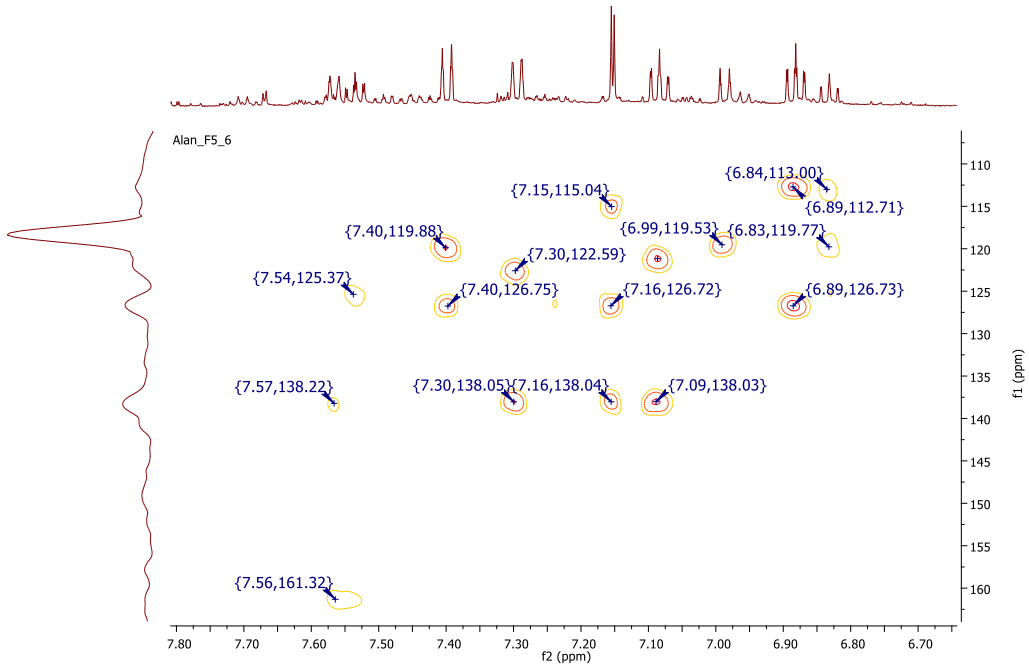


Figure S4 (a) HSQC and **(b)** HMBC of F5 fraction measured in CD_3CN-d^3 (^1H: 600 MHz; ^{13}C: 150 MHz).

7.1. Author Contributions

Conceptualization, N.C.M. and E.T.; Formal analysis, N.C.M. and A.B.; Funding acquisition, E.T.; Investigation, N.C.M., A.B. and A.C.P.; Resources, N.P.L. and E.T.; Writing—original draft, N.C.M. and A.B.; Writing—review & editing, A.C.P., N.P.L. and E.T.

7.2. Funding

This research was funded by the Research Division of Universidad de La Sabana grant ING 139–2013, COLCIENCIAS Scholarship 6172–2014 (National Doctoral Grant for Natalia Conde-Martínez) and the Brazilian foundation FAPESP (2017/17648-4; 2016/13292-8).

7.3. Acknowledgments

The authors thank the Dealership Big Group S.A. (Salinas Manaure) for their help during the sample collection. The collection and study of the strain used in this research were covered under the permission granted by the Ministerio de Ambiente y Desarrollo Sostenible de Colombia to Universidad de La Sabana, through the Contrato Marco de Acceso a Recursos Genéticos y sus Productos Derivados No. 117 (Otrosí No. 2 of 05/04/2017).

7.4. Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

CHAPTER III: Draft genome of the PHB producer *Vibrio diabolicus* A1SM3, a moderately halophilic bacterium

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In preparation

1. ABSTRACT

The genome analysis of the bacterial strain A1SM3, isolated from a sediment sample from a pond with a salinity of 4% in the Manaure Solar Saltern in La Guajira, Colombia, was done by considering the cytotoxic and antibacterial activity, in parallel to the capacity of polyhydroxybutyrates (PHB) production previously reported. This study aimed to analyze the sequence of the whole genome of strain A1SM3 to assign a taxonomic classification and to establish the genetic potential of this strain. The phylogenetic analysis of the strain revealed that is closely related to *Vibrio diabolicus*, a marine bacterium isolated for the first time from a deep-sea hydrothermal vent and widely studied for exopolysaccharides production with biotechnological value. As already known, the genome of *Vibrio* species is divided into two chromosomes (3.4 Mb and 1.8 Mb). The annotation of genes involved in secondary metabolism was performed using antiSMASH (3.0) finding the presence of the known ectoine, arylpolyenes, vibrioferrin, and PHB biosynthetic gene clusters among other putative clusters. The analysis was focused on the last one, due to the relevance as biodegradable biopolymer. As far as we know, this is the first report on the PHB biosynthetic gene cluster annotation in a strain belonging to *Vibrio diabolicus* species.

Keywords: polyhydroxybutyrate, PhaC synthase, *Vibrio diabolicus*, solar saltern, antiSMASH.

2. INTRODUCTION

The study of marine microorganism has been increasing due to their biological potential and their biotechnological applications (Giddings & Newman, 2015). Among the marine environment, the *Vibrionaceae* family is widely distributed (F. Thompson, Austin, & Swings, 2006), and within this family, the most abundant species belong to genus *Vibrio* which has been widely recognized for their pathogenic relevance (F. Thompson et al., 2004). However, during the past decade, the production of bioactive molecules interesting for medical uses and biotechnological applications has been described for different species belonging to this family (Mansson et al., 2011). One of the limitations to develop studies about these species is the ambiguity of taxonomic assignments using the traditional 16S rRNA gene sequence approach, which have led to propose the use of other genes (*ftsZ, gapA, gyrB, mreB, pyrH, recA, rpoA, topA* and *fur*) for Multi-Locus Sequencing Analysis (MLSA) to solve this problem (Machado, Cardoso, Giubergia, Rapacki, & Gram, 2017). However, due to the significant improvements in genome sequencing, different methods of phylogenetic analysis and species circumscription based on whole-genome analysis have been developed such as the calculation of the average nucleotide identity (ANI) and the tetranucleotide signature correlation index (Richter & Rosselló-Mora, 2009). The ANI value is currently the best *in silico* parameter equivalent to the former DNA-DNA hybridization, the gold standard technique for prokaryotic species circumscription at the genomic level (Richter & Rosselló-Mora, 2009). An ANI value of >94% of similarity is considered the threshold for circumscribe a prokaryotic species (Richter & Rosselló-Mora, 2009). Moreover, the tetranucleotide signature correlation index (TETRA) compares the frequency of occurrence of the tetranucleotide sequences within a genome, both parameters must have a correlation and help to clarify if different strains belong to the same species (Richter & Rosselló-Mora, 2009). These parameters can be calculated in the web platform JSpeciesWS against a database with more than 32.000 prokaryotic genomes (Richter, Rosselló-Móra, Oliver Glöckner, & Peplies, 2015).

Within the *Vibrio diabolicus* subgroup, there are a few characterized specimens. The first member described from this group*, V. diabolicus* CNCM 1-1629, was isolated from the deep-sea hydrothermal vent Polychaete annelid *Alvinella pompejana*, and has been widely studied for the production of an exopolysaccharide with biotechnological uses especially for human health (Goudenège et al., 2014; Raguénès, Christen, Guezennec, Pignet, & Barbier, 1997). Recently, a comparative genomic analysis was carried out to understand the ubiquity and diversity within this subgroup (Turner et al., 2018). The genome analysis of the 12 closed strains belonging to this subgroup, mainly conformed by representatives classified as *V. diabolicus*, *V. antiquarius* and *V alginolyticus*, was performed, and led to establish that 2 strains former

identified as *V. antiquarius*, and 4 strains of *V. alginolyticus*, were reclassified as taxonomic synonyms of *V. diabolicus* (Turner et al., 2018).

Previous studies with *V. diabolicus* A1SM3 strain revealed its potential as a producer of cytotoxic compounds against human cervix epithelial cancer cell line, SiHa (ATCC® HTB – 35 ™), antibacterial metabolites against MRSA, and as well, as producer of poly-(3)-hydroxybutyrate (PHB) analogs (Conde-Martínez et al 2019). The latter compounds are the most common polyhydroxyalkanoates (PHA) produced and accumulated by prokaryotes and have been widely studied for its biotechnological potential as an alternative solution to replace plastic due to their biodegradability properties (Ganapathy et al., 2018). It has been widely reported that marine prokaryotes produce and accumulate PHB granules intracellularly as carbon and energy reserves (Ganapathy et al., 2018). Additionally, some species from *Vibrio* spp. have been described as producers of PHB like *Vibrio azureus* BTKB33 (Sasidharan et al., 2015; Sasidharan, Varghese, & Bhat, 2013), *Vibrio harveyi* (Sun et al., 1994), *Vibrio* sp. BM-1 (Wei, Chen, Wu, & Janarthanan, 2011), *Vibrio* sp. KN01 (Numata & Doi, 2012) and other related marine species (Chien et al., 2007).

The biosynthetic pathway for the PHA and the specific one for PHB have been widely described (Madison & Huisman, 1999) and involves three enzymatic reactions catalyzed by the different enzymes. The first reaction is the condensation of two acetyl coenzyme A (acetyl-CoA) molecules to obtain acetoacetyl-CoA by a β-ketoacyl-CoA thiolase. The second reaction consists in the reduction of the acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by the action of an acetoacetyl reductase. The last reaction involves the polymerization of the (R)-3-hydroxybutyryl-CoA by the poly-(R)-hydroxyalkanoic acid synthase. The genes encoding these enzymes, are organized in an operon-like cluster in most of the bacterial genomes and are named as *pha* or *phb* (for polyhydroxubutyrates) as follows: *phaA* or *phbA*, for the ketothiolase; *phaB* or *phbB* for the acetoacetyl-CoA reductase, *phaC* or *phbC* for PHA synthase, and so on, according to the gene occurrence in the clusters for each bacterial genome. Related to this biosynthetic gene clusters (BGC), it has been described that differs among species (Madison & Huisman, 1999) and that the PHA synthase represents the key enzyme in the biosynthesis of the PHA. There have been described four classes of PHA synthases, the class I and II are formed by one type of subunit (PhaC) with molecular masses from 31 kDa to 73 kDa. The difference between them is related to the substrate specificity, class I PHA synthase polymerase the CoA thioesters of short carbon chain length hydroxy alkanoic acids (C3-C5), meanwhile, the class II PHA synthase uses medium carbon chain length hydroxy alkanoic acids (C5-C14) as substrates (Rehm, 2003; Sagong, Son, Choi, Lee, & Kim, 2018). On the other hand, the class III and IV have two different types of subunits, the PhaC subunit, and the PhaE subunit, and uses short carbon chain length hydroxy alkanoic

acids (C3-C5) (Kutralam-Muniasamy, Corona-Hernandez, Narayanasamy, Marsch, & ´ Erez-Guevara, 2017; Rehm, 2003).

Considering the biological and biotechnological potential of this strain, this study aimed to analyze the genome sequence of the strain A1SM3 to perform the phylogenetic analysis with related strains, to establish its functional potential, and to annotate the biosynthetic gene cluster associated with the production of PHB.

3. MATERIALS AND METHODS

3.1. Bacterial culture and DNA extraction

The strain A1SM3 was isolated from a sediment sample from a pond at 4% salinity (measured as % of NaCl) at the Solar Saltern in the Dealership Big Group Salinas S.A. located in Manaure, La Guajira Colombia (latitude 11°46′32.0″ N, longitude 72°27′27.4″ W). The strain was growth in media containing (M3 medium): 40 g/L NaCl, 20 g/L $MgSO_4$ $7H_2O$, 1 g/L KCl, 0.3 g/L KH_2PO_4, 0.5 g/L yeast extract, 0.5 g/L peptone, 0.5 g/L casamino acids, 0.5 g/L glucose, 0.5 g/L starch and 0.3 g/L sodium pyruvate (Antón et al., 2002) and incubated at 30 °C. This strain is deposited in the Collection of Microorganisms of Universidad de La Sabana (USAB- BIO). The growth under different concentration of NaCl was evaluated in ranges of 0.5% to 20%. Medium without the addition of NaCl was evaluated and no growth was observed. TCBS (Thiosulfate Citrate Bile Salts Sucrose) media was used as selective growth media for *Vibrio* (Oxoid Limited, Hampshire, England). API 20E and API 50CH (BioMCrieux SA, Craponne, France) were used to determine the phenotypic characteristics according to the manufacturer's instructions. For these tests, the NaCl concentration of the tested cell suspensions was adjusted at 4% according to the best growth condition for the strain. Catalase and oxidase activity was also measure and the antibiotic susceptibility profile was established using commercial antibiotic discs (Oxoid, Hampshire, England) including vancomycin (5 µg), amoxicillin (10 µg), penicillin G (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), ampicillin (10 µg), tetracycline (30 µg), clindamycin (2 µg), cefoxitin (30 µg), nalidixic acid (30 µg) and rifampicin (5 µg) by disk diffusion tests (CLSI, 2012b). Genomic DNA was extracted according to the modification of the Marmur procedure (Urdiain et al., 2008).

3.2. Whole genome sequencing and assembly

The whole genome sequencing was performed following the shotgun strategy using the Illumina HiSeq2000 platform. Paired-end (PE) reads of 100 bp were generated, with a total count of 9.281.634 pairs.

Quality filtering step removed bases at the ends below Q30 and the minimum length threshold was set to 80 bases; 8.981.932 read pairs were kept after filtering. Singletons were excluded from the dataset. Hiseq PE reads were merged using FLASH (Magoč & Salzberg, 2011) and 82.761 were successfully extended.

The genome assembly was made using SPAdes version 3.12 (Bankevich et al., 2012) with default settings and careful option testing two k-mers parameters: i) 33, 55 and 77 bases; and ii) 33, 55, 77 and 99 bases. Extended reads as well as filtered PE reads were used for the *de novo* assembly. The contig set obtained with the first parameters (k-mers 33, 55 and 77 bases) generated slightly longer contigs and was used for genome scaffolding and annotation. Contigs with less than 7,000 bases were excluded from the dataset and 17 were kept for further. Nonetheless, this assembly strategy failed to assemble the compete 16S rDNA gene. Alternatively, the second assembly with k-mers 33, 55, 77 and 99 bases, generated a contig with the whole 16S rDNA gene which was used for phylogenetic analysis only.

Contigs were compared using BLASTN with the reference genome of *Vibrio diabolicus* FDAARGOS_96 (accession assembly: GCA_002953335.1). The results indicated that chromosome 2 was fully assembled in one single contig, contig1; while most of the rest of the contigs were related to chromosome 1. There was no evidence of contigs that could be related to episomal elements such as plasmids. For chromosome 1 scaffolding analysis, the program ABACAS was used (Assefa, Keane, Otto, Newbold, & Berriman, 2009) with the same genomic reference. All remaining contigs were included within the pseudochromosome model of chromosome 1, 98.4% of the remaining assembled bases, except for contig 14 (37.349 bp). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession PRJNA516395. The version described in this paper is version SDKB01000000.

3.3. Phylogenetic analysis

Small Ribosomal subunit rDNA gene 16S was extracted from the second genome assembly (k.mers 33, 55, 77 and 99) using the rRNA gene finder RNAmmer (Lagesen et al., 2007). Initial classification of the strain A1SM3 was performed with the Ribosomal Database Project RDP Classifier (Cole et al., 2014). For tree reconstruction, the 16S rDNA gene sequences of closest relatives were aligned with MAFFT software version 7.0 and exported to MEGA 7.0 software to calculate the distance matrix and build the tree using the neighbor-joining algorithm (Saitou & Nei, 1987) with 1000 bootstrap replicates. Additionally, the genome similarity was established with the online service of JSpeciesWS (Richter et al., 2015) by the calculation of

the Average Nucleotide Identity (ANI) values and the correlation indexes of their tetranucleotide signature between the closest genomes of *Vibrio* species.

3.4. Genome annotation

The genome annotation was performed using the same reference genome (GCA_002953335.1) with RATT tool (Otto, Dillon, Degrave, & Berriman, 2011). The algorithm was able to automatically transfer 83.5% of the gene models in chromosome 1 and 94% in chromosome 2. Manual curation of relevant gene models was carried out with the genome visualizer ARTEMIS. Global comparisons of the predicted peptide sequences was performed using the GGSEARCH tool (Mackey, Haystead, & Pearson, 2002). The amino acid sequence of each putative peptide was globally aligned with the corresponding ortholog annotating a total of 2680 and 1530 protein-coding genes for chromosome 1 and 2, respectively. A total of 1546 and 465 CDS showed similarity values greater than 80% for each chromosome, respectively. On the other hand, automatic annotation of the assembled genome was made by web-based tool PATRIC with the RAST toolkit (RASTtk) (Wattam et al., 2017) which uses the subsystems categories to establish functional assignments by chromosome.

Additionally, the search for secondary metabolite biosynthetic gene clusters (BGC) was carried out using the online platform antiSMASH 3.0 (Weber et al., 2015). Domain search for the PHB gene cluster annotated by antiSMASH was analyzed by BLAST comparison in Uniprot (https://www.uniprot.org/) and the domain annotation was performed in InterPro (https://www.ebi.ac.uk/interpro). To corroborate the identification of the PHB biosynthetic gene cluster, the CDS of the annotated genome with RATT tool were analyzed in the KEGG Automatic Annotation Server (KAAS) to provide functional annotation with the manually curated KEGG genes database (Moriya, Itoh, Okuda, Yoshizawa, & Kanehisa, 2007). The result of this annotation was the KEGG orthology (KO) assignment for the CDS that were used to reconstruct the biosynthetic pathway by KEGG mapper (https://www.kegg.jp/kegg/mapper.html).

On the other hand, multiple sequence alignment of PhaC synthase amino acid sequences was performed with Clustal Omega 1.2.4 (www.uniprot.org/align/). The phylogenetic tree was reconstructed using the Maximum Likelihood (ML) method with MEGA 7.0 software (Kumar, Stecher, & Tamura, 2016) based on the JTT matrix-based model (Jones, Taylor, & Thornton, 1992). The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1.000 replicates. The amino acid sequences aligned were selected considering the extensive revision made by Rehm (2003) and Kutralam-Muniasamy et al. (2017) on the PHA synthases that have been cloned and assigned. Additionally, the amino acid

sequences of the PhaC synthase of 4 strains of *Vibrio diabolicus* (including A1SM3) were extracted using the annotation of the BGC in the whole genome made by antiSMASH.

4. RESULTS

4.1. Genome assembly and phylogenetic analysis

The whole genome sequencing experiment generated a total of 9.281.634 read pairs, after quality filtering, 8.981.932 pairs remained and were assembled using SPAdes package. The draft genome assembly of strain A1SM3 was comprised of 17 contigs (> 7.000 bases) with a total sequence length of 4.980.821 bp and 45 % GC content. The N50 value of the assembly was 801.201 bp and the length of the largest contig was 1.822.771 bp. The sequencing depth of the filtered assembled set ranged between 78X and 85X. Considering that *Vibrio* species have 2 chromosomes, it was possible to establish that the largest assembled sequence (contig 1) corresponds to the sequence of chromosome 2 according to the alignment of contigs against the reference genome of *V. diabolicus* (accession number: GCA_002953335.1).

Keeping in mind the syntheny for chromosome 1 in *Vibrio* species, the contig reordering and mapping for chromosome 1 was made with the algorithm ABACAS using the remaining 16 contigs. This assembly contained more than 97% of the expected coverage compared to the reference. The ABACAS tool generated a sequence of 3.401.980 bp (including 15 GAPs) which includes 15 of the 16 filtered contigs. Only contig 14 (37.439 bp length) could not been assigned into the assembly.

The phylogenetic analysis of the 16S rDNA sequence classified the strain A1SM3 into the genus *Vibrio,* within the *V. harveyi* clade where *V. diabolicus* and *V. alginolyticus* were the closest relatives (Figure 1). The pairwise comparison of the whole genome against the *Vibrio* spp. relatives was performed using JSpeciesWS (Richter et al., 2015). First, the phylogenetic neighbors were identified by searching the nearest tetranucleotides correlation (TCS tool), and a total of 13 genomes were selected. The calculated tetranucleotide correlation index (Table 1, values in brackets) suggested that this strain belong to the *V. diabolicus* species (values greater than 0.999). Afterward, the ANI values were calculated (Table 1 and Table S1) and the results showed that genomes from the different *V. diabolicus* strains, and the strain A1SM3, have an ANI value greater than 94%, which is the threshold for species assignment (Rosselló-Móra & Amann, 2015). It is noteworthy that some strains namely *V. alginolyticus* V2, *V. antiquarius* EX25, and *V. alginolyticus* E0666 had ANI values greater than 94%, and for this reason were recently reclassified within the *V.*

diabolicus group in an extensive comparative genomic analysis (Turner et al., 2018). All the results above confirm that the strain A1SM3 belongs to *V. diabolicus* and the strain was named as *Vibrio diabolicus* A1SM3.

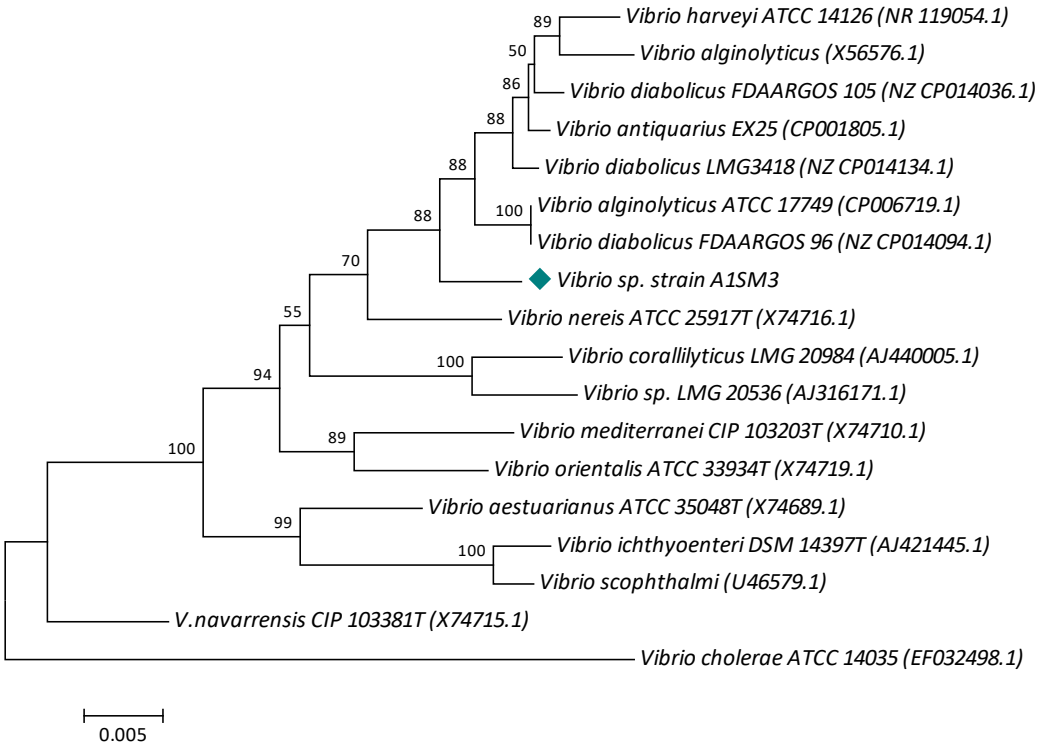

Figure 1. Phylogenetic tree reconstruction based on 16S rRNA gene sequences from strain A1SM3 and its closest phylogenetic neighbors using the neighbor-joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969). The number at nodes denote the bootstrap based on 1000 replicates. Bar, 0.005 substitutions per site.

Table 1. The pairwise comparison of average nucleotide identity (ANI) and correlation indexes of their tetranucleotides signatures (in bracket) between *V. diabolicus* A1SM3 and the genomes of the closest relatives obtained from JSpeciesWS (Richter et al., 2015).

	Strain	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1)	*V. diabolicus* A1SM3	*	97.90 (0.999)	97.69 (0.999)	98.01 (0.999)	97.58 (0.999)	97.79 (0.999)	97.99 (0.999)	97.91 (0.999)	91.61 (0.998)	91.50 (0.998)	91.57 (0.998)	91.59 (0.998)	91.64 (0.998)	91.62 (0.997)
(2)	*V. diabolicus* CNCM I-1629 (GCA_001048675.1)	97.89 (0.999)	*	97.67 (0.999)	97.88 (0.999)	97.62 (0.999)	97.69 (0.999)	97.92 (0.999)	97.82 (0.999)	91.49 (0.998)	91.38 (0.998)	91.50 (0.998)	91.63 (0.998)	91.63 (0.998)	91.47 (0.997)
(3)	*V. alginolyticus* V2* (GCA_001010935)	97.68 (0.999)	97.68 (0.999)	*	97.74 (0.999)	97.44 (0.999)	97.50 (0.999)	97.67 (0.999)	97.75 (0.999)	91.71 (0.998)	91.62 (0.998)	91.63 (0.998)	91.74 (0.998)	91.71 (0.998)	91.71 (0.998)
(4)	*V. antiquarius* EX25* (GCA_000024825)	97.99 (0.999)	97.93 (0.999)	97.74 (0.999)	*	97.65 (0.999)	97.81 (0.999)	97.98 (0.999)	97.97 (0.999)	91.54 (0.998)	91.45 (0.998)	91.53 (0.998)	91.54 (0.998)	91.62 (0.998)	91.52 (0.998)
(5)	*V. alginolyticus* E0666* (GCA_000341445.1)	97.63 (0.999)	97.67 (0.999)	97.50 (0.999)	97.68 (0.999)	*	97.64 (0.999)	97.69 (0.999)	97.61 (0.999)	91.83 (0.998)	91.68 (0.998)	91.78 (0.998)	91.83 (0.998)	91.81 (0.998)	91.82 (0.997)
(6)	*V. diabolicus* FDAARGOS_96 (GCA_002953335.1)	97.7 (0.999)	97.63 (0.999)	97.45 (0.999)	97.72 (0.999)	97.50 (0.999)	*	97.67 (0.999)	97.62 (0.999)	91.43 (0.997)	91.37 (0.998)	91.52 (0.998)	91.55 (0.998)	91.60 (0.998)	91.43 (0.997)
(7)	*V. diabolicus* LMG 3418 (GCA_002953355.1)	97.95 (0.999)	97.94 (0.999)	97.66 (0.999)	97.92 (0.999)	97.60 (0.999)	97.77 (0.999)	*	97.83 (0.999)	91.67 (0.998)	91.52 (0.998)	91.56 (0.998)	91.61 (0.998)	91.65 (0.998)	91.65 (0.998)
(8)	*V. diabolicus* FDAARGOS_105 (GCA_002953395.1)	97.85 (0.999)	97.79 (0.999)	97.74 (0.999)	97.91 (0.999)	97.56 (0.999)	97.64 (0.999)	97.85 (0.999)	*	91.49 (0.998)	91.46 (0.998)	91.41 (0.998)	91.54 (0.998)	91.53 (0.998)	91.49 (0.998)
(9)	*V. alginolyticus* ATCC 17749 (GCA_000354175.2)	91.59 (0.998)	91.52 (0.997)	91.71 (0.998)	91.41 (0.998)	91.80 (0.998)	91.49 (0.997)	91.57 (0.998)	91.48 (0.998)	*	98.49 (0.999)	98.36 (0.999)	98.55 (0.999)	98.50 (0.999)	99.99 (1.000)
(10)	*V. alginolyticus* 12G01 (GCA_000153505.1)	91.53 (0.998)	91.46 (0.998)	91.69 (0.998)	91.43 (0.998)	91.74 (0.998)	91.42 (0.998)	91.51 (0.998)	91.49 (0.998)	98.54 (0.999)	*	98.35 (0.999)	98.49 (0.999)	98.49 (0.999)	98.52 (0.999)
(11)	*Vibrio* sp. OY15 (GCA_000742455.1)	91.38 (0.998)	91.41 (0.998)	91.54 (0.998)	91.37 (0.998)	91.62 (0.998)	91.48 (0.998)	91.37 (0.998)	91.38 (0.998)	98.26 (0.999)	98.23 (0.999)	*	98.30 (0.999)	98.31 (1.000)	98.24 (0.999)
(12)	*V. alginolyticus* RM-10-2 (GCA_001267615)	91.54 (0.998)	91.66 (0.998)	91.73 (0.998)	91.44 (0.998)	91.76 (0.998)	91.62 (0.998)	91.58 (0.998)	91.55 (0.998)	98.42 (0.999)	98.36 (0.999)	98.28 (0.999)	*	98.36 (1.000)	98.42 (0.999)
(13)	*V. alginolyticus* BSW8 (GCA_000834155)	91.59 (0.998)	91.67 (0.998)	91.72 (0.998)	91.50 (0.998)	91.78 (0.998)	91.62 (0.998)	91.57 (0.998)	91.56 (0.998)	98.45 (0.999)	98.38 (0.999)	98.36 (1.000)	98.40 (1.000)	*	98.43 (0.999)
(14)	*V. alginolyticus* ATCC 17749 (GCA_000354175)	91.63 (0.997)	91.52 (0.997)	91.79 (0.998)	91.48 (0.998)	91.85 (0.998)	91.53 (0.997)	91.63 (0.998)	91.56 (0.998)	99.96 (1.000)	98.48 (0.999)	98.35 (0.999)	98.5 (0.999)	98.49 (0.999)	*

* These strains have been reclassified as taxonomic synonyms of *Vibrio diabolicus* (Turner et al., 2018)

4.2. Phenotypic characterization

Vibrio diabolicus A1SM3 showed Gram-negative stain results and grows on M3 agar as non-pigmented, circular, smooth and convex colonies and grows on thiosulphate citrate bile salt sucrose (TCBS) as yellow colonies typically described for *Vibrio* spp. that ferment sucrose. Additionally, growth was observed between 0.5 and 9.0% NaCl concentration with best growth at 4.0% NaCl. At this concentration, the strain showed a swarming motility pattern on the M3 agar surface, after 48h of incubation. The API 20E test showed positive results for the utilization of L-lysine, L-ornithine, gelatin, glucose, mannitol, saccharose and amygdalin, the production of indole and for Voges Proskauer reaction. The results of carbon utilization are shown in Table 2 and compared against *V. diabolicus* CNCM 1-1629 and other related *Vibrio* species. These results established the similarity between *V. diabolicus* A1SM3 and *V. diabolicus* CNCM 1-1629, unlike other *Vibrio* species. The only difference between both strains was the ability of *V. diabolicus* A1SM3 to metabolize salicin and cellobiose.

Table 2. Phenotypic characteristics of *V. diabolicus* A1SM3 compared with different *Vibrio* species determined by API 50CH.

	Carbohydrate	*Strain A1SM3*	*V. diabolicus* CNCM 1-1629*	*V. nereis**	*V. mytili**	*V. parahaemolyticus* ATCC 17802 **
1	Glycerol	+	+	-	+	-
2	L-Arabinose	-	-	-	+	w
3	Ribose	+	+	+	+	-
4	D-Xylose	-	-	-	+	-
5	Galactose	+	+	-	+	+
6	Glucose	+	+	+	+	+
7	Fructose	+	+	+	+	+
8	Mannose	+	+	+	+	+
9	Mannitol	+	+	-	+	+
10	N-acetyl-glucosamine	+	+	+	+	+
11	Amygdalin	+	+	-	+	-
12	Arbutin	-	-	-	+	-
13	Esculin	-	-	-	+	ND
14	Salicin	+	-	-	+	-
15	Cellobiose	+	-	-	+	-
16	Maltose	+	+	+	+	+
17	Sucrose	+	+	+	+	-
18	Trehalose	+	+	+	+	+
19	Melezitose	-	-	-	+	-
20	Starch	+	+	+	+	+
21	Glycogen	+	+	-	+	+
22	Gentiobiose	-	-	-	+	-
23	D-Turanose	-	-	+	+	-
24	Gluconate	+	+	+	+	+

* Data obtained from Raguénès et al (1997) ** Data obtained from Yang, Liu, Luo, & Pan (2015). ND: data no available. w: weak.

4.3. Genome annotation and biosynthetic gene cluster analysis

The genome annotation of *V. diabolicus* A1SM3 using the RAST toolkit (RASTtk) from PATRIC detected 2927 and 1637 CDS in chromosome 1 and 2, respectively. Within the annotated CDS, 2860 and 1594 were genus-specific, from which 2411 and 1286 encoded proteins with functional assignments (Table S2). One of the advantages of use RASTtk on PATRIC is that the set of biologically related functional roles are co-curated and classify into biological subsystems associated with a single biological process (Brettin et al., 2015). Within the proteins with functional annotation, 47% were assigned into superclass distribution by subsystems (Figure 2), and the other 53% were not assigned.

The functional analysis for each chromosome (Figure 2) showed that the genes encoding for protein processing function, DNA and RNA processing are mainly present in chromosome 1. In the other way, the genes associated with proteins involved in energy process and stress response, defense and virulence are mainly found in chromosome 2 (Figure 2).

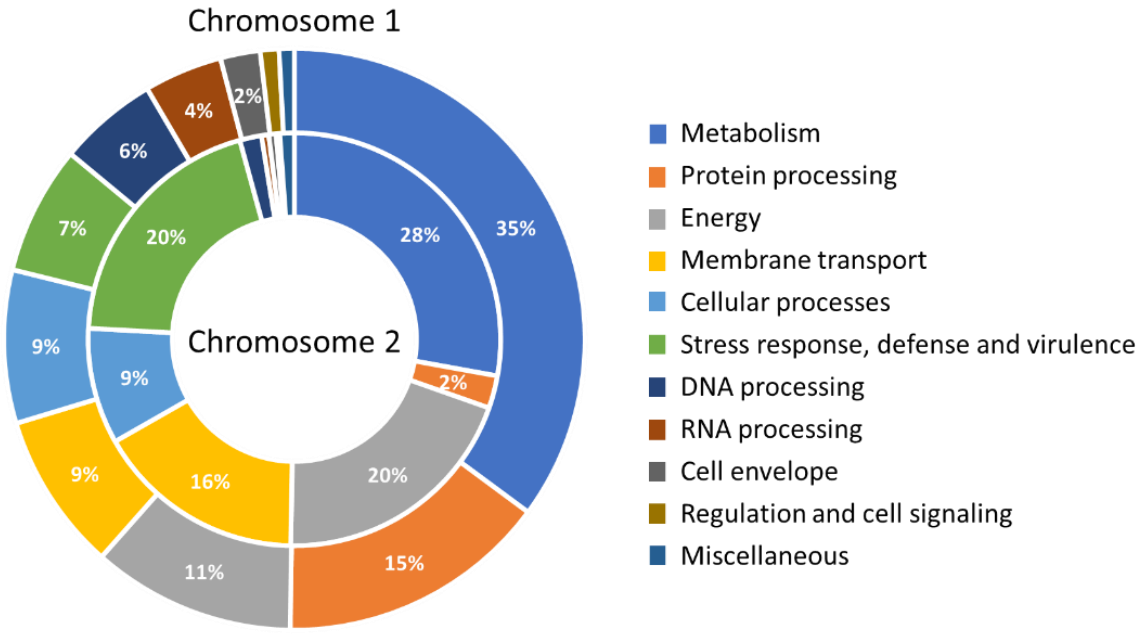

Figure 2. Functional assignments for the annotation of *V. diabolicus* A1SM3 using the subsystem distribution by chromosome.

The draft genome was analyzed using antiSMASH 3.0 to establish whether *V. diabolicus* A1SM3 could be a potential source of compounds with bioprospecting interest. The results showed a total of 43 predicted biosynthetic gene clusters (BGC), 12 of them were found in chromosome 2 (Table S2). From this predicted BGC, only 7 showed similarity with already characterized BGC (Table 3) in the MIBiG data repository (Medema et al., 2015). The last result showed that *V. diabolicus* A1SM3 have the potential to produce ectoine, a compound produced by halophilic bacteria to maintain turgor pressure under salt-stress condition (Müller & Köcher, 2011) that have been widely investigated for their biotechnological applications (J. Yin, Chen, Wu, & Chen, 2014), and only reported for *V. antiquarius* EX25 (Naughton, Blumerman, Carlberg, & Boyd, 2009) to best of our knowledge. This cluster showed high similarity with the already known BGC from the moderately halophilic bacteria *Methylomicrobium alcaliphilum* (BGC0000854) and *Methylophaga thalassica* (BGC0000856) widely studied as a moderate halophilic methylotrophic bacteria (Reshetnikov et al., 2011). Additionally, the strain display a genetic potential to produce aryl polyenes, bacterial pigments studied for their structural similarity with the carotenoids and its protective function

against light-induced damage (Schöner et al., 2016). This cluster showed a similarity of 90% with the biosynthetic gene cluster APE Vf (BGC0000837) from *Vibrio fischeri*. As well, the putative BGC of vibrioferrin showed a similarity of 100% with the genes of the cluster from *V. alginolyticus* (BGC0000947) responsible for the biosynthesis and transportation of vibrioferrin, a marine siderophore (Amin, Green, Küpper, & Carrano, 2009). Is important to highlight that *V. diabolicus* A1SM3 has not been studied yet to produce these metabolites. Finally, the polyhydroxyalkanoate (PHA) gene cluster showed 50% similarity with the PHA BGC from *Burkholderia* sp. DSM 9242 (Figure 3a) (de Andrade Rodrigues et al., 2000).

Table 3. Biosynthetic genes cluster annotated by antiSMASH and ClusterFinder algorithm in the draft genome from *Vibrio diabolicus* A1SM3.

Gene cluster Type	Chromosome	Number of clusters	Algorithm	Most similar known BGC (% genes showing similarity)
Arylpolyene	1	1	antiSMASH	APE_Vf (90%)
Bacteriocin	1	1	antiSMASH	-
Fatty acid	1	4	ClusterFinder	-
Ectoine	1	1	antiSMASH	Ectoine (100%)
Putative	1	23	ClusterFinder	-
Saccharide	1	3	ClusterFinder	O&K-antigen (29%)
Saccharide-Siderophore	2	1	ClusterFinder	Vibrioferrin (100%)
PHB	2	1	ClusterFinder	Polyhydroxyalkanoate (50%)
Putative	2	8	ClusterFinder	-
Total general		43		

Considering a previous report of the production of polyhydroxybutyrates (PHB) by *V. diabolicus* A1SM3 (Conde-Martínez et al., 2019), we evaluate the divergence of this genes and the BGC annotation was confirmed by BLAST search using the amino acid sequence of the 4 genes identified within the cluster against UniProt. Additionally, InterPro database was used to annotate the protein domains for each CDS (Figure 3a). The BLAST results reveal that the CDS Ctg1_336 encodes a protein with a length of 246 amino acids that belongs to the acetoacetyl-CoA reductase family (InterPro accession number IPR011283) and showed 99.6% identity with the SDR family NAD(P)-dependent oxidoreductase from *V. diabolicus* (UniProt entry: A0A2L2KC36). This protein family is involved in the polyhydroxybutyrate biosynthetic process (GO: 0042619) and in the oxidation-reduction process (GO: 0055114). The CDS Ctg1_337 encoding a protein with 403 amino acids length that belongs to the thiolase family (InterPro accession number IPR002155) with 99.8% identity with the subfamily of the acetyl-CoA acetyltransferases from *V. antiquarius* EX25 (Unipro entry A7JYQ1) (Figure 3a). The CDS Ctg1_338 (Figure 3a) encodes a protein of 115 amino acids length member of the phasin protein family, subfamily 3 (InterPro accession number IPR014176) with 100%

identity with different strains from *Vibrio parahaemolyticus* (UniProt entries: A0A0F6LLC5, Q87GW2, S5IZ14, A6B0K8, among others). These proteins are surface proteins that cover the PHA storage granules in bacteria and determines their size (Madison & Huisman, 1999). Finally, the CDS Ctg1_339 encodes for a protein of 591 amino acids length that belongs to the poly-(R)-hydroxyalkanoic acid synthase, class I according to InterPro (accession number IPR010963), which showed 100% identity with poly(3-hydroxyalkanoate) synthase from *Vibrio parahaemolyticus* (UniProt entry: A0A0M9C8D6), the most relevant enzyme in the PHA biosynthetic process (GO: 0042619) (Figure 3a).

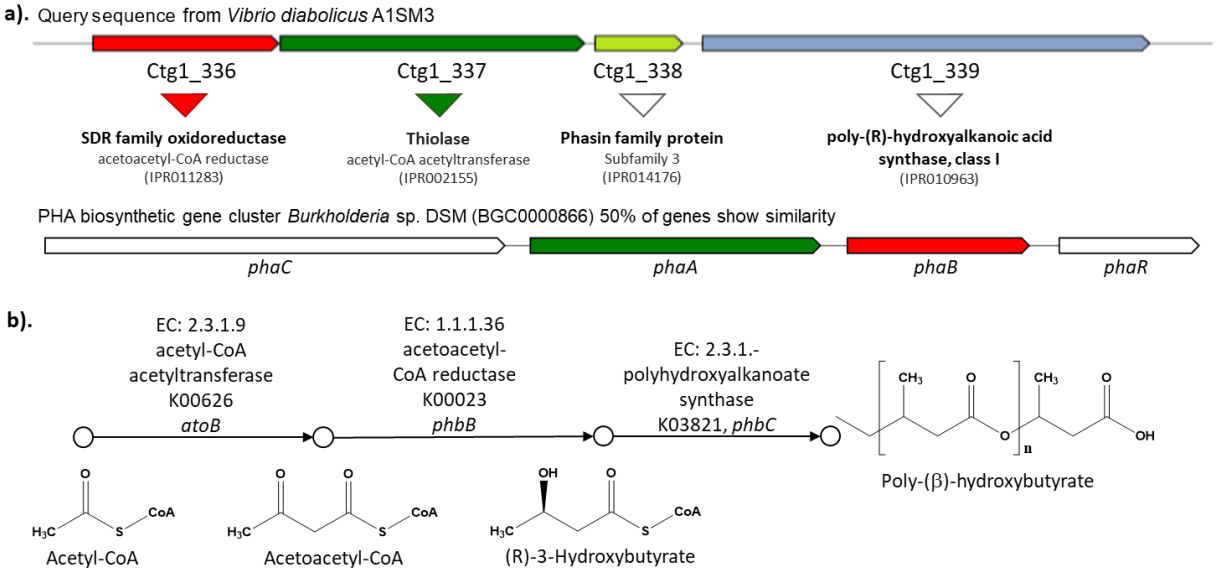

Figure 3 a). PHB biosynthetic gene cluster from *V. diabolicus* A1SM3 with domain annotation (according to Interpro) compared with the BGC of PHB founded in *Burkholderia* sp. DSM 9242. Genes with the same color were the genes that showed similarities between the two species. **b).** The biosynthetic pathway for PHB production annotated by KAAS and reconstructed by KEGG Mapper. (https://www.ebi.ac.uk/interpro/).

On the other hand, the automatic genome annotation with KAAS showed consistent results with the annotation by antiSMASH and protein domain annotation. With this tool, the biosynthetic pathway of PHB beginning with acetyl-CoA was annotated and the enzymes involved in the process were assigned according to the EC and K number (Figure 3b). Three enzymes were annotated corresponding to the CDS Ctg1_337, Ctg1_336, and Ctg1_339, respectively. The phasin protein (Ctg1_338) was not annotated with this tool because is not necessary for the biosynthesis of the PHB although plays an important role in the accumulation and granule process (Figure 3b).

According to the classification of the PHA synthases, class I corresponds to an enzyme consisting of only one type of subunit (PhaC) with molecular masses between 61-73 kDa. This agrees with the molecular mass calculated for the PhaC synthase from *V. diabolicus* A1SM3 (67.2 kDa). Besides, the substrate

preference for short chain carbon fatty acids (C3-C5) as the 3-hydroxy butanoic acid accords with the chemical structure of the butyrate fragment in PHB polymer produced by this strain (molecular formula: $C_4H_6O_2$) and identified by MS/MS (Conde-Martínez et al., 2019).

The phylogenetic reconstruction based on the multiple sequence alignment of the amino acid sequence of PhaC synthase confirmed its classification within the class I (Figure 4). Overall, 19 amino acid residues are identical in all PHA synthases aligned, including the catalytic triad residues (Cys-319, Asp-480, and His-508) previously described and reported for the class I PhaC synthase model of *Ralstonia eutropha* (Rehm, 2003).

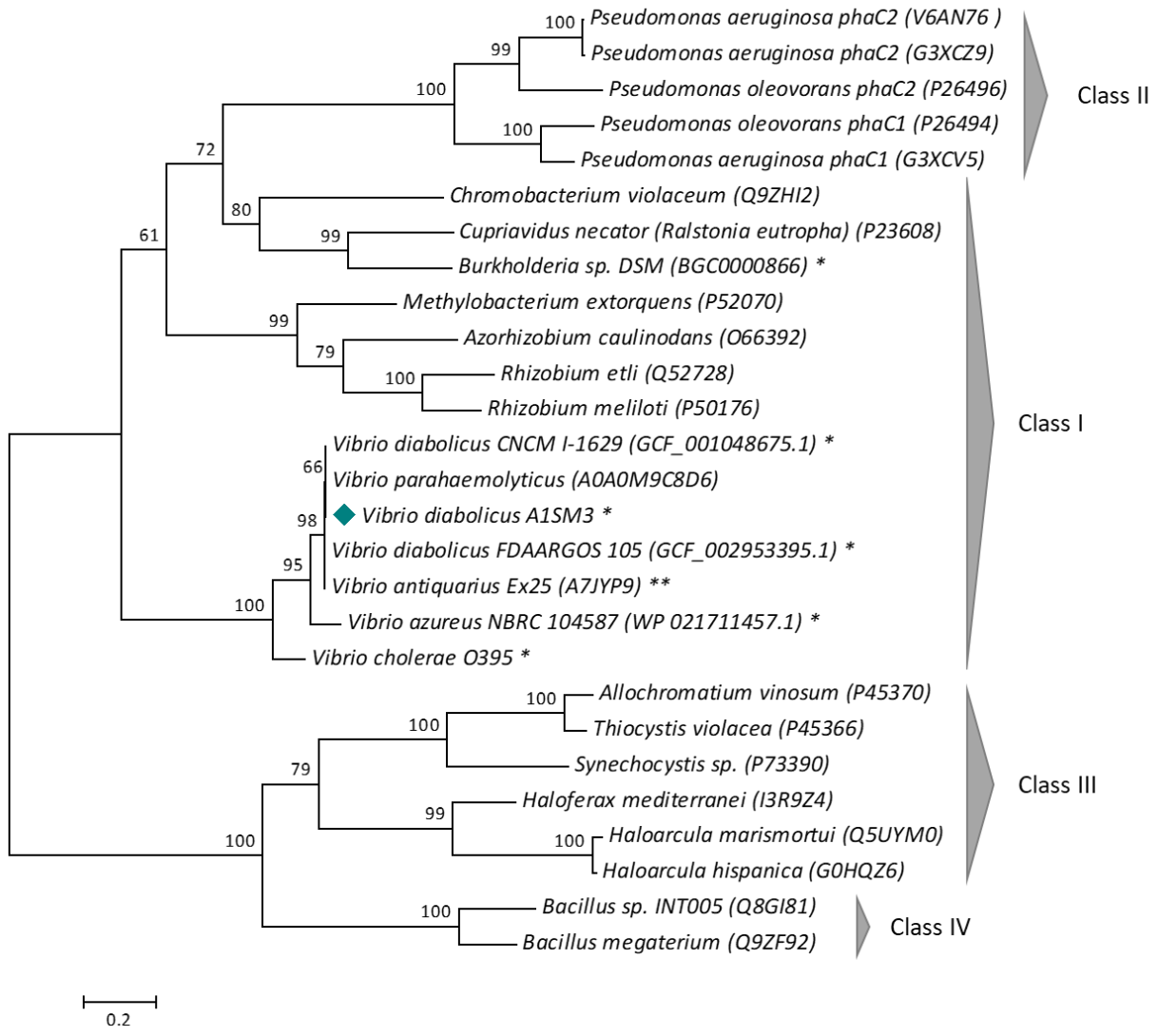

Figure 4. Molecular phylogenetic analysis of the amino acid sequence of the PhaC synthase. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The analysis involved 27 amino acid sequences. *These sequences were obtained in this study by the analysis of the whole genome

sequence in antiSMASH. **This strain was reclassified as a taxonomic synonym of *V. diabolicus* subgroup (Turner et al., 2018).

The main characteristic described for this BGC is the high divergence present among several bacterial genera, evidenced in a different gene organization and occurrence (Kutralam-Muniasamy et al., 2017). However, the amino acid sequence of the enzymes had shown a high degree of conservation (C. S. K. Reddy, Ghai, Rashmi, & Kalia, 2003). As an example, even though the PhaC synthase from *Burkholderia* sp. DMS 9242 and *V. diabolicus* A1SM3 belongs to class I and showed 42% of identity, the ordering of the genes and the genes conforming the operon-like cluster differ between them, for *Burkholderia* sp. DMS 9242 the BGC is formed and organized as follow; *phaC, phaA, phaB*, and the *phaR* gene; the former, a transcriptional regulator gene that regulates the expression of the synthase (Figure 3a). Meanwhile, the gene occurrence in the BGC described here for *V. diabolicus* A1SM3 is *phaB, phaA, phaP* (phasin protein) and *phaC*. However, the ordering of this BGC among species of *V. diabolicus* (analyzed in this work with antiSMASH), showed the same ordering of the BGC than *V. diabolicus* A1SM3 (Figure 5). Additionally, the similarity between the amino acid sequence of the PhaC synthase of *V. diabolicus* A1SM3 and the amino acid sequences from other *Vibrio* species (Figure 5, values in brackets) were 81% identity with *V. cholerae* O395 sequence and 89% identity with *V. azureus* and 99 - 100% identity with the other *V. diabolicus* strains.

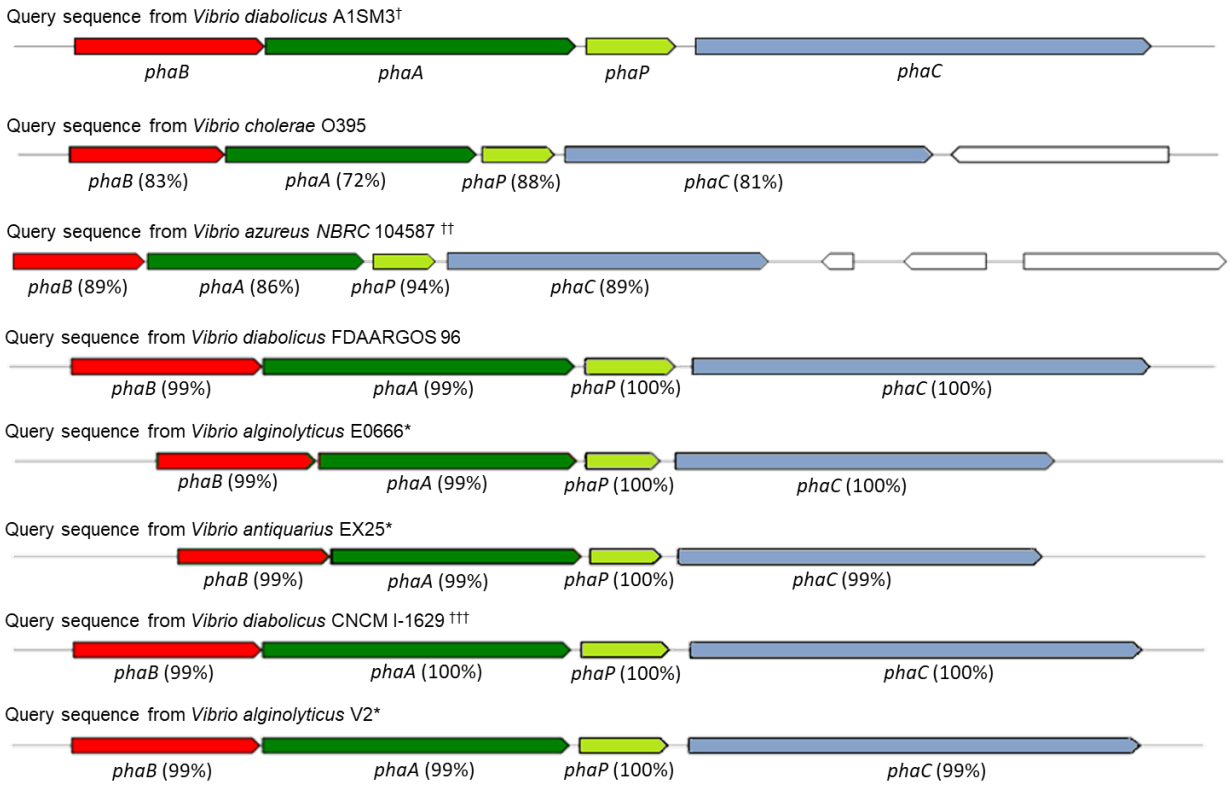

Figure 5. PHA biosynthetic gene cluster annotated in the whole genome sequences of *Vibrio* species by antiSMASH. Values in bracket are the percentage of identity compared with the amino acid sequence from *Vibrio diabolicus* A1SM3. †PHB production reported by Conde-Martínez et al., (2019). ††PHB production reported by Sasidharan et al., (2015). †††No PHB accumulation reported by Raguénès et al., (1997).

5. DISCUSSION

The advances in sequencing techniques led to the taxonomic classification of strain A1SM3 within *Vibrio diabolicus* species using comparative genomics. Additionally, the comparison between the phenotypic characterization with the type strain CNCM-1-1629 (Raguénès et al., 1997) were consistent with the reported in this work.

Regarding the phylogenetic tree of the PhaC synthases for *Vibrio* species, the cluster together showed a correlation among some species within this genus. However, is important to notice that only a few reports regarding the experimental study of this enzyme in *Vibrio* species had been reported, so further studies are necessary to characterize the biochemistry of this enzyme. Additionally, this study led to stablish that the amino acid sequence of the PhaC synthase among the *V. diabolicus* strains showed a high degree of conservation and belongs to class I. Furthermore, the same degree of conservation was observed for *phaA*,

phaB and *phaP* genes. On the other hand, for the PHB producer *V. azureus* BTKB33 (Sasidharan et al., 2015), the BGC annotation for PHB production, led to classify the PhaC synthase as class I which agrees with the previous classification reported by Sasidharan, Bhat, & Chandrasekaran (2016), showing additionally, to be the nearest reported PhaC synthase to *V. diabolicus* A1SM3.

It is noteworthy that the strain *V. diabolicus* CNCM 1-1629 was previously studied for the accumulation of PHB with negative results (Raguénès et al., 1997), contrary to the results obtained for *V. diabolicus* A1SM3 and in spite of the presence of the PHB biosynthetic gene cluster annotated for both genomes in this study. This difference could be explained by the types of methodologies used to determine the production of PHB. For strain A1SM3, a chemical identification of the PHB family was made by a different approach (HPLC-MS/MS) than the reported by Raguénès et al (1997), where the strain CNCM 1-1629 was tested by the PHB method from Gauthier and Breittmayer, using *V. nereis* as the positive control (Gauthier & Breittmayer, 1992). Though, another explanation could be related to the composition of the growth media; the strain CNCM 1-1629 was grown in marine 2216 medium (Difco) supplemented with 3% glucose for the PHB accumulation assays. However, previous results with *V. diabolicus* A1SM3 cultures (Conde-Martínez et al., 2019) showed the non-production of PHB in growth media supplemented with glucose. Additionally, this result agrees with the reported by Chien et al., (2007) for a marine *Vibrio* spp. that showed the lower growth and PHB production in the culture media containing glucose as carbon source compared with the media supplemented with glycerol, sucrose, sodium acetate and sodium succinate.

In conclusion, it is noteworthy that all the strains of *V. diabolicus* analyzed in this work has a genetic potential to produce PHB, however, as far as we know, none of them had been reported to produce them. This approach showed that the classical bioprospection potential evaluation merged with the genome analysis of the strains within *V. diabolicus* group, lead to extrapolate the new biosynthetic potential of the analyzed strains as PHB producers.

6. REFERENCES

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7. SUPPLEMENTARY INFORMATION

Table S1. The pairwise comparison of average nucleotide identity (ANI) in nucleotides between *V. diabolicus* A1SM3 and the genomes of the closest relatives obtained from JSpeciesWS (Richter et al., 2015).

Strain	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
(1) *V. diabolicus* A1SM3	*	4677939 / 5002894	4659492 / 5002894	4736445 / 5002894	4537585 / 5002894	4578872 / 5002894	4682502 / 5002894	4723467 / 5002894	4396741 / 5002894	4281766 / 5002894	4333700 / 5002894	4409159 / 5002894	4391312 / 5002894	4380114 / 5002894
(2) *V. diabolicus* CNCM I-1629 (GCF_001048675.1)	4674438 / 5132517	*	4648070 / 5132517	4665178 / 5132517	4546071 / 5132517	4678182 / 5132517	4713582 / 5132517	4693737 / 5132517	4370807 / 5132517	4275361 / 5132517	4416625 / 5132517	4464866 / 5132517	4474156 / 5132517	4358949 / 5132517
(3) *V. alginolyticus* V2* (GCA_001010935)	4659061 / 5068299	4653606 / 5068299	*	4674850 / 5068299	4515144 / 5068299	4577488 / 5068299	4659300 / 5068299	4757509 / 5068299	4452111 / 5068299	4366775 / 5068299	4383978 / 5068299	4466126 / 5068299	4477030 / 5068299	4440068 / 5068299
(4) *V. antiquarius* EX25* (GCA_000024825)	4777268 / 5089025	4688363 / 5089025	4710137 / 5089025	*	4577155 / 5089025	4661022 / 5089025	4725015 / 5089025	4758712 / 5089025	4410641 / 5089025	4314973 / 5089025	4378755 / 5089025	4400686 / 5089025	4402852 / 5089025	4378123 / 5089025
(5) *V. alginolyticus* E0666* (GCF_000341445.1)	4557391 / 5048917	4565025 / 5048917	4525836 / 5048917	4571047 / 5048917	*	4566989 / 5048917	4572754 / 5048917	4555121 / 5048917	4324273 / 5048917	4258499 / 5048917	4305127 / 5048917	4329033 / 5048917	4313292 / 5048917	4303896 / 5048917
(6) *V. diabolicus* FDAARGOS_96 (GCF_002953335.1)	4635646 / 5173621	4717940 / 5173621	4630960 / 5173621	4676567 / 5173621	4602005 / 5173621	*	4685227 / 5173621	4653423 / 5173621	4399940 / 5173621	4303048 / 5173621	4447434 / 5173621	4473755 / 5173621	4458963 / 5173621	4367658 / 5173621
(7) *V. diabolicus* LMG 3418 (GCF_002953355.1)	4730178 / 5131801	4750329 / 5131801	4696426 / 5131801	4734493 / 5131801	4593883 / 5131801	4662630 / 5131801	*	4766184 / 5131801	4455136 / 5131801	4330308 / 5131801	4399191 / 5131801	4439904 / 5131801	4443728 / 5131801	4423099 / 5131801
(8) *V. diabolicus* FDAARGOS_105 (GCF_002953395.1)	4759133 / 5426154	4722714 / 5426154	4793092 / 5426154	4761602 / 5426154	4575394 / 5426154	4645808 / 5426154	4760661 / 5426154	*	4483270 / 5426154	4376198 / 5426154	4439697 / 5426154	4492794 / 5426154	4463663 / 5426154	4455675 / 5426154
(9) *V. alginolyticus* ATCC 17749 (GCF_000354175.2)	4450561 / 5146637	4411895 / 5146637	4497631 / 5146637	4429768 / 5146637	4340493 / 5146637	4393218 / 5146637	4471876 / 5146637	4484679 / 5146637	*	4743372 / 5146637	4732118 / 5146637	4921790 / 5146637	4803306 / 5146637	5069409 / 5146637
(10) *V. alginolyticus* 12G01 (GCF_000153505.1)	4341171 / 5160431	4326627 / 5160431	4417413 / 5160431	4338177 / 5160431	4286869 / 5160431	4309005 / 5160431	4360974 / 5160431	4408260 / 5160431	4742141 / 5160431	*	4626232 / 5160431	4732156 / 5160431	4688774 / 5160431	4712260 / 5160431
(11) *Vibrio* sp. OY15 (GCF_000742455.1)	4365348 / 5198910	4447018 / 5198910	4404625 / 5198910	4366092 / 5198910	4322779 / 5198910	4412291 / 5198910	4385964 / 5198910	4401134 / 5198910	4717403 / 5198910	4597187 / 5198910	*	4812656 / 5198910	4778855 / 5198910	4696873 / 5198910
(12) *V. alginolyticus* RM-10-2 (GCA_001267615)	4432004 / 5284103	4488440 / 5284103	4473523 / 5284103	4392074 / 5284103	4332577 / 5284103	4441090 / 5284103	4428186 / 5284103	4478863 / 5284103	4898009 / 5284103	4710354 / 5284103	4808589 / 5284103	*	4863837 / 5284103	4864195 / 5284103
(13) *V. alginolyticus* BSW8 (GCA_000834155)	4408398 / 5190632	4487653 / 5190632	4481576 / 5190632	4392252 / 5190632	4313260 / 5190632	4426368 / 5190632	4429381 / 5190632	4439457 / 5190632	4779857 / 5190632	4672928 / 5190632	4773353 / 5190632	4861194 / 5190632	*	4759530 / 5190632
(14) *V. alginolyticus* ATCC 17749 (GCA_000354175)	4385484 / 5049668	4357689 / 5049668	4438585 / 5049668	4352992 / 5049668	4288211 / 5049668	4316019 / 5049668	4389489 / 5049668	4405501 / 5049668	5043888 / 5049668	4675420 / 5049668	4691377 / 5049668	4857218 / 5049668	4762756 / 5049668	*

Table S2. Genome annotation of *Vibrio diabolicus* A1SM3 by RAST toolkit (RASTtk) from PATRIC

Protein features	*V. diabolicus* A1SM3	
	Chromosome 1	Chromosome 2
Hypothetical proteins	516	351
Proteins with functional assignments	2411	1286
Proteins with EC number assignments	782	306
Proteins with GO assignments	643	266
Proteins with Pathway assignments	546	231
Proteins with PATRIC genus-specific family (PLfam) assignments	2860	1594
Proteins with PATRIC cross-genus family (PGfam) assignments	2861	1594

Table S3. Biosynthetic gene clusters annotated by antiSMASH.

Cluster	Type	Biosynthetic gene cluster (product)	similarity (% of genes)	Chromosome (contig)	Coordinates (From - To)	
Cluster 1	Cf_saccharide	O&K-antigen	29	1	156102	232016
Cluster 2	Cf_putative	-		1	446140	457073
Cluster 3	Cf_putative	-		1	609742	618778
Cluster 4	Cf_putative	-		1	734731	742772
Cluster 5	Cf_putative	-		1	828549	831665
Cluster 6	Arylpolyene	APE_Vf	90	1	885879	929465
Cluster 7	Cf_putative	Menaquinone	16	1	955352	964007
Cluster 8	Cf_putative	-		1	1081216	1084033
Cluster 9	Cf_putative	-		1	1126496	1134824
Cluster 10	Cf_putative	-		1	1134916	1143399
Cluster 11	Cf_putative	-		1	1145661	1151384
Cluster 12	Cf_putative	-		1	1232820	1255635
Cluster 13	Cf_putative	-		1	1354903	1362705
Cluster 14	Cf_putative	-		1	1419257	1423682
Cluster 15	Ectoine	Ectoine	100	1	1429265	1439651
Cluster 16	Cf_saccharide	-		1	1498240	1529807
Cluster 17	Cf_putative	-		1	1711599	1714318
Cluster 18	Cf_putative	-		1	1769993	1826922
Cluster 19	Cf_putative	-		1	1846609	1855988
Cluster 20	Cf_fatty_acid	-		1	2046542	2067492
Cluster 21	Cf_putative	-		1	2183041	2188505
Cluster 22	Cf_fatty_acid	-		1	2227919	2249130
Cluster 23	Cf_putative	-		1	2250878	2255590
Cluster 24	Cf_fatty_acid	-		1	2362775	2383764
Cluster 25	Cf_putative	-		1	2670736	2674028
Cluster 26	Cf_putative	-		1	2805885	2815414
Cluster 27	Cf_putative	-		1	2863319	2870902
Cluster 28	Bacteriocin	-		1	3164080	3174949
Cluster 29	Cf_putative	-		1	3175924	3180312
Cluster 30	Cf_putative	-		1	3220521	3225144
Cluster 31	Cf_putative	-		1	3272186	3281303
Cluster 32	Cf_putative	-		2	253385	255771
Cluster 33	Cf_putative	Polyhydroxyalkanoate	50	2	359944	364153
Cluster 34	Cf_putative	-		2	519028	534378
Cluster 35	Cf_putative	Fengycin	13	2	548029	562115
Cluster 36	Cf_fatty_acid	-		2	867336	888430
Cluster 37	Cf_putative	-		2	1039669	1044267
Cluster 38	Cf_putative	-		2	1126828	1130550
Cluster 39	Cf_putative	-		2	1252596	1255886
Cluster 40	Cf_saccharide	-		2	1288335	1312483
Cluster 41	Cf_putative	-		2	1391488	1404100
Cluster 42	Cf_saccharide-Siderophore	Vibrioferrin	100	2	1593237	1632082
Cluster 43	Cf_putative	-		2	1801907	1808133

GENERAL DISCUSSION

Solar salterns are recognized as a challenging environments for microorganisms considering the range of salinity to which they are exposed, starting from slightly greater salinity than the sea and reaching up to 36%, at the saturation point for NaCl, that, combined with the exposure to UV-radiation, turn microorganisms inhabiting these system into polyextremophiles. The study on marine environment despite being one of the most relevant environments for drug discovery (G. Romano et al., 2017) is still facing important challenges mainly for the isolation of marine microorganisms, considering that most of them have been shown to be uncultivable (Rosselló-Móra & Amann, 2015). This study was an example of how the merging of different techniques belonging to distinct areas of knowledge as applied microbiology, analytical chemistry, and genomics led to characterize the bacterial communities from Manaure Solar Saltern with relevant bioprospecting potential and to identify the metabolites responsible for it.

With the aim of increasing the possibility to detect the production of antibacterial and cytotoxic compounds, a culturing strategy consisted on the use of several liquid media to enrich different microbial populations in the mixed cultures was proposed. Once the bioactivities evaluated were detected in the crude extracts of the mixed cultures, a classical procedure to isolate the bacterial strains from the mixed cultures was carried out. Three of the forty mixed cultures evaluated displayed positive results, one of them, named as A1SM3-36 showed both antibacterial and cytotoxic activities tested. From this mixed culture, the isolation and characterization of the bacterium *V. diabolicus* A1SM3, a known marine specie with an unexpected biological potential, was performed. This strain showed the capacity to produce antibacterial and cytotoxic compounds, which has not been reported previously for the specie, as far as we know. Thus, this strategy demonstrates a faster and effective way to select bioactive consortia, which could lead to the isolation of the microorganism responsible of the bioactivity. However, it is noteworthy that this could be achieved because the bioactive metabolite is produced during bacterial growth and not in response to the presence of other microorganisms in the mixed culture, as it could be the case for the mixed culture A1SM1-29 (mentioned in chapter I), considering that the bioactivity was lost when the isolates were evaluated individually. On the other hand, further work is needed to characterize the mixed culture named as C8SM3-40 to determine the community composition and the metabolites responsible for the cytotoxic activity of the crude extract.

Beyond the importance of determine the community composition in the mixed cultures, there are different approaches that combined with our strategy could lead to improving the natural product

discovery. One example of these approaches is the combination with micro-fermentations, an unexplored approach proposed by Romano, Jackson, Patry, & Dobson (2018), to enhance the biodiscovery process by increasing the culture conditions evaluated, and the microorganisms or consortia to be studied. Additionally, the integration of MS/MS, NMR and molecular biology techniques proved to be an effective and helpful strategy to the identification of the producer microorganism and the bioactive metabolites, which represent a complete study on natural products biodiscovery.

The bioactive potential of the crude extract of *V. diabolicus* A1SM3 evidenced in the bioautography assay reveals that at least three different compounds showed antibacterial activity against MRSA and the analysis of the enriched bioactive fractions by MS/MS led to the identification of the isotrisindoline as one of the bioactive compounds. The development of a biotechnological process to produce and isolate isotrisindoline from *V. diabolicus* A1SM3 cultures could be a great opportunity to innovate in the natural product field, focusing on its production for a pharmacological application. With this goal, the following question was envisaged, how the composition and salinity of the growth media affect the isotrisindoline production in *V. diabolicus* A1SM3? This question was answered from the proposed experiments with this strain grown in different media, using mixtures variation of carbon and nitrogen sources, that combined with molecular networking based on the MS/MS data of the crude extracts, revealed the correlation between the occurrence of tryptophan in the nitrogen source with the biosynthesis of isotrisindoline.

It is noteworthy that the bis(indolyl)methane moiety characteristic in isotrisindoline molecule has been widely investigated for exhibiting a wide range of biological and pharmacological activities (Li et al., 2016) and especially its isomer, the trisindoline, that has been widely investigated for their anticancer activity (Kamal, Srikanth, Khan, Shaik, & Ashraf, 2010; Praveen, Parameswaran, & Majik, 2015; B. Reddy et al., 2012; Yoo et al., 2008), and recently for its α-glucosidase inhibitory activity associated with type II diabetes (G. Wang et al., 2017). In spite of isotrisindoline only has a few studies on bioactivities, it would be interesting to study how the variation in the position of the indole moieties on the oxindole ring, affects their bioactivity by means of molecular docking. This analyses could evidence the interaction between the active sites in the enzymes involved in the inhibition process, as was evidenced in the inhibitory activity for the α-glucosidase with the trisindoline, which showed to be a lead molecule for further research for developing novel anti-diabetic agents (G. Wang et al., 2017).

On the other hand, the GNPS platform allowed the analysis through the visualization of the complex MS/MS data and led to the identification of PHB analogs. Additionally, based on the experiments with

different mixtures of the nutrient sources, it was evidenced that, contrary on the most studies on PHB production, the culture media supplemented with glucose does not have a positive result to *V. diabolicus* A1SM3 regarding bacterial growth and PHB production, which agrees with the reported for another marine *Vibrio* spp. strain (Chien et al., 2007). This metabolomic approach helped to uncover additional biotechnological potential and gave information about the culture conditions that may improve the exploitation of this strain as PHB producer.

Once the bioprospecting potential of *V. diabolicus* A1SM3 was experimentally established, the sequencing of the whole genome was performed. The aim of this analysis was to establish whether this strain could have additionally functional potential that could be further studied under experimental conditions. The use of automatic annotation tools such as antiSMASH was helpful to determine the presence of known gene cluster that has been associated with biosynthetic pathways (Weber et al., 2015). This analysis revealed the potential to produce ectoine and arylpolyenes, compounds that have been widely investigated for their biotechnological applications but have not been described for this specie. Additionally, based on the experimental data obtained and the analysis using antiSMASH, the biosynthetic pathway of the PHB production was correlated with the genes involved, and the classification of the PhaC synthase for *V. diabolicus* A1SM3 as class I was established (Chapter III).

Regarding the biosynthetic pathway for isotrisindoline, the annotation in the genome of *Vibrio diabolicus* A1SM3 of the *tnaA* and the IMPD homolog genes, previously linked to the biosynthesis of isotrisindoline supports the hypothetical pathway proposed by Takeshige et al. (2015). This pathway starts with the conversion of tryptophan to indole by means of the tryptophanase (coded by *tnaA* gene). Then, the oxidation of the indole to indoxyl (3-oxyindole) and finally, the IMPD homolog oxidize it to obtain isatin, the precursor of the isotrisindoline and trisindoline isomer, by non-enzymatic reactions (N. R. Kwon et al., 2008; Yoo et al., 2008). However, the oxidation step for indole remains unknown considering that the *ipoA* gene coding the oxygenase linked to the biosynthesis of trisindoline is still unannotated in the genome of *V. diabolicus* A1SM3.

Considering the information obtained and analyzed in this thesis, the strain *V. diabolicus* A1SM3 still have a genetic potential to be experimentally established, and further studies are necessary to establish the production yield of PHB and the characterization of this biopolymer to establish their potential industrial application. Finally, the result of the integration of the culture media variation strategy with molecular networking and the genome analysis of *V. diabolicus* A1SM3, demonstrated that all the strains from *V.*

diabolicus (analyzed in this thesis) have also the potential to produce PHB, an unexplored biotechnological application for this specie until this research. Additionally, the Manaure Solar Salter proves to be a source of marine bacterial with a wide bioprospecting potential that includes *V. diabolicus* A1SM3, the *Virgibacillus* spp. strains, and the isolates from the mixed culture C8SM3-40 that still need characterization. As far as we know, this is the first published research about this environment in Colombia. As a result of this thesis, new research questions are posed to develop in future studies, related to enhancing the production yield of isotrisindoline and PHB to develop a biotechnological process that allow the sustainable exploitation of this bacterial resource.

OVERALL CONCLUSION AND PERSPECTIVES

The integration of different techniques from different areas of knowledge such as microbiology, chemistry, and bioinformatics, enables the advances in applied sciences and a better understanding of the bioprospection of microorganisms found in nature. The results reported in this thesis had shown the identification of a marine strain with multiple biological activities and biotechnological applications isolated from the unexplored Manaure Solar Saltern in La Guajira, Colombia. On the other hand, for future work with this strain, it is important to establish the production yield of PHB by *Vibrio diabolicus* A1SM3 and to study their biocompatibility for different applications and the biodegrading properties that has been widely described for this biopolymer. Additionally, further experiments to study the genes involved in the biosynthesis of the isotrisindoline could be proposed through a directed mutagenesis approach on the *tnaA* and IMPD homolog gene to corroborate their role, and additional experiments will be needed to establish which genes are the responsible for the indole oxidation. Besides, the description of the functional potential in the genome analysis creates the expectation to study how this strain could be used as a producer of antioxidant compounds (arylpolyenes) or the ectoine derivates. Finally, there are still more than 600 microorganisms in the collection of microorganisms isolated from a range of different salinities between 4% and 36% of NaCl from Manaure Solar Saltern in the Bioprospecting Research Group that are waiting to be studied for their bioprospecting potential.

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