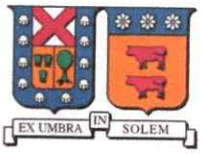


Bioprospecting for actinomycetes producing antimicrobial compounds isolated from marine sediments by characterizing genetic clusters involved in the biosynthesis of secondary metabolites

Tesis Doctoral
Entregada a la
Universidad de Chile
en cumplimiento parcial de los requisitos
para optar al Grado de Doctor

Doctorado en Ciencias mención Microbiología

Facultad de Ciencias

Por

Agustina Natalia Undabarrena Canusso

Enero, 2017

Directores de Tesis
Dr. Michael Seeger Pfeiffer & Dra. Beatriz Cámara Herrera

FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

INFORME DE APROBACIÓN

TESIS DE DOCTORADO

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por la candidata

Agustina Natalia Undabarrena Canusso

Ha sido aprobada por la comisión de Evaluación de la Tesis como requisito para optar al grado de Doctor en Ciencias mención Microbiología, en el examen de Defensa Privada de Tesis rendido el día 5 Enero 2017

Directores de Tesis:

Dr. Michael Seeger Pfeiffer .....

Dra. Beatriz Cámara Herrera .....

Comisión de Evaluación de la Tesis

Dra. Margarita Carú .....

Dra. Gloria Levicán .....

Dr. Francisco Chávez .....

Dr. Carlos Santiviago .....

FOR MY FAMILY

... AND THE OCEAN

*'**Thich Nhat Hanh**, un filósofo y monje budista vietnamita, escribe sobre cómo disfrutar de una buena taza de té.*

Debemos estar completamente atentos al presente para disfrutar de una taza de té.

Solo siendo conscientes del presente, nuestras manos sentirán el calor de la taza.

Sólo en el presente aspiraremos el aroma del té, saborearemos su dulzura y llegaremos a apreciar su exquisitez.

Si estamos obsesionados por el pasado, o preocupados por el futuro, dejaremos escapar la oportunidad de disfrutar una buena taza de té.

Cuando miremos el interior de la taza, su contenido ya habrá desaparecido.

Con la vida ocurre lo mismo.

Si no vivimos plenamente el presente, en un abrir y cerrar de ojos la vida se nos habrá escapado.

Habremos perdido sus sensaciones, su aroma, su exquisitez y su belleza...

Y sentiremos que ha transcurrido a toda velocidad.

El pasado ya es pasado. Aprendamos de él y dejémoslo atrás.

El futuro, ni tan siquiera ha llegado. Hagamos planes para el futuro pero no perdamos el tiempo preocupándonos por él. Preocuparse no sirve de nada.

Cuando dejemos de pensar en lo que ya ha ocurrido,

cuando dejemos de preocuparnos por lo que todavía no ha pasado,

estaremos en el presente.

Sólo entonces empezaremos a experimentar la alegría de vivir.'

Ham sa Vam

Autobiography

Nací un 17 de mayo de 1988 en Buenos Aires, Argentina. Mis padres, Luis y Alejandra, me bautizaron con el nombre de Agustina Natalia Undabarrena Canusso, y soy la mayor de dos hermanos. Viví en Buenos Aires durante mi infancia hasta el año 1996, donde realicé la enseñanza básica en el colegio Norbridge School. Luego por motivo del trabajo de mi padre, nos mudamos a Santiago, Chile, donde viví mi adolescencia. Terminé la enseñanza básica y media en el colegio Bradford School. Durante mis años en el colegio me apasioné por las ciencias naturales y en especial por la Biología. Es por esto que el año 2006 ingresé a la Pontificia Universidad Católica para estudiar Licenciatura en Biología. Al graduarme en el año 2010 realicé diversos trabajos en laboratorios de investigación en el área de microbiología clínica y ambiental, hasta que ingresé al Doctorado en Ciencias con mención en Microbiología de la Universidad de Chile el año 2012. Realicé mi trabajo de Tesis Doctoral en el Laboratorio de Microbiología Molecular y Biotecnología Ambiental, en la Universidad Técnica Federico Santa María, en Valparaíso, a cargo de los tutores el Dr. Michael Seeger Pfeiffer y la Dra. Beatriz Cámara Herrera. Tras 4 años de arduo trabajo al fin he alcanzado mis objetivos académicos para presentarme a la obtención del grado de Doctora.

Acknowledgements

I am deeply grateful to the following for making this dissertation possible:

Prof. **Dr. Michael Seeger** and **Dra. Beatriz Cámara**, for being excellent research supervisors and providing me the freedom of perform every crazy idea that came to my mind. For their patience, encouragement and support that even extends beyond the confines of work. For showing me that science should be every day enjoyed, and for teaching me how to keep going on even when the darkness appeared. For letting me know that in every corner, something amazing is awaiting to be discovered.

Thanks to all my labmates of **LMMBA**, speacially to the **Actino Team**, that held me every day. For their constant understanding and support, and for being a great team inside and outside the lab. For your cares and concerns, and for being there for me in every opportunity that I needed. Specially for helping me getting settled when I first arrived to Valparaiso, having no one here. I would not have done it without you all.

To our bioinformatic support, **Dr. Juan Ugalde** and **Dr. Eduardo Castro-Nallar**, that helped me to unveil the fascinating world of *omics* and were by my side understanding and thinking how this story was all connected.

To my commission professors: **Dra. Margarita Carú, Dra. Gloria Levicán, Dr. Francisco Chávez** and **Dr. Carlos Santiviago**, that were all along this road with advices and ideas to improve and make this Ph.D thesis better, in every presentation.

To our international colaborators: **Dr. Fabrizio Beltrametti** and **Dr. Edward Moore**, for teaching me and helping me to get my experiments on track.

To the **GIGA Lab**, from University of Liége, especially to **Dr. Joseph Martial**, for making my internship in Belgium possible. To **Dra. Cecile Vanderweerdt** and **Dr. Edwin de Pauw** labs, for teaching me enormously. For adopting me as they had known me since ever.

Most importantly, to **my family** and **friends**, who gives me constant and unconditional love. *Only love is real*. To you I dedicate this work.

Funding

This work was supported by the "Comisión Nacional de Investigación Científica y Tecnológica" (FONDECYT #11121571 and #1110992) and Swedish Research Council N° 2013-6713 with additional funding from the Universidad Técnica Federico Santa María (USM131342, 131562). Finally, we acknowledge Conicyt "Ph.D. fellowship", Conicyt "Gastos Operacionales #21120621", Conicyt "Beca de pasantía en el extranjero" along with PIEA (UTFSM).

Abbreviations

A: Adenylation domain
ACP: Acyl Carrier Protein
Ala: Alanine
ANI: Average Nucleotide Identity
ASW: Artificial Sea Water
AT: Acyl Transferase domain
BASU: *Bacillus subtilis*
BGCs: Biosynthetic Gene Clusters
C: Condensation domain
COGs: Cluster of Orthologous Genes
Cy: Cyclization domain
DH: Dehydratase domain
DMSO: Dimethyl sulphoxide
DNA: Desoxyribonucleic acid
E: Epimerization domain
ENFA: *Enterococcus faecalis*
ER: Enoyl Reductase domain
ESCO: *Escherichia coli*
ESI-FT ICR: Electrospray Ionization – Fourier Transform – Ion Cyclotron Resonance
F: N-formylation domain
HPLC: High Pressure Liquid Chromatography
KLPN: *Klebsiella pneumoniae*
KR: Ketoreductase domain
KS: Ketosynthase domain
LIMO: *Listeria monocytogenes*
LVEM: Low Voltage Electron Microscopy
MA: Marine Agar
MALDI-TOF: Matrix assisted Laser Desorption/Ionization – Time of Flight
MIBiG: Minimum Information about a Biosynthetic Gene Cluster
MILU: *Micrococcus luteus*
MLSA: Multi-Locus Sequence Analysis
MNP: Marine Natural Product
MRSA: Methicillin-Resistant *Staphylococcus aureus*
MS: Mass Spectrometry
MT: N-methylation domain
NMR: Nuclear Magnetic Resonance
NP: Natural Product
NRP: Non-ribosomal Peptide
NRPS: Non-ribosomal Peptide Synthetase
Orn: Ornithine
Ox: Oxidoreductase domain
PCP: Peptidyl Carrier Protein

PCR: Polymerase Chain Reaction
PHAs: Polyhydroxyalkanoates
PKS: Polyketide Sythase
PSAU: *Pseudomonas aeruginosa*
R: Reductase domain
RNA: Ribonucleic acid
SAEN: *Salmonella enterica*
STAU: *Staphylococcus aureus*
STEP: *Staphylococcus epidermidis*
TE: Thioesterase domain
Thr: Threonine
TLC: Thin Layer Chromatography
Trp: Tryptophan
Tyr-NO_2: Nitro-tyrosine
Val: Valine

Table of Contents

Figure Index

Table Index

1 Abstract

Bioactive compounds are increasingly required for diverse biotechnological applications. Among them, the development of new drugs is one of the major relevances due to the steady rise in the number of antibiotic-resistant bacterial pathogens. The *phylum Actinobacteria,* represent the most prominent group of microorganisms for the production of bioactive compounds, notably antibiotics, antifungal and antitumor agents. Many bioactive natural products with applied potential have been isolated from actinomycetes derived from sea sediments. Therefore, an excellent alternative to discover the potential of novel natural products is provided by the marine environment. Marine ecosystems are particularly suited for bioprospecting, a process that aims to identify and isolate natural compounds with the aid of its genetic material. In this line, the marine coasts of Chile remain poorly explored and contain a high diversity of microorganisms, many of which must still to be described. The bioprospection of new actinomycetes isolates from poorly explored marine environments can lead to the discovery of novel bioactive compounds with biotechnological potential. In this context, the exploration of the cultivable diversity of actinomycetes in marine sediments from a remote fjord of Chile, the Comau fjord located in Northern Chilean Patagonia, was proposed. Twenty five actinomycetes comprising 10 genera were isolated and characterized, for their tolerance to abiotic stress, marine adaptations, susceptibility to commercial antibiotics, biochemical assays and phylogenetic relationships. Five actinomycetes are proposed to be novel species. Moreover, antimicrobial activity among the actinomycetes was assessed, showing a promising potential to produce bioactive compounds.

A great number of bioactive molecules belong to the group of polyketides, nonribosomal peptides or a combination of both. These metabolites are a vast group of structurally diverse natural compounds produced by a variety of microorganisms. The polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) biosynthetic systems are organized in genetic clusters, and thus, their bioinformatic detection through whole genome sequencing is possible. Therefore in this study, five actinomycetes presenting antimicrobial properties and genes encoding for PKS/NRPS were selected for their subsequent whole genome sequencing in order to study these metabolic routes involved in the synthesis of bioactive compounds. In particular, one strain, *Streptomyces* sp. H-KF8, presented 26 biosynthetic gene clusters (BGCs), where two of them corresponded to NRPS routes. These metabolic pathways were genetically characterized, and prediction of the chemical structure of their products was proposed. Notably, a large number of these clusters have no similarities with other known clusters, suggesting that *Streptomyces* sp. H-KF8 possess novel metabolic routes involved in the synthesis of novel compounds.

Finally, a conventional approach for the extraction of the compound(s) using organic solvents and subsequent bio-guided fractionation of the crude extract of *Streptomyces* sp. H-KF8 was performed, to gain insights into the biological activity and chemical nature of the antimicrobial molecules that are being produced by this bacterium. Mass spectrometry techniques such as ESI FT ICR MS, MALDI-TOF MS and Imaging MS, were used to detect molecules that are being expressed under antagonistic interactions, and to provide the monomer composition of the selected masses. These candidates molecules were subsequently compared to the bioinformatic data of the sequenced genome of *Streptomyces* sp. H-KF8, to unveil the link between the genomic-metabolic relationship. Specifically, a connection between the NRPS

cluster #6 and the metabolites produced by *Streptomyces* sp. H-KF8 was accomplished. Proposed mechanism of action of this antimicrobial metabolite along with tailouring reactions that may modify the peptidic core are also described.

Altogether, these results suggest that Chilean Patagonian fjords are suitable environments for bioprospecting for novel species of actinomycetes with promising antimicrobial activity. Genome sequencing was a fundamental tool to stablish the genetic potential to produce bioactive compounds. *Streptomyces* sp. H-KF8 demonstrated to harbour novel biosynthetic gene clusters for the synthesis of secondary metabolites, where one of them, the cluster #6, showed a sustained correlation among the predicted bioinformatic and the experimental data, suggesting the production of a novel antimicrobial compound of a glycopeptide nature.

1 Resumen

Los compuestos bioactivos son ampliamente requeridos para diversas aplicaciones biotecnológicas. Entre ellas, el desarrollo de nuevas drogas es de mayor relevancia debido al dramático aumento de bacterias patógenas resistentes a antibióticos. El *phylum Actinobacterias* representan el grupo de microorganismos más prominente en relación a la producción de compuestos bioactivos, como antibióticos, antifúngicos y antitumorales, entre otros. Numerosos productos naturales bioactivos con potencial aplicable han sido aislados de actinomycetes derivadas de sedimentos marinos. Por esta razón, una excelente alternativa para descubrir nuevos productos naturales puede proveer del ambiente marino. Los ecosistemas marinos son particularmente apropiados para la bioprospección, un proceso que tiene como objetivo identificar y aislar compuestos naturales a partir de su material genético. En esta línea, las costas de Chile aún permanecen inexploradas y contienen una alta diversidad de microorganismos, muchos de los cuales todavía no han sido descritos. La bioprospección de nuevos aislados de actinomycetes obtenidos de ambientes marinos inexplorados, podría llevar al descubrimiento de compuestos bioactivos novedosos con gran potencial biotecnológico. En este contexto, la exploración de la diversidad cultivable de actinomycetes desde sedimentos marinos del remoto fiordo de Comau, ubicado en la Patagonia Norte de Chile, fue propuesto. Veinticinco actinomycetes que comprenden 10 géneros fueron aislados y caracterizados por su tolerancia a estrés abiótico, adaptaciones marinas, susceptibilidad a antibióticos comerciales, ensayos bioquímicos y relaciones filogenéticas. De éstos, cinco aislados son propuestos como nuevas especies. Adicionalmente, la actividad antimicrobiana entre todos los aislados fue evaluada, mostrando un prometedor potencial para producir compuestos bioactivos.

Un gran número de moléculas bioactivas pertenecen al grupo de los policétidos, péptidos no-ribosomales o a la combinación de ambos. Estas moléculas son un vasto grupo de compuestos naturales con diversas estructuras producidos por una gran variedad de microorganismos. Las policétido sintasas (PKS) y las sintetasas de péptidos no-ribosomales (NRPS) son sistemas biosintéticos que están organizados en agrupamientos genéticos, por lo tanto, su detección bioinformática a través de la secuenciación de genomas completos es posible. En este estudio, cinco actinomycetes que presentaron propiedades antimicrobianas y genes que codificaban para PKS/NRPS, fueron seleccionadas para la subsecuente secuenciación de sus genomas completos con el fin de estudiar sus rutas metabólicas involucradas en la síntesis de compuestos bioactivos. En particular, la cepa *Streptomyces* sp. H-KF8 presentó 26 agrupamientos biosintéticos (BGCs), donde dos de ellos correspondieron a rutas de NRPS. Estas vías metabólicas fueron caracterizadas genéticamente, y la predicción de la estructura química de sus productos fue propuesta. Notablemente, un gran número de estos BGCs no tuvieron similitud con otros BGCs conocidos, lo cual sugiere que *Streptomyces* sp. H-KF8 posee nuevas rutas metabólicas involucradas en la síntesis de nuevos compuestos.

Finalmente, una aproximación convencional para la extracción del (o los) compuesto(s) usando solventes orgánicos y un subsecuente fraccionamiento del extracto crudo de *Streptomyces* sp. H-KF8, fue llevado a cabo para ganar conocimiento acerca de la actividad biológica y la naturaleza química de las moléculas antimicrobianas producidas por esta cepa. Técnicas de espectrometría de masas tales como ESI FT ICR MS, MALDI-TOF MS e Imaging MS, fueron usadas para detectar moléculas presentes bajo interacciones antagónicas, y para proporcionar información sobre la composición de los monómeros de masas seleccionadas. Las moléculas candidatas fueron comparadas contra los datos bioinformáticos del genoma

secuenciado de *Streptomyces* sp. H-KF8 para revelar la relación genómica-metabolómica. Específicamente, una conexión entre el agrupamiento NRPS #6 y los metabolitos producidos por *Streptomyces* sp. H-KF8 fue lograda. Además, un mecanismo de acción propuesto para el metabolito antimicrobiano, en conjunto con las reacciones de modificación post-ensamblaje las cuales modifican el esqueleto peptídico, también son descritas.

En conjunto, estos resultados sugieren que los fiordos de la Patagonia Chilena son ambientes apropiados para la bioprospección de nuevas especies de actinomycetes con actividad antimicrobiana prometedora. La secuenciación del genoma fue una herramienta fundamental para establecer el potencial genético para la producción de compuestos bioactivos. *Streptomyces* sp. H-KF8 demostró poseer nuevos BGCs para la síntesis de metabolitos secundarios donde uno de ellos, el agrupamiento NRPS #6, mostró tener una sostenida correlación entre la predicción bioinformática y los datos experimentales, sugiriendo la producción de un nuevo compuesto antimicrobiano de naturaleza glicopeptídica.

2 Introduction

2.1 The problem - Multi-drug Resistant Bacteria

The discovery of penicillin by Alexander Fleming in 1928, it purification by Florey and Chain in 1939, and subsequent industrial production in 1942, marketed the beginning of the 'Golden Age of Antibiotics' -covering from the 1940s to the 1970s- in which commercialization of several life-saving drugs was achieved (Knight *et al.*, 2003). Antimicrobials are active by targeting essential components of bacteria metabolism, such as inhibition of cell wall synthesis (*e.g.*, β-lactams), DNA gyrase (*e.g.*, quinolones), DNA-directed RNA polymerase (*e.g.*, rifampicin), protein synthesis (*e.g.*, aminoglycosides) and competing with enzymes (*e.g.*, sulphonamides) (Coates *et al.*, 2002). The emergence and dissemination of multi-drug resistant bacteria coupled to the lack of new antibiotics with new modes of action are today's one of the main challenges to treat infectious diseases, since all classes of antibiotics have seen the emergence of bacterial resistance, thus limiting their use (Genilloud, 2014). One of the major concerns is the emergence of multi-drug resistances in the so-called **ESKAPE** pathogens, referring to ***Entercoccus faecium***, ***Staphylococcus aureus***, ***Klebsiella pneumoniae***, ***Acinetobacter baumannii***, ***Pseudomonas aeruginosa*** and ***Enterobacter*** spp., which represent one of the main causes of death worldwide (Livermore, 2009). The phenomena of resistance could be due to an innate property of the bacterium (*e.g.*, biofilm production), consequence of mutation (*e.g.*, efflux pump) or acquired by horizontal gene transfer (Hogan & Kolter, 2002). The molecular mechanisms of bacterial resistance involves drug inactivation, modification of the site of action, modification of the permeability of the cell wall, overproduction of the target enzyme and the bypass of the inhibited steps (Opal, Mayer & Madeiros, 2000). Factors that determine whether resistance develops are

complex and interdependent, and includes the action mechanism, if the drug has a time- or concentration-dependent mode of action, the potency against the population of bacteria and the magnitude and duration of the available concentration (Coates *et al.*, 2002). The improper use of antibiotics in medical treatment, as well as their indiscriminate use in agriculture, livestock and aquaculture, has lead today to a discouraging scenario where more than 70% of bacteria are resistant to commercial antibiotics most commonly used (Brown *et al.*, 2006). The introduction of new compounds into therapy have decreased significantly over the past years, and no new class of broad-spectrum compounds has been discovered since 1960. Therefore, we are facing a critical global decline in antimicrobial research, with an urgent need to find novel therapeutic compounds.

2.2 Bioprospecting *phylum Actinobacteria* for natural product discovery

Natural Products (NPs) play a significant role in drug discovery. In fact, 78 % of antibiotics commercially marketed from 1982 to 2002 have originated from NPs (Peláez, 2006). Takin into consideration only year 2014, 25 % of the approved new chemical entities are from natural or natural-derived products (Newman & Cragg, 2016). On the contrary, chemical combinatorial approaches that develop molecules of synthetic origin have been unable to compete with NPs (Payne *et al.*, 2007). Antimicrobial-producing organisms belong to the three several domains of life, including microorganisms, fungi, invertebrates and plants, although secondary metabolite production rates and chemical structures differ considerably among them. Microorganisms provide >60,000-80,000 metabolites, where 47 % are biologically active, mainly showing antibiotic, antitumor, antifungal, antiparasitical and antiviral effects (Bérdy, 2012). The *phylum Actinobacteria* is well-known for contributing with 45 % of all microbial metabolites. More than

5,000 compounds have been reported and contributed to the development of 90 % commercial antibiotics (Jose & Jha, 2016). The class *Actinobacteria*, accounts for 7,000 compounds reported in the Dictionary of Natural Products (Jensen *et al.*, 2005a). Most antibiotics derive from the *Streptomyces* genus (75 %), which have drawn attention since the discovery of the antibiotic streptomycin in the early 1940s. Streptomycin was the first compound of a plethora of bioactive secondary metabolites derived from members of the genus *Streptomyces*, which would become established as the most prolific bacterial antibiotic producers delivering the majority of antibiotic drugs still in use today (Bérdy, 2005). However, productivity of classical screening methods decreased during the 1970s, due to the frequent re-discovery of known compounds as efforts were placed in obtaining soil isolates from similar sampling sites (Fenical, 1993). In this context, diverse unexplored natural habitats were later considered for the isolation of novel members of actinomycetes, where the marine ecosystem gained focus for bioprospecting.

Oceans cover up to 70% of the Earth's surface and harbour a largely untouched biodiversity (Donia & Hamann, 2003; Haefner, 2003). Marine environments are highly diverse in terms of abiotic selective pressures, which are the driving force for ecological adaptations that is reflected in the genetic and metabolic physiological traits of microorganisms. Thus, marine actinomycetes are particularly suited to explore in order to find novel Marine Natural Products (MNP) (Lam, 2006; Bull & Stach, 2007). Marine environment provide an established ecological niche for actinomycetes (Das, Lyla & Khan, 2006; Ward & Bora, 2006). Isolation of actinomycetes have been characterized from diverse marine sources, such as mangrove forests (Hong *et al.*, 2009; Baskaran, Vijayakumar & Mohan, 2011; Lee *et al.*, 2014); marine sponges (Kim, Garson & Fuerst, 2005; Montalvo *et al.*, 2005; Zhang *et al.*, 2006; Jiang *et al.*, 2007; Sun *et al.*, 2015); corals (Hodges, Slattery & Olson, 2012; Kuang *et al.*, 2015; Mahmoud & Kalendar, 2016; Pham *et al.*,

2016); sea cucumbers (Kurahashi *et al.*, 2010); pufferfishes (Wu *et al.*, 2005) and seaweed (Lee *et al.*, 2008). Notably, actinomycetes are predominant in marine sediments (Mincer *et al.*, 2002; Magarvey *et al.*, 2004; Jensen *et al.*, 2005b; León *et al.*, 2007; Gontang, Fenical & Jensen, 2007; Bredholdt *et al.*, 2007; Maldonado *et al.*, 2008; Duncan *et al.*, 2014; Yuan *et al.*, 2014), whereas also in deep-sea sediments (Colquhoun *et al.*, 1998; Pathom-Aree *et al.*, 2006).

Marine bioprospecting from both macro- and micro-organisms has led to at least 30,000 MNPs, and the number of new compounds from marine-derived sources accounted to 164 in 2014 (Blunt *et al.*, 2009). Marine actinomycetes have been described as an emerging source for novel bioactive molecules (Fiedler *et al.*, 2005; Lam, 2007; Bull & Stach, 2007; Joint, Mühling & Querellou, 2010; Zotchev, 2012; Subramani & Aalbersberg, 2012; Valliappan, Sun & Li, 2014). Antibiotics produced by actinomycetes have been evolving for ~1 billon years where its fitness has been tested by the ability to penetrate other microbes and inhibit target enzymes, macromolecules or molecular structures (Baltz, 2008). The novelty in MNPs derives from unique chemical structures and metabolic pathways that are found due to the specific evolution of microorganisms in marine environments (Knight *et al.*, 2003). Actinomycetes produce almost exclusively some classes of structurally complex compounds, such as macrocyclic lactones, lactams, cyclopeptides, depsipetides and polycyclic quinone-based molecules (Bérdy, 2012). Therefore, the importance of cultivating these microorganisms is crucial for a viable opportunity to biodiscovery (Joint, Mühling & Querellou, 2010).

Actinomycetes NP research for the discovery of novel antibiotics involves an integral approach of genetics, genomics and metabolomics areas, including: i) the isolation and dereplication of isolates; ii) prediction and identification of novel compounds; iii) enhancing

production titers of potential compounds; iv) uncovering genome information and associated biosynthetic potential; v) collection and processing of genome data; vi) mining, editing and heterologous expression of cryptic gene clusters and vi) comprehensive metabolic profiling (Jose & Jha, 2016). Altogether, these studies reflect the importance of pursuing marine actinomycetes as a prolific source for novel MNP discovery.

2.2.1 Generalities of the *phylum Actinobacteria*

The *phylum Actinobacteria* comprises Gram-positive organisms with a high G+C content and constitutes one of the largest *phyla* within *Bacteria* (Gao & Gupta, 2012). Taxonomy of the *pylum Actinobacteria* has been controversial. Originally, they were historically considered as an intermediary between fungi and bacteria, due to their mycelial morphology, and radial colony growth, resembling fungi (Krassilnikov, 1941; Barka *et al.*, 2016). Based on 16S rRNA gene phylogeny, there are now 6 proposed classes, comprising 5 basal ones with one or two orders each (*Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria* and *Thermoleophilia*) and the main class *Actinobacteria*, that comprises 15 orders (Ludwig *et al.*, 2012). Recently the phylogeny has been revised in light of complete genome sequences, proposing that the orders *Frankiales* and *Micrococcales* should be split into coherent entities (Sen *et al.*, 2014). Nevertheless, the classification is still under constant changing. Briefly, it includes more than 3,000 species, where the different genera among this *phylum* exhibit an enormous diversity in terms of morphology, physiology and metabolic capabilities (Barka *et al.*, 2016). Surprisingly, the class *Actinobacteria* contains both the most deadly bacterial pathogen (*i.e.*, *Mycobacterium* genus) and the microorganisms that are the most important for antibiotic production (*i.e.*, *Streptomyces* genus) (Doroghazi & Metcalf, 2013). The morphologies of their species varies from cocoid (*e.g.*,

Micrococcus) or rod-coccoid (*e.g.*, *Arthrobacter*) to fragmenting hyphal forms (*e.g.*, *Nocardia*) or highly-differentiated branched mycelia (*e.g.*, *Streptomyces*) (Ventura *et al.*, 2007). Although not ubiquitous, spore formation is common along actinomycetes, and spore ranges from motile zoospores to specialized propagules. They also produce numerous extracellular metabolic enzymes and a wide variety of secondary metabolites (Gao & Gupta, 2005). In nature, actinomycetes are widely distributed in both terrestrial and aquatic ecosystems, where they play an important ecological role in the nutrient recycling of refractory biomaterial by decomposition and humus formation (Ventura *et al.*, 2007; Barka *et al.*, 2016).

Specifically, the *Streptomyces* genus has been the focus of study in the continuous anti-microbial search for producing two-thirds of all known antibiotics (Bérdy, 2005). In general, *Streptomyces* are characterized for slow growth and development. *Streptomyces* possess a complex developmental life cycle (Flärdh & Buttner, 2009). In solid substrate, it is well-studied that they grow as a substrate mycelium made of multiple hyphae that grow by tip extension and branch through the soil in search for nutrients (Chater *et al.*, 2010). They acquire nutrients by secreting enzymes that break down insoluble organic polymers, such as chitin and cellulose. On the contrary, little is known about *Streptomyces* morphogenetic in liquid media. Recently, a new developmental model was proposed, which involves programmed cell death phenomena of the mycelium to form four morphological classes (pellets, clumps, branched hyphae and non-branched hyphae) of differentiated mycelium without hydrophobic layers (Yagüe *et al.*, 2013). As *Streptomyces* are non-motile bacteria ubiquitous in different natural environments, they must compete with other fast-growing microorganisms in order to have access for nutrients. To confront this, *Streptomyces* are armed with a wide range of secondary metabolites that help them to survive under adverse conditions (Ruiz *et al.*, 2010; Sánchez *et al.*, 2010).

2.2.2 *Phylum Actinobacteria* in marine ecosystems

The first marine actinomycete isolated was *Rhodococcus marinonascens* reported in 1984 (Helmke & Weyland, 1984). Members belonging to approximately 50 genera have been isolated from various marine sources (Goodfellow & Fiedler, 2010). These include isolates from novel genera such as *Demequina* (Yi, Schumann & Chun, 2007), *Marinactinospora* (Tian *et al.*, 2009a), *Marisediminicola* (Li *et al.*, 2010), *Miniimonas* (Ue *et al.*, 2011), *Paraoerskovia* (Khan *et al.*, 2010), *Phycicococcus* (Lee, 2006), *Phycicola* (Lee *et al.*, 2008), *Salinibacterium* (Han *et al.*, 2003), the obligate marine *Salinispora* genus (Maldonado *et al.*, 2005), *Sciscionella* (Tian *et al.*, 2009b) and *Serinicoccus* (Yi *et al.*, 2004), as well as new species within known genera such as *Arsenicococcus* (Hamada *et al.*, 2009), *Dermacoccus* (Pathom-aree *et al.*, 2006b), *Kocuria* (Seo *et al.*, 2009), *Nocardiopsis* (Chen *et al.*, 2016), *Saccharomonospora* (Liu *et al.*, 2010), *Streptomyces* (Pimentel-elardo & Scheuermayer, 2009), *Williamsia* (Pathom-aree *et al.*, 2006a) and *Verrucosispora* (Liao *et al.*, 2009; Dai *et al.*, 2010). The huge cultivable diversity of microorganisms discovered and the subsequent studies related to these are clear evidence that actinomycetes are active components of marine microbial communities (Genilloud, 2014).

The marine environment contains several ecosystems, from the sea surface, down to the habitats on and under the sea floor. Actinomycetes are widely distributed along these niches, forming stable communities within various marine ecosystems such as the surface microlayer, the water column, associated to marine free-swimming vertebrates or sessile invertebrates, in marine snow, depth sediments and below sea subfloor (Ward & Bora, 2006). Despite that relatively little is known concerning their ecological role in marine environments, actinomycetes from several marine sources have been reported to decompose agar, alginates and laminarin,

cellulose, chitin, oil and other hydrocarbons. Also, they have been implicated in the decay of wood submerged in seawater (Goodfellow & Williams, 1983). The living conditions to which marine actinomycetes had to adapt during evolution ranges from extremely high pressure, anaerobic conditions, fluctuating temperatures and salinity, and high acidic pH conditions (Lam, 2006). Microorganisms sense, adapt and respond quickly to their environment and compete for defense and survival (Zhang *et al.*, 2005). As marine environmental surroundings are markedly different from terrestrial ones, it has been proposed that marine actinomycetes have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds. A major number of actinomycetes have been associated with soft bodied or sedentary lifestyle marine organisms such as marine sponges and corals, due to their need for chemical weapons for defense and survival. These compounds help them to dissuade predators, to keep competitors at bay or to paralyze their prey (Haefner, 2003). In general, secondary metabolites have survival functions such as: i) competitive weapons used against other organisms; ii) metal transporting agents; iii) agents of symbiosis; iv) differentiation effectors or v) communicating signal molecules (Demain & Fang, 2000). Thereof, these molecules play important ecological roles in their natural environments, as signal molecules, facilitating intra- or inter-species interactions within microbial communities, related to virulence, colonization, motility, stress response and biofilm formation (Romero *et al.*, 2011). Research has taken advantage from these unique molecules and their bioactive potential, to discover novel anti-infective compounds with antibacterial, antifungal and/or antitumoral properties, and apply them in current clinical challenges (Gulder & Moore, 2010).

2.2.3 *Phylum Actinobacteria* in Chile

Chile has a variety of natural environments that have not been explored and, to date, scarce studies involving actinomycetes have been carried out. Isolation of *Streptomyces* from soil collected from Easter Island (Vézina, Kudelski & Sehgal, 1975) and from sand collected from the Atacama Desert, Northern Chile (Santhanam *et al.*, 2012a,b, 2013) were reported. However, the Chilean vast coast has remained largely unexplored, and to date, there are only two studies reporting the isolation of marine actinomycetes: one carried out with sediments from Chiloe Island (Hong *et al.*, 2010) and a recent report from our laboratory, carried out in Valparaíso Central Bay (Claverías *et al.*, 2015).

In this thesis, the bioprospection of marine actinomycetes isolated from the National Marine Protected Area of Huinay at the Comau fjord was proposed. Northern Patagonia Chilean fjords are unique in terms of biogeography and remains remote and largely unstudied as rich sources for untapped novel microorganisms. Fjords have unique biogeographic characteristics such as a relatively narrow inlet, with significantly eroded sea floor and communication with the open sea (Bredholdt *et al.*, 2007). The Comau fjord is a pristine area unique by its geological nature. It is comparatively smaller than other fjords in Chile, and also one of the deepest (Ugalde *et al.*, 2013). Comau fjord is characterized by steep slopes, with surrounding mountains that have a height up to 2,000 m with a dense extratropical rainforest covering from the sea to the top (Lagger *et al.*, 2009). An annual precipitation of 5,600 mm provides a fresh water input crucial in providing minerals, which also decrease the salinity of the surface water (Silva, 2006; Lagger *et al.*, 2009). The surface water temperatures ranges between 5 and >20 °C, whereas the main

water body has temperatures ranging between 8-12°C (Lagger *et al.*, 2009; Sobarzo, 2009), that sustains a thermohaline circulation (Bustamante, 2009).

This is the first report on bioprospection of actinomycetes by a cultivable-dependent survey in this unique ecosystem. Only a metagenomic study had been carried out in the Comau fjord, where a microbial mat was analyzed, in which 1% of community reads was represented by the *phylum Actinobacteria* (Ugalde *et al.*, 2013). This metagenomic study reveals the presence of the *phylum* in this ecosystem. Since the Chilean Patagonia sustains an autochthonous biodiversity, it is hypothesized that it may harbour novel actinomycetes producing novel anti-infective compounds, due to novel metabolic routes not present in their terrestrial counterparts.

2.2.4 Biosynthetic routes for metabolite production in *phylum Actinobacteria*

Metabolic routes by which bacteria display secondary metabolites synthesis are diverse. However, due to their role in ecological adaptation for survival, evolution has grouped these genes in the so-called Biosynthetic Gene Clusters (BGCs). A typical secondary metabolite BGC involves genes for the scaffold synthesis, scaffold modification, resistance and efflux, along with positive and negative regulators (**Figure 1**). These genes are organized as clusters in the genomes, allowing coordinated expression of the proteins required for the synthesis of a specific secondary metabolite (Zotchev, 2014).

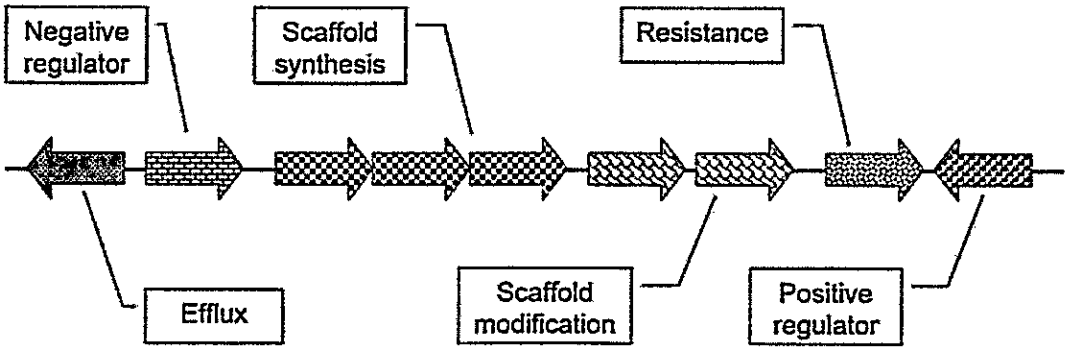


Figure 1: Organization of a typical secondary metabolite biosynthesis gene cluster (Zotchev, 2014).

Secondary metabolites biosynthetic machinery utilizes precursors from primary metabolism to build a molecular skeleton with the help of scaffold-synthesizing enzymes. Expression of genes for such enzymes is usually regulated by a positive regulator that responds to particular environmental signals. Scaffold modification enzymes add chemical groups to the skeleton, such as sugars, hydroxyl-, formyl-, methyl- and amino-groups, among others (Zotchev, 2014). To avoid self-toxicity, several resistance mechanisms are implemented: one depends on the active efflux of the metabolite by a specific transporter which can be repressed by a negative regulator; another employs a gene encoding a resistance protein, which can be an enzyme modifying the molecular target of the secondary metabolite or an enzyme modifying the secondary metabolite itself, rendering it inactive (Hopwood, 2007).

It has been demonstrated that BGCs are widespread in diverse bacterial species from geographically distinct locations (Morlon *et al.*, 2015). Nevertheless, the distribution along the *phyla* is not homogeneous. Actinomycetes are known to harbour a high number (17 or more) of BGCs (Bentley *et al.*, 2002; Udwary *et al.*, 2007), in comparison to other bacteria such as *Bacillus*,

Pseudomonas and *Ralstonia* genera, that have four or less of these gene clusters (Kunst *et al.*, 1997; Stover *et al.*, 2000; Salanoubat *et al.*, 2002).

A large number of antimicrobial compounds are produced by a group of BGCs called non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), or a combination of both (NRPS/PKS) (Fischbach & Walsh, 2006). These enzymes synthesise a surprisingly diverse secondary metabolite structures, and are organized in assembly lines that work in an iterative and coordinated fashion to form linear oligomers by the use of sequential chemical condensation reactions (Cane, Walsh & Khosla, 1998; Walsh, 2004; Fischbach & Walsh, 2006). Each module in a cluster is responsible for chain extension through recognition, activation and incorporation of specific substrates. Monomer units are used as building blocks, and usually are acyl-CoA thioesters for PKS systems and amino acids (proteinogenic and non-proteinogenic) for NRPS systems, which are enabled by a phosphopantetheine group that acts as a 'swing-arm' co-factor (Salomon, Magarvey & Sherman, 2004).

In PKS systems, sequential condensation of carboxylic acids resembles the synthesis of fatty acids in bacteria. There are three major classes of PKS systems, characterized by their mode of synthesis and structural type of product. Type I PKSs, are multienzyme complexes that are organized into individual, linear modules, each of which is responsible for a single, specific chain elongation process and post-condensation modification of the resulting β-carbonyl. Type II PKSs are complexes of monofunctional proteins that build polyketide chains by their iterative use of a single set of distinct enzymes to construct polyketide chains, which are then cyclized to produce small molecules containing aromatic ring systems. Type III PKSs contain one protein, one domain and one active site to carry out the three central reactions of chain initiation, elongation and

cyclization (Salomon, Magarvey & Sherman, 2004). The actions of the essential core domains consists of an initiation module that starts with an acyl transferase domain (AT) for the selection of an activated acyl-CoA monomer. Then, the AT domain transfers the acyl-CoA to the swinging arm, the acyl carrier protein (ACP), which in turn transfers the chain to the upstream ketosynthase domain (KS) which catalyses the decarboxylation of the carboxylic acid and the subsequent Claisen condensation. Optional domains acting upon the newly formed β-carbonyl are the ketoreductase (KR) domain, which reduces the carbonyl to a hydroxyl group; the dehydratase (DH) domain, which dehydrates the alcohol to form a double bond; and an enoyl reductase (ER) domain that is responsible for the reduction of the double bond to a fully saturated methylene. The complete elongated and functionalized chain is often transferred to a final thioesterase domain (TE) that catalyses the hydrolytic release of a linear compound and can be coupled with cyclization to generate macrolactone structures (**Figure 2**) (Salomon, Magarvey & Sherman, 2004; Fischbach & Walsh, 2006; Donadio, Monciardini & Sosio, 2007).

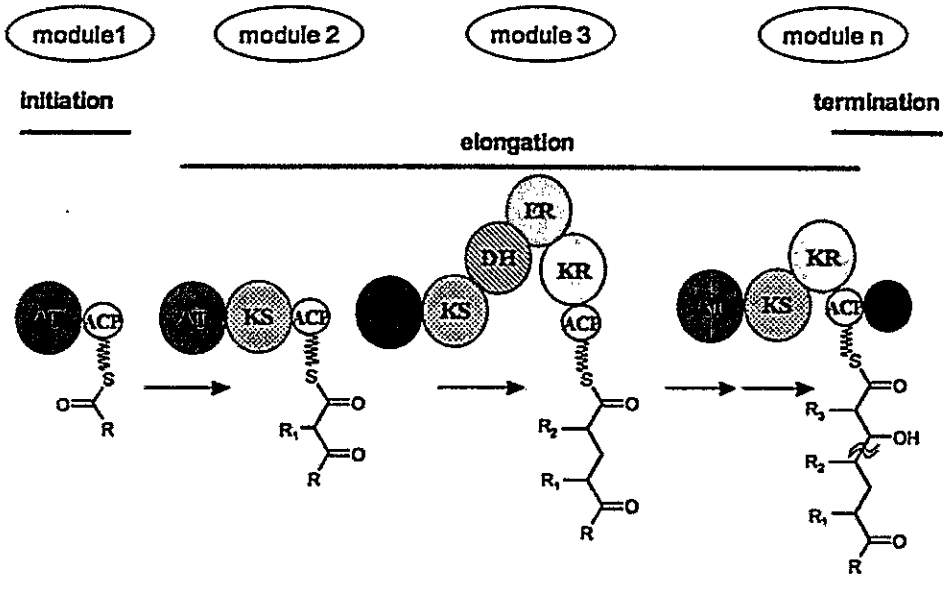


Figure 2: Basic steps during synthesis of polyketides (Donadio, Monciardini & Sosio, 2007).

NRPS systems are large, multifunctional enzyme complexes that build chains from individually selected building blocks. NRPS enzymes are also organized into modules, each of which is responsible for one cycle of elongation by incorporation of a single amino acid into the growing peptide chain. Each elongation module contains three essential domains. First, the adenylation domain (A) selects a specific amino acid and activates it as an amino acyl adenylate. Then, the activated amino acid is transferred to the swinging arm of the peptidyl carrier protein (PCP) domain. Finally, the condensation (C) domain catalyses the peptide bond formation between amino acids in adjacent modules. The chain is successively elongated and released by the action of an integrated TE domain or by a separate TE generating either a linear or a cyclic peptide (**Figure 3**). Additional structural diversity is accomplished by auxiliary domains such as epimerization (E), *N*-methylation (MT), cyclization (Cy), oxidoreductase (Ox), *N*-formylation (F) and reductase (R) domains. Compounds synthesized by NRPSs can be distinguished by the presence of non-proteinogenic, branched and D-amino acids and are often cyclic in structure (Salomon, Magarvey & Sherman, 2004; Fischbach & Walsh, 2006; Donadio, Monciardini & Sosio, 2007).

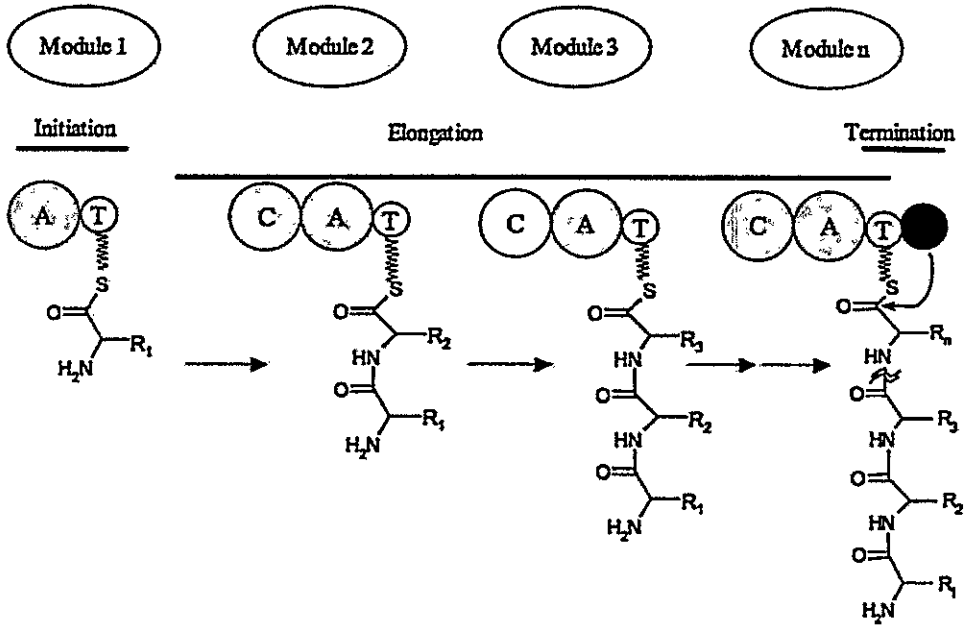


Figure 3: Basic steps during non-ribosomal synthesis of peptides (Donadio, Monciardini & Sosio, 2007).

Due to the structural organization of these modular enzymatic systems, the chemical structure of these molecules can be determined by the order of genes within the operon (Li *et al.*, 2009). This is known as the *colinearity rule* goberned by the *Stachelhaus code*, which is the basis for the *in silico* prediction of the substrates that bind to each enzymatic module (Stachelhaus, Mootz & Marahiel, 1999). Therefore, the final product of each BGC may be predicted, and it is possible to have insights into their biological activities, when compared to online databases. Predictions can be made only with PKS type I and NRPS systems, due to the linearity of their assemblies (Donadio, Monciardini & Sosio, 2007).

2.2.5 Genome mining for BGCs in *phylum Actinobacteria*

Recently, there has been a burst in applying whole-genome sequencing methodologies into the search for novel NP. This has played a fundamental role opening new strategies for unrevealing the mechanisms of secondary metabolite biosynthesis, especially in actinomycetes, mainly due to the complexity and time-consuming traditional approaches that still cannot cover their BGCs as a whole. In several *Streptomyces* strains, genomics has helped to gain insights into their complex physiological and metabolic versatility. *Streptomyces* have linear chromosomes (Hopwood, 1999); and their genome sizes are among the largest genomes found within the microbial world (Weber *et al.*, 2003). The number of predicted coding sequences is positively correlated with genome size (Ohnishi *et al.*, 2008), and approximately nearly 5% of their genomes are devoted to the synthesis of secondary metabolites (Ikeda *et al.*, 2003). The ability to produce a wide variety of bioactive molecules is based on the fact that they contain the largest numbers of BGCs, and particularly a high number of PKS and NRPS systems (Challis, 2008). Due to these interesting properties, nearly 700 species and 30,000 strains of *Streptomyces* have been identified (Euzéby, 2011). Recently, the complete genomes of 19 species have been sequenced, and more than 125 draft genomes are available in GenBank database (Harrison & Studholme, 2014). Genome mining has become a powerful tool to unveil the biotechnological potential of *Streptomyces* species, where BGCs can be identified through the AntiSMASH platform (Weber *et al.*, 2015) and even the chemical structure of the core molecules can be predicted. Information about BGCs, pathways and its metabolites are gathered together in the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) database which is suitable for comparison purposes (Medema *et al.*, 2015). Genome mining has positioned as a fundamental bioinformatic-approach

in the NP field towards drug discovery (Challis, 2008; Jensen *et al.*, 2013; Doroghazi & Metcalf, 2013; Antoraz *et al.*, 2015; Tang *et al.*, 2015; Katz & Baltz, 2016).

2.2.6 Metabolites from *phylum Actinobacteria*

Recent research focusing on marine actinomycetes have yielded numerous novel biologically active compounds, and more importantly, a surprisingly much more improved rate of discovery of new compounds in contrast to their terrestrial counterparts (Bernan, Greenstein & Maiese, 1997). Perhaps the most well-known example among actinomycetes is the marine *Salinispora pacifica* that has been shown to produce at least four novel polyketides: salinipyrones A and B, and the pacificanones A and B (Oh *et al.*, 2008). *Salinispora* was the first marine-obligate genus discovered (Maldonado *et al.*, 2005), due to its specific requirement of ionic sodium for growth (Mincer *et al.*, 2002). So far, three species have been described, *S. tropica*, *S. arenicola* and *S. pacifica*. It has been demonstrated that these species harbour species-specific genomic islands enriched in genes associated with secondary metabolite biosynthesis. Although they are closely related, different metabolic products among them are produced, thus linking secondary metabolism to ecological diversification (Penn *et al.*, 2009). The metabolite salinosporamide A obtained from *S. tropica* (Udwary *et al.*, 2007) is an active cytotoxic inhibitor of the 20S proteasome causing cell apoptosis (Feling *et al.*, 2003). Biosynthesis of salinosporamide A involves a PKS type I-catalyzed condensation of an acetate starter unit with an unusual extender, a chloroethylmalonyl-CoA. The Cl atom is crucial for the proteasome-inhibiting activity (Eustáquio *et al.*, 2009). Currently, salinosporamide A is under phase I of clinical trial as a potent antitumoral drug (Fenical *et al.*, 2009). Genome mining of *S. tropica* has led to the identification of at least 19 BGCs for secondary metabolites (Udwary *et al.*, 2007; Penn *et al.*, 2009).

Several reports have highlighted the importance of culturing marine actinomycetes as prolific sources of NPs, with the subsequent discovery of novel bioactive compounds (Zotchev, 2012). Examples of metabolites produced by marine actinomycetes involve the dermacozines, a new structural class of phenazine derivatives obtained from *Dermacoccus abyssi* sp. nov. isolated from a Mariana Trench sediment at a depth of 10,898 m (Pathom-aree *et al.*, 2006b; Abdel-Mageed *et al.*, 2010) and the antibiotic kocurin active against methicillin-resistant *Staphylococcus aureus* (MRSA), which is a thiazolyl peptide obtained from sponge-derived *Kocuria* and *Micrococcus* isolates (Palomo *et al.*, 2013). Also, different marine *Streptomyces* species have been demonstrated to produce a diverse myriad of novel compounds. Examples include the polycyclic tetramic acid macrolactam ikatugamycin, which showed antifungal and antibacterial activity and was isolated from an off-shore sediment nearby Utonde, in Equatorial Guinea (Lacret *et al.*, 2015); the novel champacyclin, an NRPS-octapeptide isolated from marine sediments of the Baltic Sea (Pesic *et al.*, 2013); the new antibiotic anthracimycin, isolated from Santa Barbara marine sediments in California, which have showed a significant activity towards *Bacillus anthracis* (Jang *et al.*, 2013); and the novel alkaloid xinghaiamine A, with broad spectrum antibacterial and cytotoxic activities which is a sulfoxide-containing compound never seen before in microorganisms (Jiao *et al.*, 2013). Interestingly, there are additional unique functional groups solely found in marine natural products (Piel *et al.*, 2000; Engelhardt *et al.*, 2010; Li *et al.*, 2011; Jiao *et al.*, 2013). Additionally, incorporation of halogenated groups is more abundant in MNP, which is in part due to the fact that marine environments are especially rich in chlorine and bromine elements (Zhang *et al.*, 2005).

To date, diverse antibiotics currently still used in clinic have been identified to be synthesized by PKS and NRPS metabolic routes from soil actinomycetes. The erythromycin, is a

PK produced by *Streptomyces griseus* (Staunton & Wilkinson, 1997) and the tetracyclin is a PK produced by *Streptomyces rimosus* (Chopra & Roberts, 2001). Vancomycin is a NRP produced by *Nocardia orientalis* (Reynolds, 1989). Diverse studies in marine-derived actinomycetes demonstrate that they represent rich sources for novel bioactive compounds with many therapeutic applications, where NRPS and PKS routes are fundamental to contribute to chemical diversity and biological specificity (Goodfellow & Fiedler, 2010; Subramani & Aalbersberg, 2012; Antoraz *et al.*, 2015; Katz & Baltz, 2016)

A combinatorial strategy to exploit the ability of marine actinomycetes to produce bioactive metabolites was pursued. This Ph.D. study involves isolation of marine actinomycetes from an underexplored environment and the screening of their antimicrobial activities, with the aim to perform genome sequencing to selected isolates in order to find the metabolic routes; and the subsequent analytical detection and identification of one compound produced by a particular *Streptomyces* strain (**Figure 4**).

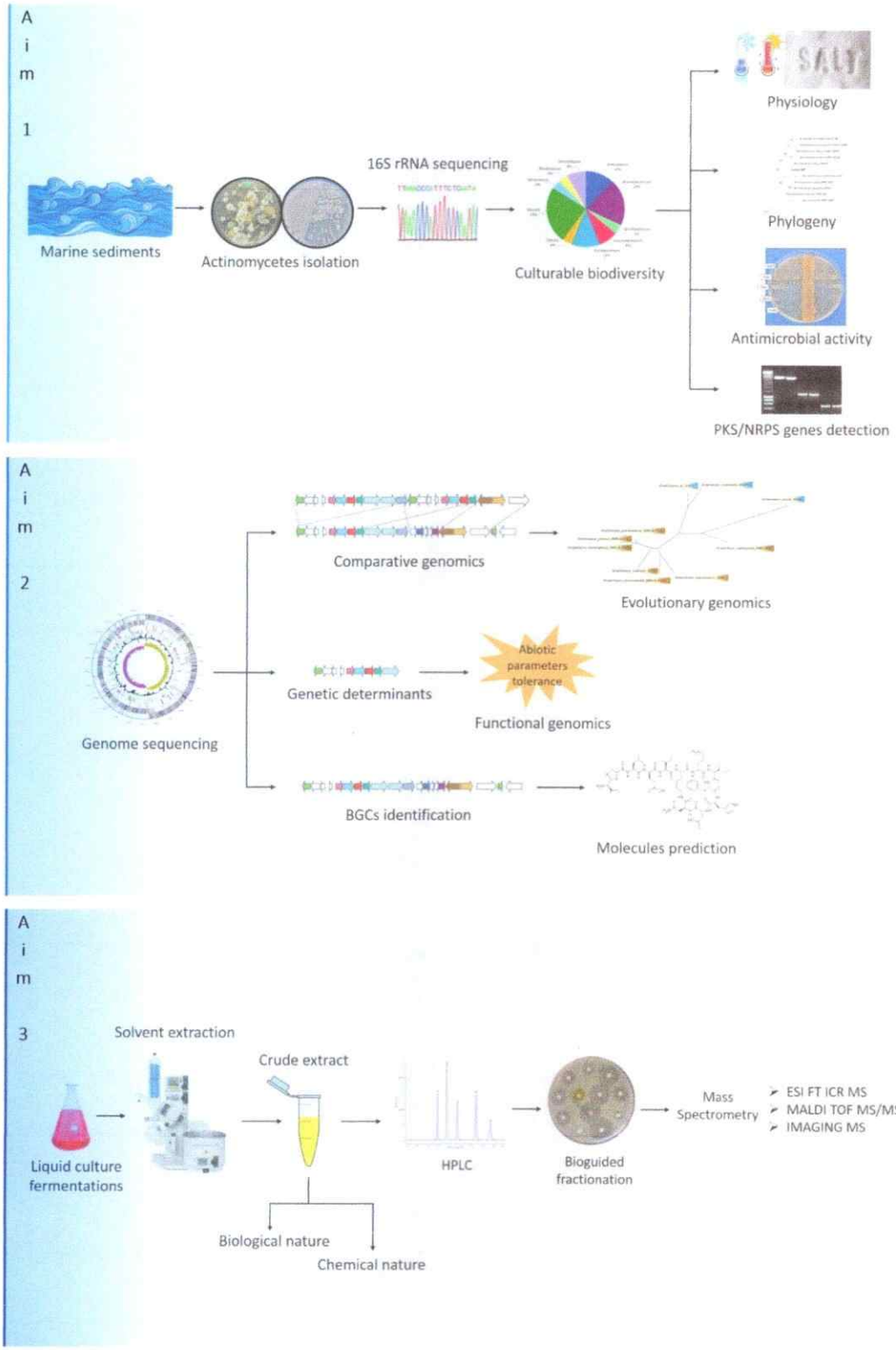


Figure 4: Overall bioprospection strategy to access the potential of marine actinomycetes to produce antimicrobials compounds.

3 Hypothesis

- Actinomycetes with antimicrobial activity isolated from marine sediments from Comau Fjord harbour novel biosynthetic gene clusters of non-ribosomal peptides (NRP) which synthetize bioactive secondary metabolites.

4 Aims

4.1 Principal Aim

Identification and characterization of biosynthetic gene clusters involved in the synthesis of non-ribosomal peptides (NRP) secondary metabolites in actinomycetes with antimicrobial activity isolated from marine sediments of Comau Fjord.

4.2 Specific Aims

4.2.1 Isolation and characterization of actinomycetes from marine sediments of Comau fjord and evaluation of their antimicrobial activity.

4.2.2 Determination of the presence of genes encoding for PKS and NRPS and identification of PKS and NRPS biosynthetic clusters in actinomycetes that produce antimicrobial metabolites.

4.2.3 Characterization of antimicrobial metabolites produced by actinomycetes that have PKS and NRPS biosynthetic gene clusters and bioinformatic prediction of their structure.

5 Results

5.1 Aim 1: Isolation and characterization of actinomycetes from marine sediments of Comau fjord and evaluation of their antimicrobial activity

Results involving this aim are part of a publication that will be presented as Chapter I.

Chapter I: Exploring the diversity and antimicrobial potential of marine *Actinobacteria* from the Comau fjord in northern Patagonia, Chile. Undabarrena A., Beltrametti F., Claverías F., González M., Moore E.R.B., Seeger M. & Cámara B. (2016) *Frontiers in Microbiology* 7:1135. DOI: 10.3389/fmicb.2016.01135.

5.2 Aim 2: Determination of the presence genes encoding for PKS and NRPS and identification of their biosynthetic gene clusters in actinomycetes that produce antimicrobial metabolites

Results involving this aim are part of two publications that will be presented as Chapter II and Chapter III, respectively.

Chapter II: Genome sequence of *Streptomyces* sp. H-KF8, a marine actinobacteria isolated from a Northern Chilean Patagonian fjord. Undabarrena A., Ugalde J.A., Castro-Nallar E., Seeger M. & Cámara B. (2017) *Genome Announcements* 5:e01645-16. DOI: 10.1128/genomeA.01645-16.

Chapter III: Genomic data mining of the marine actinobacteria *Streptomyces* sp. H-KF8 unveils insights into multi-stress related genes and metabolic pathways involved in antimicrobial synthesis. Undabarrena A., Ugalde J.A., Seeger M. & Cámara B. (2017) *PeerJ* 5:e2912. DOI: 10.7717/peerj.2912.

5.3 Aim 3: Characterization of antimicrobial metabolites produced by actinomycetes that harbour PKS and NRPS biosynthetic clusters and bioinformatic prediction of their structure

Results involving this aim will be presented in this thesis as Chapter IV.

Chapter IV: Chemical detection of antimicrobial compounds in *Streptomyces* sp. H-KF8: unveiling their connection between its NRPS biosynthetic gene clusters.

Chapter I: Exploring the diversity and antimicrobial potential of marine *Actinobacteria* from the Comau fjord in Northern Patagonia, Chile

frontiers in Microbiology

ORIGINAL RESEARCH
published: 19 July 2016
doi: 10.3389/fmicb.2016.01135

Exploring the Diversity and Antimicrobial Potential of Marine Actinobacteria from the Comau Fjord in Northern Patagonia, Chile

*Agustina Undabarrena[1], Fabrizio Beltrametti[2], Fernanda P. Claverías[1], Myriam González[1], Edward R. B. Moore[3,4], Michael Seeger[1] and Beatriz Cámara[1]**

[1] Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Departamento de Química & Centro de Biotecnología Daniel Alkalay Lowitt, Universidad Técnica Federico Santa María, Valparaíso, Chile, [2] Actygea S.r.l., Gerenzano, Italy, [3] Culture Collection University of Gothenburg (CCUG), Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [4] Department of Infectious Diseases, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden*

OPEN ACCESS

Edited by:
Learn-Han Lee, Monash University Malaysia Campus, Malaysia

Reviewed by:
Atte Von Wright, University of Eastern Finland, Finland
Polpass Arul Jose, Central Salt and Marine Chemicals Research Institute, India

***Correspondence:**
Beatriz Cámara
beatriz.camara@usm.cl

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *23 May 2016*
Accepted: *07 July 2016*
Published: *19 July 2016*

Citation:
Undabarrena A, Beltrametti F, Claverías FP, González M, Moore ERB, Seeger M and Cámara B (2016) Exploring the Diversity and Antimicrobial Potential of Marine Actinobacteria from the Comau Fjord in Northern Patagonia, Chile. Front. Microbiol. 7:1135. doi: 10.3389/fmicb.2016.01135

Bioprospecting natural products in marine bacteria from fjord environments are attractive due to their unique geographical features. Although, *Actinobacteria* are well known for producing a myriad of bioactive compounds, investigations regarding fjord-derived marine *Actinobacteria* are scarce. In this study, the diversity and biotechnological potential of *Actinobacteria* isolated from marine sediments within the Comau fjord, in Northern Chilean Patagonia, were assessed by culture-based approaches. The 16S rRNA gene sequences revealed that members phylogenetically related to the *Micrococcaceae*, *Dermabacteraceae, Brevibacteriaceae, Corynebacteriaceae, Microbacteriaceae*, *Dietziaceae*, *Nocardiaceae,* and *Streptomycetaceae* families were present at the Comau fjord. A high diversity of cultivable *Actinobacteria* (10 genera) was retrieved by using only five different isolation media. Four isolates belonging to *Arthrobacter*, *Brevibacterium*, *Corynebacterium* and *Kocuria* genera showed 16S rRNA gene identity <98.7% suggesting that they are novel species. Physiological features such as salt tolerance, artificial sea water requirement, growth temperature, pigmentation and antimicrobial activity were evaluated. *Arthrobacter*, *Brachybacterium*, *Curtobacterium*, *Rhodococcus,* and *Streptomyces* isolates showed strong inhibition against both Gram-negative *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella enterica* and Gram-positive *Staphylococcus aureus*, *Listeria monocytogenes*. Antimicrobial activities in *Brachybacterium*, *Curtobacterium,* and *Rhodococcus* have been scarcely reported, suggesting that non-mycelial strains are a suitable source of bioactive compounds. In addition, all strains bear at least one of the biosynthetic genes coding for NRPS (91%), PKS I (18%), and PKS II (73%). Our results indicate that the Comau fjord is a promising source of novel *Actinobacteria* with biotechnological potential for producing biologically active compounds.

Keywords: cultivable actinobacteria, antimicrobial activity, Comau fjord, marine sediments, Northern Patagonia

INTRODUCTION

The increased prevalence of multi-drug resistance pathogens along with the rapid development of cross resistances with new antibiotics is the driving force in the identification and production of novel therapeutic agents (Livermore, 2009). All classes of antibiotics have seen emergence of resistance compromising their use; hence there is an urgent need for new bioactive compounds (Genilloud, 2014). The traditional approach consisting of isolation and cultivation of new microorganisms of underexplored habitats is still rewarding (Axenov-Gribanov et al., 2016), and has brought to the identification, production and commercialization of most of the antibiotics (Newman and Cragg, 2012). Despite the chemically synthetic efforts, natural environments are still the main source for the discovery of novel antibiotics (Fenical and Jensen, 2006; Bull and Stach, 2007). Although, the diversity of life in terrestrial environments is well reported, the highest biodiversity is in the world's oceans (Donia and Hamann, 2003). Oceans are strongly complex habitats in terms of pressure, salinity and temperature variations (Fenical, 1993), therefore marine microorganisms have to develop physiological traits including chemically complex biosynthesized metabolites to ensure their survival in this highly dynamic habitat. Research has taken advantage from these unique molecules to discover novel bioactive compounds with antibacterial, antifungal and/or antitumor properties, and apply them in current clinical challenges (Gulder and Moore, 2010).

In this scenario, bacteria from the *phylum Actinobacteria* are a prominent source of biologically active natural compounds, since they are well known for their capacity to biosynthesize versatile secondary metabolites (Katz and Baltz, 2016). Actinobacteria are one of the major phyla of the domain *Bacteria* (Goodfellow and Fiedler, 2010). It encompasses high GC-content Gram-positive bacteria that includes 17 orders (Gao and Gupta, 2005; Sen et al., 2014). Surprisingly, the class *Actinobacteria* contains both the most deadly bacterial pathogen (i.e., *Mycobacterium* genus) and the microorganisms that are the most important for antibiotic production (i.e., *Streptomyces* genus) (Doroghazi and Metcalf, 2013). *Streptomyces* are responsible for two-thirds of all known antibiotics. In addition, several other important biologically-active compounds have been found, including antitumoral, antifungal, herbicidal, and antiparasitic compounds (Bérdy, 2005). Due to the extensive sampling of soil *Streptomyces*, the rate of discovery of novel metabolites is decreasing (Fenical, 1993), which is the reason why bioprospecting efforts are currently being developed in marine underexplored ecosystems.

Marine environments are an established ecological niche for actinobacteria (Das et al., 2006; Ward and Bora, 2006). Cultivable actinobacteria from marine habitats have been characterized from mangrove forests (Hong et al., 2009; Baskaran et al., 2011; Lee et al., 2014a,b; Ser et al., 2015, 2016), marine sponges (Kim et al., 2005; Montalvo et al., 2005; Zhang et al., 2006; Jiang et al., 2007; Sun et al., 2015), corals (Hodges et al., 2012; Kuang et al., 2015; Mahmoud and Kalendar, 2016; Pham et al., 2016), sea cucumbers (Kurahashi et al., 2010), pufferfishes (Wu et al., 2005), and seaweed (Lee et al., 2008). Notably, actinobacteria are predominant in marine sediments (Mincer et al., 2002; Magarvey et al., 2004; Jensen et al., 2005; Bredholdt et al., 2007; Gontang et al., 2007; León et al., 2007; Maldonado et al., 2008; Duncan et al., 2014; Yuan et al., 2014) and also in deep sea sediments (Colquhoun et al., 1998; Pathom-Aree et al., 2006). Marine actinobacteria have been described as an emerging source for novel bioactive molecules (Lam, 2006; Joint et al., 2010; Subramani and Aalbersberg, 2012; Zotchev, 2012). The majority of these secondary metabolites are produced by polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) metabolic pathways (Salomon et al., 2004). Notably, it is reported that actinobacteria have a higher number of these biosynthetic genes (Donadio et al., 2007).

The extensive coast of Chile is a promising biome to explore marine actinobacterial communities, and in this context, the bioprospecting of sediments of a marine protected area, the Comau fjord, in the Chilean Northern Patagonia was proposed. The Comau fjord is a pristine area unique by its geological nature. It is comparatively smaller than other fjords in Chile, and also one of the deepest (Ugalde et al., 2013); characterized by steep slopes, with surrounding mountains that have a height of up to 2000 m with a dense extratropical rainforest covering from the sea to the top (Lagger et al., 2009). The aim of this study was to isolate marine actinobacteria from this unique ecosystem. The cultivable diversity of actinobacterial strains along with their environmental adaptation traits was investigated, and their ability to produce antibacterial activity against model strains was assessed.

MATERIALS AND METHODS

Environmental Samples

Sampling was performed in the Marine Protected Area of Huinay in January 2013, located in the Commune of Hualaihué, in the Los Lagos Region, Chile. Samples were collected from marine sediments within the Comau Fjord in the Northern Patagonia. Four different coastal locations were sampled in front of Lilihuapi Island (42°20, 634′S; 72°27, 429′W), Tambor Waterfall (42°24, 161′S; 72°25, 235′W), Punta Llonco (42°22, 32′S; 72°25, 4′W), and in front of Lloncochaigua River mouth (42°22, 37′S; 72°27, 25′W) (**Figure 1**). Underwater samples were collected by Huinay Scientific Field Station scuba divers, dispensing samples directly from marine sediments into sterile 50 mL tubes. Marine sediments were taken from subtidal zones at different depths, ranging from 0.25 to 26.2 m. Salinity was measured at each sampling site, and ranged from 5 $\mu g\ L^{-1}$ in the coast in front of Lloncochaigua River mouth, where there is a meaningful input of fresh water, to 31 $\mu g\ L^{-1}$ in the coast of Lilihuapi Island, located further away from continental land. Samples were maintained on ice until transported to the laboratory, where they were stored at 4°C.

Isolation of Actinobacteria

Samples were both plated directly or serially diluted (10^{-4} and 10^{-6}) before plating on selective media for the isolation of actinobacteria. Five selective media were used as previously reported (Claverías et al., 2015): M1 Agar (Mincer et al., 2002), ISP2 and NaST21Cx Agar (Magarvey et al., 2004),

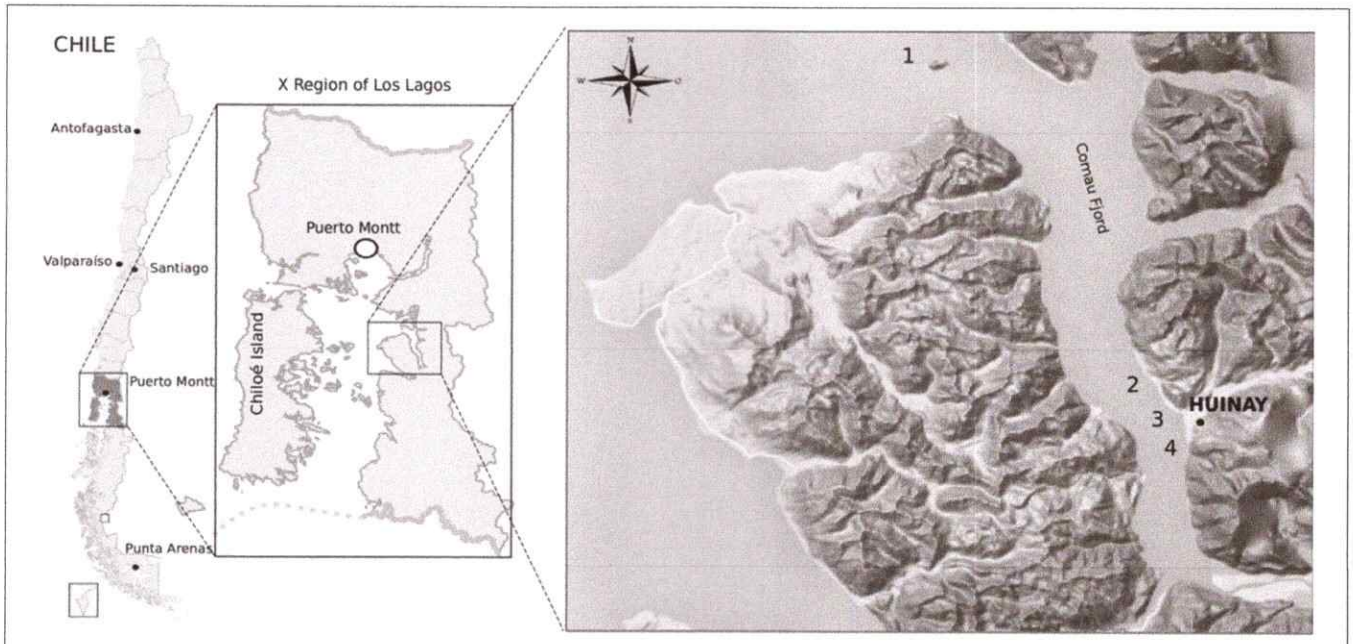


FIGURE 1 | Geography of sampling sites for actinobacteria isolation from the Comau fjord in Northern Patagonia, Chile. Map of sampling locations within the Comau fjord (Los Lagos Region). Numbers indicate the sites where marine sediments were collected at the coast close to: Lilihuapi Island (1), Punta Lloncо (2), Lloncochaigua River mouth (3), and Tambor Waterfall (4). Black dot indicates location of the Huinay Scientific Field Station.

R2A Agar (Difco), and Marine Agar (MA) 2216 (Difco). All media were amended with nalidixic acid (25 $\mu g\ mL^{-1}$), as an inhibitor of primarily fast-growing Gram-negative bacteria, and cycloheximide (100 $\mu g\ mL^{-1}$) for fungi inhibition [28]. All media with the exception of Marine Agar, were prepared with artificial sea water (ASW) (Kester et al., 1967). The agar media cultures were incubated at 30°C until visible colonies were observed, up to 1–2 months. For isolation purposes, colonies were individually streaked out onto Tryptic Soy Agar medium (TSA) prepared with ASW (TSA-ASW) and eventually transferred on new plates until pure cultures were obtained. Isolated bacteria were stored at −20 and −80°C, in 20% glycerol, TSB medium and ASW for maintenance.

Detection and Identification of Actinobacteria

A PCR-assay was conducted as a screening method for detecting actinobacterial strains among the isolates with primers targeting the V3–V5 regions of the 16S rRNA gene of actinobacteria (S-C-Act-0235-a-S-20 and S-C-Act-0878-A-19) (Stach et al., 2003). DNA extractions were performed, using a lysis method by culture boiling suspensions of bacterial cells (Moore et al., 2004). Each PCR reaction contained 1 μL of genomic DNA, 12.5 μL of GoTaq Green Master Mix (Promega) and 0.6 μM of each primer in a final reaction volume of 25 μL. The reaction started with an initial denaturation, at 95°C for 5 min, followed by 35 cycles of DNA denaturation, at 95°C for 1 min, primer-annealing, at 70°C for 1 min and extension cycle, at 72°C for 1.5 min, with a final extension at 72°C for 10 min (Claverías et al., 2015). PCR-amplicons were visualized in 2% agarose gel electrophoresis and subsequently revealed with SYBR Green staining (E-gel, Invitrogen).

Positive isolates were selected for 16S rRNA gene amplification, using universal primers 27F and 1492R (Lane, 1991). The reaction mix (50 μL) contained 1 μL of genomic DNA, 25 μL of GoTaq Green Master Mix (Promega) and 0.2 μM of each primer. The reaction started with an initial DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, primer-annealing at 55°C for 1 min and primer-extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. PCR products were sent to Macrogen Inc. (Seoul, Korea) for purification and sequencing using the conserved universal primer 800R. Retrieved sequences were manually edited and BLAST nucleotide analyses were performed with the National Center for Biotechnology Information server (NCBI) and actinobacteria were initially identified up to the genus level.

Antimicrobial Activity Tests

Bioprospecting for antimicrobial activity was initially performed using the cross-streak method as described (Haber and Ilan, 2014), with slight modifications (Claverías et al., 2015). Fresh cultures of the isolated actinobacterial strains were inoculated as a line in the middle of an agar medium plate and incubated at 30°C until notable growth was observed (7 days for mycelial strains and 5 days for non-mycelial strains). Strains were grown on TSA-ASW and ISP2-ASW media. Five reference bacteria were the target of inhibition tests: *Staphylococcus aureus* NBRC 100910^T (STAU); *Listeria monocytogenes* 07PF0776 (LIMO); *Salmonella enterica* subsp enterica $LT2^T$ (SAEN); *Escherichia*

coli FAP1 (ESCO) and *Pseudomonas aeruginosa* DSM50071^T (PSAU). Cultures were incubated at 37°C overnight and inhibition zones were ranked qualitatively as: −, no inhibition; +/−, attenuated growth of test strain in the area closest to the actinobacterial line; +, <50% growth inhibition (less than half of the bacterial line was inhibited); ++, 50% growth inhibition (half of the bacterial line was inhibited); + + +, >50% growth inhibition (more than half of the bacterial line was inhibited). All experiments were performed in duplicate, using an internal control with one of the reference strains.

Further antimicrobial tests were performed with selected isolates *Streptomyces* sp. H-KF8, *Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Kocuria* sp. H-KB5 and *Rhodococcus* sp. H-CA8f. Strains were grown in a 50 mL liquid culture in ISP2-ASW medium for 10 days for non-mycelial strains and 15 days for the mycelial strain, with continuous shaking at 30°C. Crude extracts were obtained after solvent extraction using hexane, methanol and ethyl acetate in a 1:1 ratio (v/v) for two times. Evaporation of solvent was performed with speed vacuum, and extract was dissolved in 10% dimethyl sulphoxide (DMSO) until a final concentration of 5 mg mL^{-1}. Antimicrobial assays were evaluated using 10 µL of each extract, over LB agar plates spread with the bacterial test strains STAU, PSAU, SAEN, and ESCO. Plates were incubated overnight at 37°C and inhibitions zones were checked. ISP2 medium and 10% DMSO were used as negative controls.

Detection of PKS and NRPS Biosynthetic Genes

Amplification of biosynthetic genes was carried out by PCR, using degenerate primers targeting the ketosynthase domain in PKS type I with primers KS-F (5′CCSCAGSAGCGCSTS YTSCTSGA3′) and KS-R (5′GTSCCSGTSCCGTGSGYSTCSA3′) (Gontang et al., 2010); and PKS type II with primers KSα (5′TSGRCTACRTCAACGGSCACGG3′) and KSβ (5′TACSAG TCS WTCGCCTGGTTC3′) (Ayuso et al., 2005). The adenylation domain in NRPS systems was detected with primers A3F (5′GCSTACSYSATSTACACSTCSGG3′) and A7R (5′SASGTCV CCSGTSCGGTAS3′) (Ayuso-Sacido and Genilloud, 2005). PCR programs were performed as previously described (Ayuso et al., 2005; Ayuso-Sacido and Genilloud, 2005; Gontang et al., 2010). Products were visualized in 1% agarose gels electrophoresis, and stained with GelRed (Biotium). *Streptomyces violeaceoruber* DSM 40783 was used as a control for all PCR reactions. Detection was determined as +, if the amplicon was located at the expected size (700 bp for PKS type I; 800–900 bp for PKS type II and 700–800 bp for NRPS); and −, if amplicon was absent or it was present at any other size.

Phylogenetic Analysis

Representative strains for each genus identified from partial 16S rRNA gene sequence analyses were selected for the nearly-complete sequencing of this gene, as previously described (Claverías et al., 2015). PCR products were quantified and sent to Macrogen Inc. (Seoul, Korea) for purification and sequencing, using primers 27F, 518F, 800R, and 1492R. Manual sequence edition, alignment, and contig assembling were performed using Vector NTI v10 software package (Invitrogen). Sequence contigs were analyzed performing BLAST with NCBI to determine the closest type strain match using the 16S ribosomal RNA sequence of Bacteria and Archaea database. The Neighbor-Joining algorithm (Saitou and Nei, 1987) using MEGA software version 6.0 (Tamura et al., 2013) with bootstrap values based on 1000 replications (Felsenstein, 1985) was used to construct a phylogenetic tree based on the V1-V9 region of the 16S rRNA gene sequences. The 16S rRNA gene sequences were deposited in GenBank under the following accession numbers: *Arthrobacter* sp. H-JH3 (KT799841); *Brachybacterium* sp. H-CG1 (KT799842); *Brevibacterium* sp. H-BE7 (KT799843); *Corynebacterium* sp. H-EH3 (KT799844); *Curtobacterium* sp. H-ED12 (KT799845); *Kocuria* sp. H-KB5 (KT799846); *Dietzia* sp. H-KA4 (KT799847); *Micrococcus* sp. H-CD9b (KT799848); *Rhodococcus* sp. H-CA8f (KT799849); *Streptomyces* sp. H-KF8 (KT799850) and *Streptomyces* sp. H-CB3 (KT799851).

Phenotypic Characterization of Actinobacterial Strains

For the morphological and physiological characterization of the representative strains, colony pigmentation, spore formation, growth temperatures, ASW requirement and NaCl tolerance were evaluated. Optimal colony pigmentation was observed on TSA-ASW after a 3-month incubation at 4°C. To establish the effects of temperature on growth, 10 µL of actinobacterial cultures were streaked onto TSA-ASW plates, and incubated at 4, 20, 30, 37, and 45°C. For NaCl tolerance, LB agar with 0, 1, 3.5, 5.0, 7.0, 10, and 20% (w/v) NaCl was prepared. As described previously, 10 µL of the actinobacterial cultures were streaked onto LB agar plates and incubated at 30°C. To detect the requirement of seawater on growth, ISP2 was prepared as follows: medium with Milli-Q H_2O; medium with ASW; and medium with Milli-Q H_2O supplemented with 3.5% (w/v) NaCl (equivalent to ASW NaCl concentration). Incubation times were from 10 days (for non-mycelial strains) to 14 days (for mycelial stains) at 30°C. The reference time for growth was that on which growth was observed on control plates. Results were interpreted as: +, if the strain tested was able to grow on medium-ASW but did not grow on medium/Milli-Q H_2O and on medium/Milli-Q H_2O supplemented with 3.5% NaCl; and −, if the strain tested was able to grow on all three media.

Resistance to Model Antibiotics

Representative strains of each genus were grown to exponential phase (turbidity at 600 nm of 0.3) and plated on Mueller-Hinton agar plates for antibiotic susceptibility testing. Antibiotic discs for Gram-positive bacteria (Valtek) were placed above and inhibition grown zones as diameters were measured and compared with values obtained from the Clinical and Laboratory Standards Institute (CLSI) from year 2016 to determine susceptibility (S), or resistance (R) of each antibiotic tested.

RESULTS

Isolation and Identification of Actinobacteria

Eleven marine sediment samples were collected from four different sites in Comau fjord, Northern Patagonia, Chile

(**Figure 1**). Altogether 25 marine actinobacteria were isolated. Their distribution according to the sampling site was: 40% from Lilihuapi island coast, 28% from Punta Llonco, and 16% from Loncohaigua river mouth and Tambor waterfall, each. The majority (80%) of the isolates were from sediments situated approximately 10 m deep. Only occasional isolates were obtained from deeper sediments or from the shallow locations. The *Actinobacteria* isolated belong to three suborders: *Streptomycineae*, *Micrococcineae,* and *Corynebcaterineae*; comprising eight different families. Relative abundances of the strains according to the genera isolated (**Figure 2A**) indicated that most abundant genera were *Kocuria* and *Brachybacterium*. The selective media had a major influence on the number of isolates obtained (**Figure 2B**). M1-ASW medium was the most effective regarding the number and diversity of isolates recovered. Interestingly, strains of *Brachybacterium*, *Brevibacterium*, *Micrococcus,* and *Rhodococcus* genera were isolated exclusively with this medium (**Figure 2B**).

Antimicrobial Activity Assays

Our first approach was to screen all actinobacterial strains for antimicrobial activity, using the cross-streak method, against five reference strains: STAU, LIMO, PSAU, SAEN, and ESCO (**Figure 3A**). Actinobacterial strains showed antimicrobial activity, presenting a broad spectrum of inhibition although with different inhibition patterns (**Table 1**). Inhibition of reference strains largely depended on the media where actinobacterial strains were cultivated, proving TSA-ASW to be generally better for antimicrobial activity than ISP2-ASW medium. *Arthrobacter*, *Brachybacterium, Curtobacterium,* and *Rhodococcus* isolates showed potent antimicrobial bioactivity to more than one target (**Table 1**). Regarding the Gram-negative bacteria tested, TSA-ASW-grown actinobacterial strains were able to inhibit ESCO (84%) and PSAU (24%); whereas ISP2-ASW-grown isolates inhibited up to 76 and 48%, respectively. Concerning the Gram-positive reference strains, 64% of the TSA-ASW-grown actinobacterial strains inhibited both LIMO and STAU; whereas ISP2-ASW-grown strains, 56% showed inhibition for LIMO and 36% for STAU (**Figure 3B**).

Notably, 67% of the antimicrobial activities observed with the cross-streak method were retrieved with various solvent extractions from actinobacterial liquid cultures (**Table 2**). Ethyl acetate was more effective in extracting active compounds, as crude extracts from *Rhodococcus* sp. H-CA8f, *Kocuria* sp. H-KB5 and *Brevibacterium* sp. H-BE7 presented antimicrobial activity. On the other hand, antimicrobial activity from *Arthrobacter* sp. H-JH3 was effectively extracted from the cell pellet using methanol. Crude extracts from *Rhodococcus* sp. H-CA8f showed an antimicrobial effect against all bacteria tested, confirming results obtained from the cross-streak method.

Detection of PKS and NRPS Biosynthetic Genes

The presence of biosynthetic PKS (type I and II) and NRPS genes were detected by PCR in representative actinobacterial isolates (**Table 3**). Interestingly, most isolates bear at least one biosynthetic gene of PKS or NRPS. Among them, NRPS was the predominant gene observed (91%), followed by PKS type II (73%). Only 18% of actinobacterial isolates showed the presence of PKS type I gene.

Phylogenetic Analysis

For phylogenetic analysis, the 16S rRNA gene was sequenced for selected actinobacterial isolates, representatives of each genus retrieved in sediment samples from Comau fjord. A dendogram of the estimated phylogenetic relationships is presented in **Figure 4** and the sequence similarities of selected actinobacterial strains to type strains of related species are given in **Table 3**. Four of the actinobacterial isolates are below the 98.7% sequence identity threshold and therefore may be potential candidates of new taxons. These isolates belong to *Arthrobacter* and *Kocuria* genera (*Micrococcaceae*

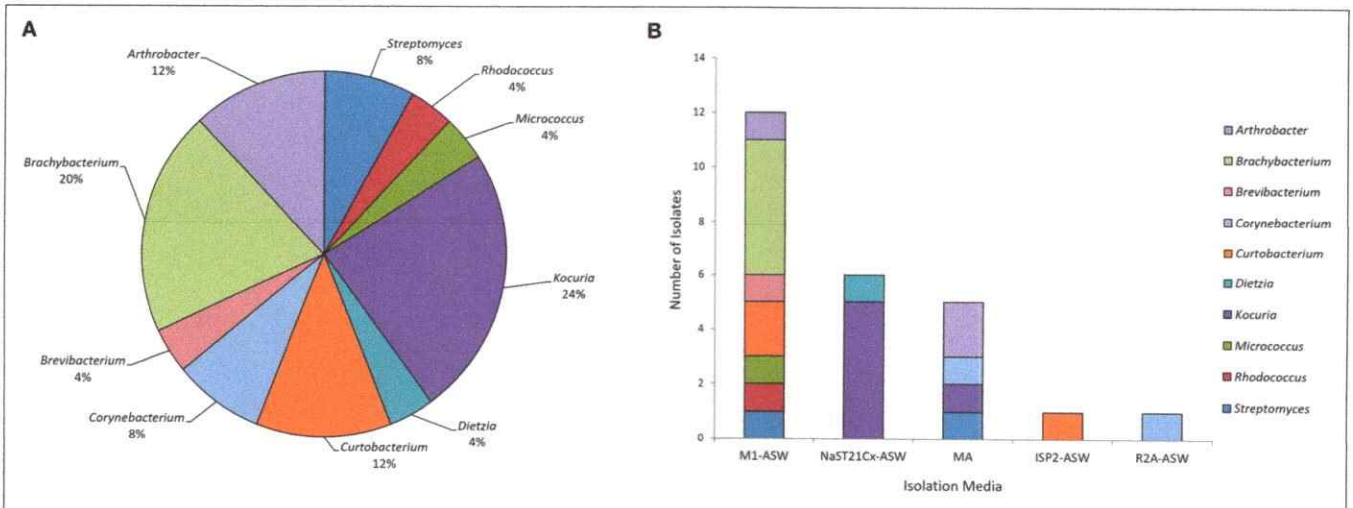


FIGURE 2 | Biodiversity of actinobacteria from the Comau fjord in Northern Patagonia. (A) Distribution of the relative abundance of the actinobacterial genera isolated. **(B)** Number of actinobacteria of various genera isolated using different culture media.

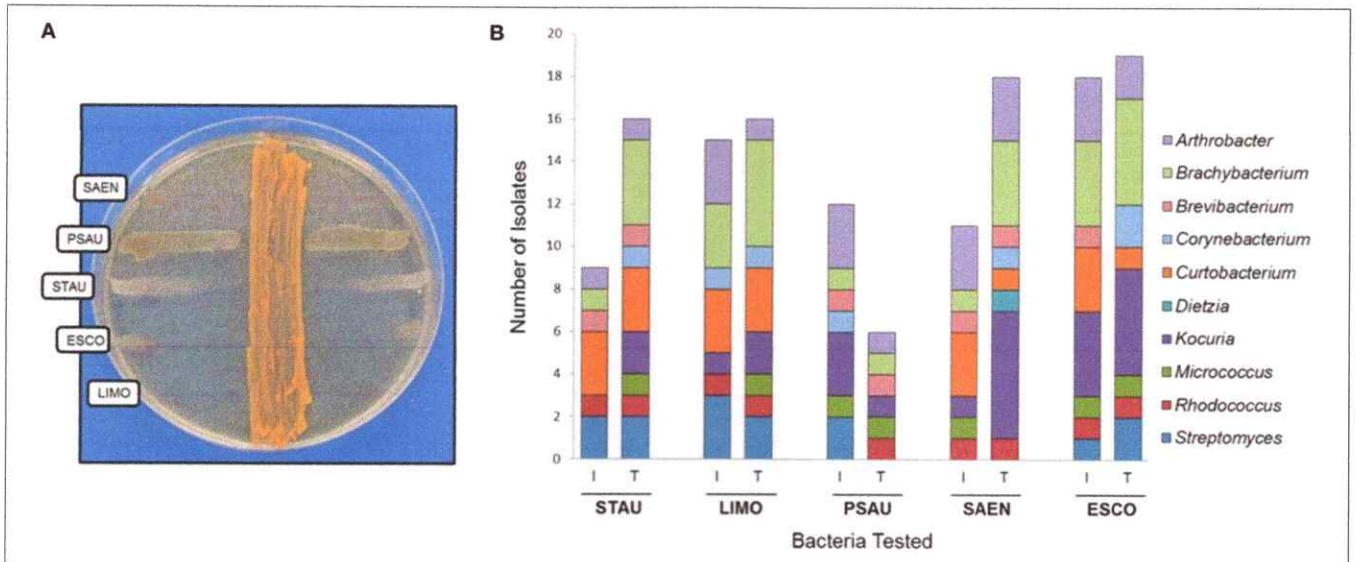


FIGURE 3 | Antimicrobial activity of actinobacterial strains from the Comau fjord in Northern Patagonia. (A) Cross-streak method of *Rhodococcus* sp. H-CA8f showing different patterns of inhibition zones with several model bacteria. **(B)** Antimicrobial activity of actinobacterial strains using the cross-streak method. STAU, *Staphylococcus aureus*; LIMO, *Listeria monocytogenes*; PSAU, *Pseudomonas aeruginosa*; SAEN, *Salmonella enterica*; ESCO, *Escherichia coli*. I, ISP2-ASW media; T, TSA-ASW media.

TABLE 1 | Antimicrobial activity of actinobacterial strains against model pathogens using the cross-streak method.

Strain	Genus	STAU		LIMO		PSAU		SAEN		ESCO	
		ISP2	TSA	ISP2	TSA	ISP2	TSA	ISP2	TSA	ISP2	TSA
H-CA8b	*Arthrobacter*	+/−	+/−	+++	+++	+++	+++	+/−	+/−	++	++
H-JH1	*Arthrobacter*	−	−	++	−	+	−	+/−	+/−	+	−
H-JH3	*Arthrobacter*	−	−	++	−	+	−	+/−	+/−	+	+/−
H-CA4	*Brachybacterium*	−	−	+++	+	−	++	−	+/−	+/−	+++
H-CD1	*Brachybacterium*	−	+/−	−	+++	−	−	−	+/−	+/−	+/−
H-CE9	*Brachybacterium*	−	+/−	+	++	−	−	+	+	++	+
H-CF1	*Brachybacterium*	++	++	+	++	−	−	−	+/−	−	+/−
H-CG1	*Brachybacterium*	−	+/−	−	+++	+/−	−	−	−	+/−	+/−
H-BE7	*Brevibacterium*	+	+/−	−	−	+	+/−	++	+	+	−
H-EH3	*Corynebacterium*	−	+/−	+/−	+++	−	−	−	−	−	+/−
H-KF5	*Corynebacterium*	−	−	−	−	+/−	−	−	+/−	−	+/−
H-BE10	*Curtobacterium*	+++	+++	+/−	+++	−	−	++	+++	+/−	++
H-CD9a	*Curtobacterium*	+	+	+/−	+	−	−	+/−	−	+/−	+/−
H-ED12	*Curtobacterium*	++	++	+	++	−	−	+/−	−	+/−	+/−
H-KA4	*Dietzia*	−	−	−	−	−	−	−	+/−	−	−
H-KA9	*Kocuria*	−	−	−	−	+/−	−	−	+/−	−	+/−
H-KA10	*Kocuria*	−	+/−	−	+/−	−	−	−	+++	+/−	+/−
H-KB1	*Kocuria*	−	−	−	−	−	−	−	+/−	+/−	+/−
H-KB5	*Kocuria*	−	−	−	−	+/−	−	−	+/−	+/−	−
H-KB6	*Kocuria*	−	+/−	+/−	+++	+/−	+/−	+	+/−	+/−	+
H-JH7	*Kocuria*	−	−	−	−	−	−	−	+	−	+/−
H-CD9b	*Micrococcus*	−	+/−	−	+++	+	+/−	+/−	−	+/−	+/−
H-CA8f	*Rhodococcus*	++	++	+++	+++	−	+++	+++	+++	+++	+++
H-CB3	*Streptomyces*	+++	+++	+/−	+/−	+/−	−	−	−	+	++
H-KF8	*Streptomyces*	+++	+++	+/−	+/−	+/−	−	−	−	+	+

−, no inhibition; +/−, attenuated growth; +, <50% growth inhibition; ++, 50% growth inhibition; +++, >50% growth inhibition. Both media were prepared with ASW.

TABLE 2 | Antimicrobial activities of crude extracts using various solvents for selected actinobacterial isolates grown in ISP2-ASW medium.

Strain	Solvent	Bacterial Test Strain			
		STAU	PSAU	SAEN	ESCO
H-KF8	Hexane	–	–	–	–
	Ethyl acetate	–	–	–	–
	Methanol	+	–	–	+
H-CA8f	Hexane	–	–	–	–
	Ethyl acetate	+	+	+	+
	Methanol	–	–	–	–
H-KB5	Hexane	–	–	–	–
	Ethyl acetate	–	+	+	+
	Methanol	–	–	–	–
H-JH3	Hexane	–	–	–	–
	Ethyl acetate	–	–	–	–
	Methanol	–	–	+	+
H-BE7	Hexane	–	–	–	–
	Ethyl acetate	–	–	+	–
	Methanol	–	–	–	–

family), *Brevibacterium* genus (*Brevibacteriaceae* family), and *Corynebacterium* genus (*Corynebacteriaceae* family) (**Table 3**). Interestingly, the psychrotolerant isolate *Kocuria* sp. H-KB5 has a 96.97% sequence identity with the type strain *K. polaris* CMS 76 or^T, a strain isolated from an Antarctic cyanobacterial mat sample (Reddy et al., 2003). Moreover, strain H-KB5 forms a separate branch within the *Kocuria* group in the phylogenetic tree (**Figure 4**). This isolate will be further characterized in a polyphasic approach to determine its taxonomic position.

Phenotypic Characterization of Isolated Actinobacterial Strains

The Comau fjord is characterized by defined zoning patterns of strong vertical and horizontal salinity gradients. The first 15 m underwater are influenced by waters of low salinity (~1.0%). Below this depth, a halocline is found that produces a constant water salinity of 3.2% (Castillo et al., 2012). In order to analyze how the salinity affects the growth of the actinobacterial isolates, NaCl tolerance was determined for each strain (**Table 3**). 82% of the representative isolates were able to grow in the presence of 1.0, 3.5, 5.0, and 7.0% (w/v) NaCl (**Figure 5**). 45% of the strains, belonging to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Curtobacterium*, and *Kocuria* genera, were able to grow in presence of 10% (w/v) NaCl (**Table 3**). None of the isolated actinobacteria was able to grow with 20% w/v NaCl.

To study adaptation to marine environments, actinobacterial strains were tested for ASW requirement. Most strains (73%), belonging to *Arthrobacter*, *Brachybacterium*, *Corynebacterium*, *Dietzia*, *Kocuria*, *Rhodococcus*, and *Streptomyces* genera were positively influenced by sea water as they required ASW for growth, suggesting marine adaptation. Interestingly, strain *Brevibacterium* sp. H-BE7, showed improved growth with both ASW and 3.5% NaCl, rather than with Milli-Q H_2O and 0% NaCl, suggesting a specific salt requirement confirmed by its growth in 10% (w/v) NaCl (**Figures 5B–D**).

As the Comau fjord deep-waters reach temperatures below 10°C, actinobacterial strains were tested for growth at different temperatures. Notably, 73% of strains belonging to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kocuria*, *Dietzia*, and *Rhodococcus*, and to a lesser extent, *Streptomyces*, were able to grow at 4°C (**Figure 6**). Moreover, pigmentation of the colonies was more intense after growth at 4°C, in comparison to 30°C (**Figures 6B–D**). Colony pigmentation of all representative actinobacteria was visualized macroscopically and detailed in **Table 3**.

Resistance to Model Antibiotics

Antibiogram experiments demonstrated that all isolated actinobacterial strains are resistant to at least one of the antibiotics tested. Furthermore, these isolates showed resistance to several antibiotics of different classes. Interestingly, strains H-JH3, H-BE7, H-KA4, H-CD9, H-CG1, H-ED12, and H-CA8f showed resistances to ≥6 antibiotics, wherein resistance to tetracycline, ciprofloxacin and oxacyllin were observed for all the actinobacterial strains. Strain H-KA4 and H- ED12 showed resistance to all antibiotics tested, whereas strain H-BE7 was susceptible only for sulfonamides (**Table 4**).

DISCUSSION

Marine actinomycetes isolated from the National Marine Protected Area of Huinay at the Comau fjord in Northern Patagonia were studied, along with their physiological and taxonomic properties, and their potential to produce antimicrobial compounds. Patagonian fjords are largely unexplored, and may provide a rich source of microorganisms producing novel anti-infective compounds. This is the first bioprospection report of cultivable actinobacteria in this unique ecosystem, where 25 actinobacteria were isolated and characterized. Two studies report the isolation of marine actinobacteria from sediments of Chile's vast coast; one from Chiloé Island (Hong et al., 2010) and a recent study performed in Valparaíso Central Bay (Claverías et al., 2015). Only a metagenomic study has been carried out with a microbial mat located in the Comau fjord, revealing that 1% of community reads was represented by the *phylum Actinobacteria* (Ugalde et al., 2013).

In this study, a lower abundance of actinobacteria associated to marine sediments was observed compared to Valparaíso Bay where actinobacterial strains belonging to 18 genera were isolated, using the same cultivating conditions (Claverías et al., 2015). Although, members of the *Rhodococcus* and *Dietzia* genera were successfully isolated from the Comau fjord, they were less represented (8%) than in Valparaiso Bay (33%). The lower actinobacterial abundance in Comau fjord could be due to the lower content of organic matter in this microhabitat that can range between 0.5 and 3.4% of organic carbon content for Northern Chilean Patagonian fjords (Sepúlveda et al., 2011).

TABLE 3 | Biogeographic and physiological characteristics of representative actinobacterial strains.

Strain	Closest Type Strain (Accession N°) (% Identity)	Biogeographic Characteristics				Physiological Characteristics				Biosynthetic genes		
		Sampling Site	Depth (m)	Salinity (ppt)	Sediment characteristics	Temperature (°C)	Salinity (%NaCl)	ASW Requirement	Pigmentation	PKS I	PKS II	NRPS
H-JH3	*Arthrobacter oxydans* DSM 20119^T (X83408) (98,26)	Lilihuapi Island	11.3	28.5	Shells and sponges	4–37	0–10	+	Bright cream	–	–	+
H-CG1	*Brachybacterium paraconglomeratum* JCM 17781^T (AB645761) (99.16)	Tambor Waterfall	6.1	30.5	Hard sediment	4–37	0–10	+	Bright yellow	+	+	+
H-BE7	*Brevibacterium oceani* BBH7^T (AM158906) (97.94)	Punta Lionco	25.1	29.5	Good water visibility	4–37	0–10	–	Bright orange	+	+	+
H-EH3	*Corynebacterium pilbarense* IMMIB WACC-658^T (FN295567) (98.10)	Loncochaigua River	0.25	5	Low tide	20–37	7	+	Bright cream	–	+	+
H-ED12	*Curtobacterium oceanosedimentum* ATCC 31317^T (GU269547) (99.02)	Punta Lionco	25.1	29.5	Good water visibility	20–45	0–10	–	Pale cream	–	+	+
H-KB5	*Kocuria polaris* CMS 76or^T (NR028924)(96.97)	Loncochaigua River	0.25	5	Low tide	4–37	0–10	+	Bright pink	–	+	+
H-KA4	*Dietzia natronolimnaea* DSM 444860^T (FJ468329) (99.06)	Tambor Waterfall	15.6	28.5	Sand, mussels and sea urchins	4–37	0–7	+	Intense orange	–	+	+
H-jCD9b	*Micrococcus luteus* NCTC 2665^T (CP001628) (99.15)	Punta Lionco	14.5	29	Shells, poor water visibility	20–37	0–7	–	Light yellow	–	–	–
H-CA8f	*Rhodococcus jianlingiae* djl-6-2^T (DQ185597) (98.84)	Lilihuapi Island	22.9	31	Shells and old nets	4–30	0	+	Light pink	–	+	+
H-KF8	*Streptomyces prasinus* NRRL B-2712^T (DQ026658) (99.92)	Punta Lionco	14.5	29	Shells, poor water visibility	4–37	0–7	+	White mycelium	–	+	+
H-CB3	*Streptomyces prasinus* NRRL B-2712^T (DQ026658) (99.86)	Tambor Waterfall	15.6	28.5	Sand, mussels and sea urchins	4–37	0–7	+	White mycelium	–	–	+

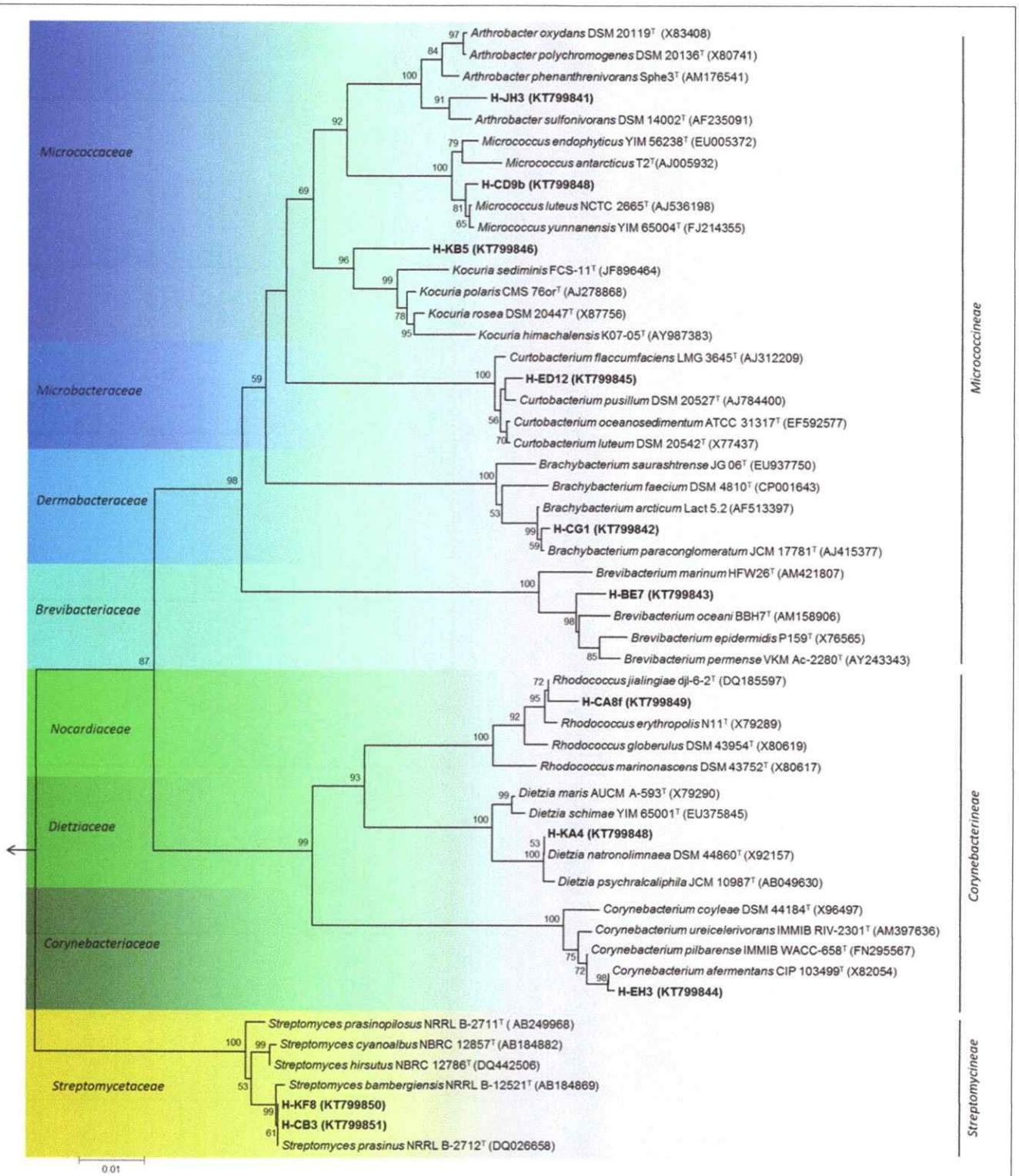


FIGURE 4 | Phylogenetic tree of representative actinobacterial strains isolated from the Comau fjord in Northern Patagonia, Chile. Neighbour-joining tree of 16S rRNA gene showing the three suborders within the *phylum Actinobacteria*. Node numbers represent the percentage of bootstrap replicates (1000 resampling) which supported the proposed branching order shown at consistent nodes (values below 50% were not shown). Gene sequence positions 55–1410 were considered, according to the *Escherichia coli* K12 (AP012306) 16S rRNA gene sequence numbering. Arrow points to the outgroup *E. coli* K12. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Scale bar corresponds to 0.01 substitutions per nucleotide positions.

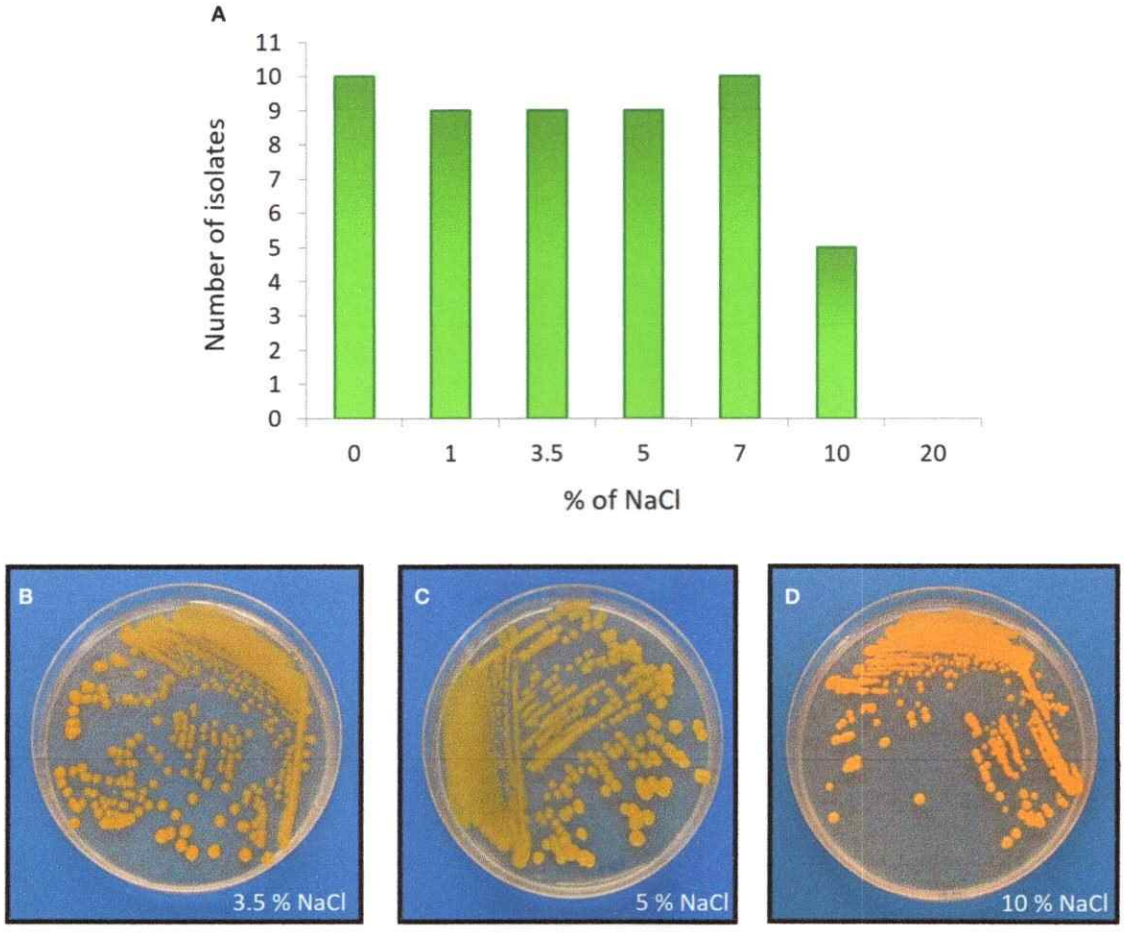


FIGURE 5 | NaCl effect on actinobacterial growth. Upper panel: **(A)** Distribution of actinobacterial isolates and their ability to grow in LB medium with various percentages of NaCl. Bottom panel: As an example, the halophilic *Brevibacterium* sp. H-BE7 grown in LB medium containing: **(B)** 3.5%; **(C)** 5%; and **(D)** 10% NaCl concentrations.

Gram-positive bacteria are more commonly observed in organic rich habitats (Fenical, 1993). Water samples from Valparaíso Bay are influenced by contamination with polycyclic aromatic hydrocarbons as well as with heavy metals (Campos et al., 1987; Palma-Fleming et al., 2008; Fuentes et al., 2015). It can also be influenced by hydrographic features such as seasonal upwelling which can supply nutrients to shallow waters (Capone and Hutchins, 2013). In contrast, the Comau fjord has a high precipitation rate that provides a fresh water input (Silva, 2006) which can affect microorganisms in marine sediments. The four sites from Comau fjord have minimal anthropogenic intervention, thereof changes in microbial communities are given almost exclusively by natural processes.

Despite the fact that a relatively low number of actinobacterial strains were retrieved from Comau fjord, a rather high cultivable biodiversity (10 genera) was observed using 5 isolation media. In comparison, the actinobacteria isolated using 11 selective media from the Trondheim fjord (Norway) belonged to 12 genera (Bredholdt et al., 2007). Also, in a culture-dependent study using sediments collected near Chiloé Island, Chile, five genera were retrieved using 7 media, being dominant the *Micromonospora* genus (Hong et al., 2010). Although, no *Micromonospora* members have been isolated in this work, this could be due to the different isolation media used. In this report, 24% of isolates were obtained from NaST21Cx medium, which is derived from ST21Cx medium by elimination of yeast extract and replacement of artificial sea water (Magarvey et al., 2004). It has been reported that media composed of relatively simple nutrients yielded more cultured actinobacteria in diverse environments (Zhang et al., 2006; Gontang et al., 2007; Qin et al., 2012). This is consistent with the negligible amount of nutrients that are actually available for marine actinobacteria within hostile ocean ecosystems (Das et al., 2006). This is the case for our study since more isolates were obtained with media containing low nutrients or complex carbon sources rather than common media constituents such as peptone and simple sugars, which are proposed to be unrealistic marine nutrients (Kurtböke et al., 2015). In this study, the major abundance of actinobacteria was found in deeper samples, which is in accordance with that observed in the Trondheim fjord (Hakvåg

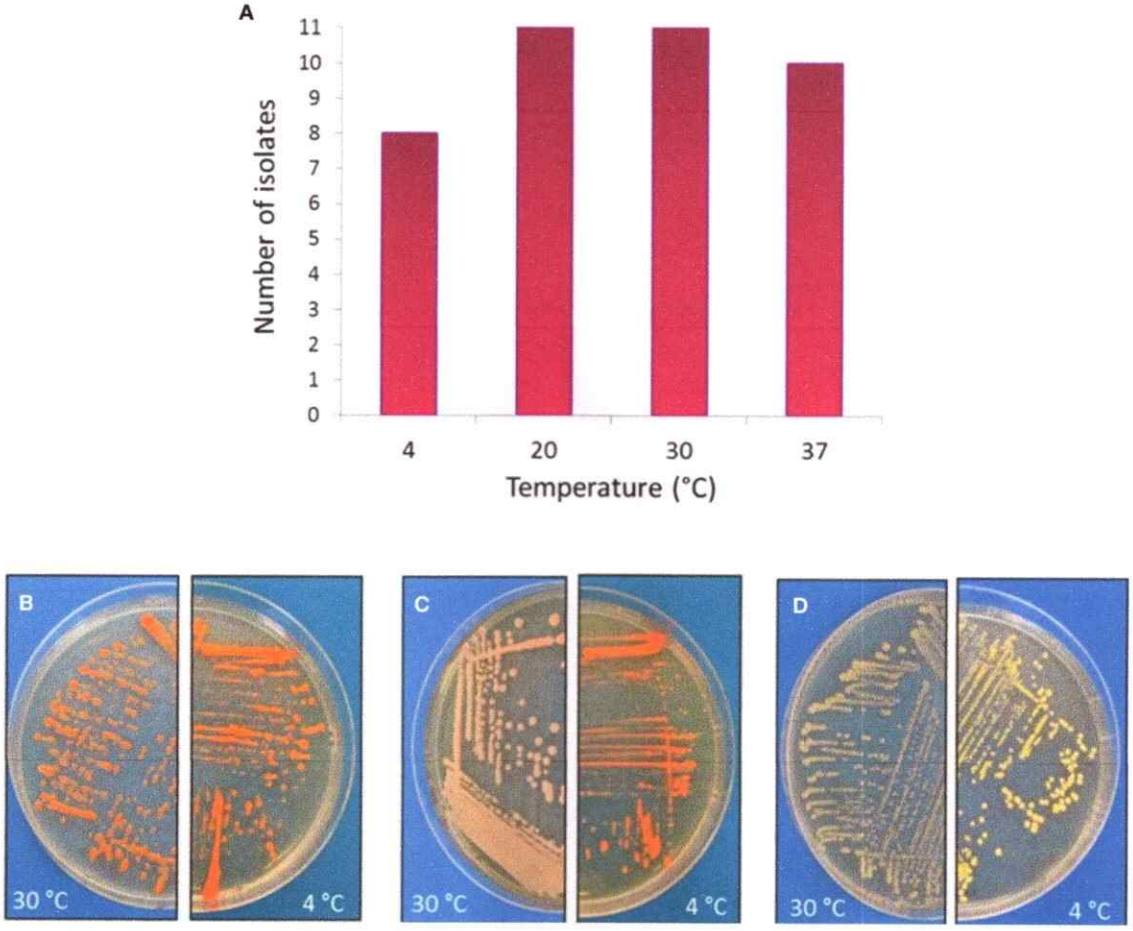


FIGURE 6 | Temperature effect on actinobacterial growth. Upper panel: **(A)** Distribution of actinobacterial isolates and their ability to grow in different temperatures. Bottom panel: As an example, actinobacterial strains grown in TSA-ASW medium at either 30°C (left) or 4°C (right), showing differences in pigmentations. **(B)** *Dietzia* sp. H-KA4; **(C)** *Kocuria* sp. H-KB5; **(D)** *Brachybacterium* sp. H-CG1.

TABLE 4 | Antibiotic resistance of selected actinobacterial strains.

Antibiotic	Class	Huinay Isolates										
		H-JH3	H-CG1	H-BE7	H-EH3	H-ED12	H-KB5	H-KA4	H-CD9	H-CA8f	H-KF8	H-CB3
Penicillin (10 UOF)	β-Lactam	R	R	R	NG	R	R	R	R	R	R	S
Chloramphenicol (30 μg)	Other	S	S	R		R	S	R	R	R	R	R
Tetracycline (30 μg)	Poliketide	R	R	R		R	R	R	R	R	R	R
Oxacyllin (1 μg)	β-Lactam	R	R	R		R	R	R	R	R	R	R
Erythromycin (15 μg)	Macrolide	R	S	R		R	S	R	S	S	R	S
Clindamycin (2 μg)	Lincosamides	R	S	R		R	S	R	S	R	S	S
Sulfa-Trimethroprim (25 μg)	Sulfonamide	R	S	S		R	S	R	S	R	S	S
Ciprofloxacin (5 μg)	Fluoro-quinolone	R	R	R		R	R	R	R	R	R	R
Cefazolin (30 μg)	Cephalosporin (1st)	R	R	R		R	R	R	R	R	S	S
Gentamicin (10 μg)	Aminoglycoside	S	R	R		R	S	R	R	R	S	S

NG, No growth; R, Resistant; S, Susceptible.

et al., 2008). Moreover, an elevated number (73%) of isolates showed an ASW requirement for growth. Evidence of isolation of seawater-dependent actinobacteria from marine sediments has been reported (Mincer et al., 2002; Maldonado et al., 2005). The fact that growth of some isolates is positively influenced by sea water can be an indicator that suggests they might be well

adapted to the marine environments (Bredholdt et al., 2007; Penn and Jensen, 2012; Yuan et al., 2014). Nevertheless, since isolates obtained from Comau fjord can also grow without NaCl, they represent novel moderate halotolerant features in actinobacteria from this pristine sampling zone. This is consistent with the fact that these isolates have to overcome the dynamics of strong salinity gradients observed within the Comau fjord.

Reports of marine actinomycetes as a source of novel secondary bioactive metabolites have been extensively recognized (Haefner, 2003; Knight et al., 2003; Fiedler et al., 2005; Fenical and Jensen, 2006; Zhang et al., 2006; Gulder and Moore, 2010; Kurtböke et al., 2015). Two screenings for antimicrobial activities were pursued in this report, and notably, inhibition of the growth of at least one of the model bacteria was observed. It is noteworthy to highlight that antimicrobial activities from non-mycelial strain (e.g., *Rhodococcus* sp. H-CA8f) outcompete the activities of mycelial-type strains. To our knowledge, this is the first report of strong antibacterial activities associated to a *Rhodococcus* isolated from marine sediments. The *Rhodococcus* strain isolated in this study has a strong activity (>50% growth inhibition) against *E. coli*, *S. enterica*, *P. aeruginosa*, and *L. monocytogenes*; whereas a *Rhodococcus* strain isolated from Valparaíso Bay sediments (Claverías et al., 2015) had only a modest activity against *E. coli*. Antimicrobial activity from marine-derived isolates, but not necessarily from sediments, includes a *Rhodococcus* isolated from South China Sea corals that presented activity against *B. subtilis*, *B. thuringiensis*, and *E. coli* (Zhang et al., 2013), whereas *Rhodococcus* strains isolated from corals of the Arabian Gulf showed activity against *S. aureus* (Mahmoud and Kalendar, 2016). In this study, antimicrobial activity of *Arthrobacter* sp. H-JH3 against *S. enterica* and *E. coli* is highlighted by its novelty. In this line, there are reports about antarctic *Arthrobacter* strains isolated from sponges that were able to inhibit the growth of *Burkholderia cepacia* complex by the production of volatile organic compounds (Fondi et al., 2012; Orlandini et al., 2014). Also, antimicrobial activity against *Vibrio anguillarum* and *S. aureus* was detected from samples collected from the Arctic Ocean (Wietz et al., 2012). Interestingly, this is the first report indicating growth inhibition of Gram-negative strains by a *Brevibacterium* isolate. Only a bacteriocin able to inhibit *L. monocytogenes*, but inactive against Gram-negative was reported for this genus (Motta and Brandelli, 2002). In contrast, antimicrobial activity against *S. enterica* was observed in crude extracts, suggesting a different mode of action.

It has been reported that most natural products with interesting biological activities are synthesized by PKS (type I or type II), NRPS, and even PKS-NRPS hybrid pathways (Fischbach and Walsh, 2006). Some pharmacologically commercial examples include the polyketide antibiotic erythromycin (Staunton and Wilkinson, 1997) and the non-ribosomal peptide antibiotic of the cephalosporin family (Aharonowitz and Cohen, 1992). In this report, a PCR-based screening was pursued for the detection of these biosynthetic genes in actinobacterial isolates, in order to explore the potential to produce secondary metabolites with biotechnological applications. Notably, 91% of the isolates tested showed the presence of at least one of the three biosynthetic genes, which confirms that these metabolic pathways are widely distributed among this *phylum* (Donadio et al., 2007). As molecular methods for analyzing these genes are useful for screening of isolates for prediction of potential bioactive molecule production (Hodges et al., 2012), future efforts will be focused in sequencing these biosynthetic genes, to gain knowledge of the novelty of the bioproducts in which they are involved in.

The marine habitat sampled in the Northern Patagonia of Chile was a promising scenario to search for novel actinobacterial strains. In this study, four putative new species are proposed: *Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Corynebacterium* sp. H-EH3 and *Kocuria* sp. H-KB5, based on numerical thresholds related to 16S rRNA gene sequences (Rosselló-Móra and Amann, 2015). In addition, representatives of *Micrococcineae*, *Corynebacterineae*, and *Streptomycineae* suborders were isolated. Interestingly, actinobacterial isolates showed sequence similarity with strains reported from colder habitats. 73% of the isolates belonging to *Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Kocuria*, *Dietzia*, *Rhodococcus*, and *Streptomyces* genera were able to grow at 4°C, suggesting a psychrotolerant adaptation which is in accordance with the water body temperature range of the Comau fjord (Lagger et al., 2009; Sobarzo, 2009), sustaining a thermohaline circulation (Bustamante, 2009). A difference in colony pigmentation was observed at low temperatures. Pigments can be enhanced under specific conditions such as climate stress, since they are part of the non-enzymatic antioxidant mechanisms in cell defense to prevent oxidative damage (Correa-Llantén et al., 2012). Another role of pigments in response to cold is to decrease the membrane fluidity to counterbalance the effects of fatty acids in Antarctic bacteria (Chattopadhyay, 2006). Pigments can also contribute to antibacterial activity, positioning them as interesting biotechnological candidates for food, cosmetic and textile industries (Rashid et al., 2014; Leiva et al., 2015).

Comparison with 16S ribosomal RNA sequences Bacteria and Archaea NCBI database, reveals only two closest type strains of marine origin: *Brevibacterium oceani* BBH7^T isolated from deep sea sediment of the Indian Ocean (Bhadra et al., 2008) and *Curtobacterium oceanosedimentum* ATCC 31317^T isolated from Irish sea marine sediments (Kim et al., 2009). In contrast, when sequences are compared with NCBI nucleotide collection database, actinobacterial isolates showed more similarity with polar marine isolates. This is the case for the psychrotolerant *Arthrobacter* sp. H-JH3, which showed a 98.82% identity with *A. scleromae* Asd M4-11 (Vardhan Reddy et al., 2009), a bacterium isolated from a melt water stream of an Arctic glacier. The psychrotolerant *Brachybacterium* sp. H-CG1 showed a high similarity (99.16%) with *B. articum* Lact 5.2 (Acc. Number: AF434185, unpublished), a bacterium isolated from a sea-ice sample from the permanently cold fjord of Wijde fjord, Spitzbergen, in the Arctic Ocean. Another interesting relation is given for strain H-CD9b from the genus *Micrococcus*, which has a 99.15% of sequence identity with the type strain *M. luteus* NCTC 2665^T (Rokem et al., 2011) that is a soil metal resistant bacterium, and a slightly more sequence identity (99.43%) with *Micrococcus* sp. strain MOLA4 (Acc. Number: CP001628, unpublished) a bacterium isolated from sea water of

North Western Mediterranean Sea. Also, strain H-CA8f, showed a higher sequence similarity (98.91%) to *Rhodococcus* sp. TMT4-41 isolated from a glacier in China (Acc. Number: JX949806, unpublished) than to its closest type strain *R. jialingiae* djl-6-2^T (Wang et al., 2010).

Antibiogram experiments demonstrated that, in general, actinobacterial strains showed resistance. Interestingly, *Curtobacterium* sp. H-ED12, *Dietzia* sp. H-KA4 and *Brevibacterium* sp. H-BE7 showed resistance to almost all antibiotics tested, possibly due to the presence of multiple biosynthetic clusters, involving different classes of antibiotic compounds. Strains H-BE7 and H-ED12 inhibited both Gram-positive and Gram-negative model bacteria, suggesting different modes of action of the antibacterial molecules produced by this strain. Thus, it seems plausible that biosynthetic pathways involving metabolites of similar nature could be present in these isolates. A typical cluster of secondary metabolism includes genes for multi-domains enzymes that carry out the synthesis of different bioactive metabolites and when this metabolite has an antimicrobial activity, it is coupled to its corresponding resistance gene (Zotchev, 2014).

To our knowledge, this is the first report of the isolation and ecophysiological characterization of actinobacteria from sediments of a Patagonian fjord. This single survey uncovered a broad cultivable diversity which provides the basis for the bioprospection of bioactive compounds. The isolation of novel actinobacterial species and the evidence that most of our isolates produced antibiotic activities supports our approach.

AUTHOR CONTRIBUTIONS

AU conceived and designed the experiments, performed the experiments, analyzed the data, prepared the manuscript. FB designed the experiments. FC performed the experiments. MG performed the sampling and experiments. EM performed the sampling and edited the manuscript. MS performed the sampling, prepared and edited the manuscript. BC performed the sampling, conceived and designed the experiments, analyzed the data, prepared and edited the manuscript.

ACKNOWLEDGMENTS

The authors thank the scuba divers and staff from Huinay Scientific Field Station. This work was supported by the Comisión Nacional de Investigación Científica y Tecnológica (FONDECYT 11121571 to BC) and Swedish Research Council N° 2013-6713 (to BC and EM) with additional funding from the Universidad Técnica Federico Santa María (USM 131342, 131562 to MS). AU, FC acknowledges Conicyt PhD fellowship (AU), Conicyt Gastos Operacionales 21120621 (AU), and PIEA> UTFSM (AU, FC).

REFERENCES

Aharonowitz, Y., and Cohen, G. (1992). Penicillin and Cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. *Annu. Rev. Microbiol.* 46, 461–495. doi: 10.1146/annurev.mi.46.100192.002333

Axenov-Gribanov, D. V., Voytsekhovskaya, I. V., Tokovenko, B. T., Protasov, E. S., Gamaiunov, S. V., Rebets, Y. V., et al. (2016). Actinobacteria isolated from an underground lake and moonmilk speleothem from the biggest conglomeratic karstic cave in Siberia as sources of novel biologically active compounds. *PLoS ONE* 11:e0152957. doi: 10.1371/journal.pone.0152957

Ayuso, A., Clark, D., González, I., Salazar, O., Anderson, A., and Genilloud, O. (2005). A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. *Appl. Microbiol. Biotechnol.* 67, 795–806. doi: 10.1007/s00253-004-1828-7

Ayuso-Sacido, A., and Genilloud, O. (2005). New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. *Microb. Ecol.* 49, 10–24. doi: 10.1007/s00248-004-0249-6

Baskaran, R., Vijayakumar, R., and Mohan, P. (2011). Enrichment method for the isolation of bioactive actinomycetes from mangrove sediments of Andaman Islands India. *Malays. J. Microbiol.* 7, 26–32.

Bérdy, J. (2005). Bioactive microbial metabolites. *J. Antibiot.* 58, 1–26. doi: 10.1038/ja.2005.1

Bhadra, B., Raghukumar, C., Pindi, P. K., and Shivaji, S. (2008). Brevibacterium oceani sp. nov., isolated from deep-sea sediment of the Chagos Trench, Indian Ocean. *Int. J. Syst. Evol. Microbiol.* 58, 57–60. doi: 10.1099/ijs.0.64869-0

Bredholdt, H., Galatenko, O. A., Engelhardt, K., Fjaervik, E., Terekhova, L. P., and Zotchev, S. B. (2007). Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. *Environ. Microbiol.* 9, 2756–2764. doi: 10.1111/j.1462-2920.2007.01387.x

Bull, A., and Stach, J. (2007). Marine actinobacteria: new opportunities for natural product search and discovery. *Trends Microbiol.* 15, 491–499. doi: 10.1016/j.tim.2007.10.004

Bustamante, M. S. (2009). "The southern Chilean fjord region: oceanographic aspects," in *Marine Benthic Fauna of Chilean Patagonia*, eds V. Häussermann, and G. Försterra (Santiago: Nature in Focus), 53–60.

Campos, V., Pinochet, H., De Gregori, I., Lund Plantat, F., Zahr, M., Delgado, D., et al. (1987). Estudios sobre contaminación del agua en la Bahía de Valparaíso. *Boletín del Inst. Salud Pública* 27, 72–83.

Capone, D. G., and Hutchins, D. A. (2013). Microbial biogeochemistry of coastal upwelling regimes in a changing ocean. *Nat. Geosci.* 6, 711–717. doi: 10.1038/ngeo1916

Castillo, M. I., Pizarro, O., Cifuentes, U., Ramirez, N., and Djurfeldt, L. (2012). Subtidal dynamics in a deep fjord of southern Chile. *Cont. Shelf Res.* 49, 73–89. doi: 10.1016/j.csr.2012.09.007

Chattopadhyay, M. K. (2006). Mechanism of bacterial adaptation to low temperature. *J. Biosci.* 31, 157–165. doi: 10.1007/BF02705244

Claverías, F. P., Undabarrena, A., González, M., Seeger, M., and Cámara, B. (2015). Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaíso bay, Chile. *Front. Microbiol.* 6:737. doi: 10.3389/fmicb.2015.00737

Colquhoun, J. A., Heald, S. C., Li, L., Tamaoka, J., Kato, C., Horikoshi, K., et al. (1998). Taxonomy and biotransformation activities of some deep-sea actinomycetes. *Extremophiles* 2, 269–277. doi: 10.1007/s007920050069

Correa-Llantén, D. N., Amenábar, M. J., and Blamey, J. M. (2012). Antioxidant capacity of novel pigments from an Antarctic bacterium. *J. Microbiol.* 50, 374–379. doi: 10.1007/s12275-012-2029-1

Das, S., Lyla, P. S., and Khan, S. A. (2006). Marine microbial diversity and ecology: importance and future perspectives. *Curr. Sci.* 90, 1325–1335.

Donadio, S., Monciardini, P., and Sosio, M. (2007). Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Nat. Prod. Rep.* 24, 1073–1109. doi: 10.1039/b514050c

Donia, M., and Hamann, M. T. (2003). Marine natural products and their potential applications as anti-infective agents. *Lancet Infect. Dis.* 3, 338–348. doi: 10.1016/S1473-3099(03)00655-8

Doroghazi, J. R., and Metcalf, W. W. (2013). Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *BMC Genomics* 14:611. doi: 10.1186/1471-2164-14-611

Duncan, K., Haltli, B., Gill, K. A., and Kerr, R. G. (2014). Bioprospecting from marine sediments of New Brunswick, Canada: exploring the relationship between total bacterial diversity and actinobacteria diversity. *Mar. Drugs* 12, 899–925. doi: 10.3390/md12020899

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.2307/2408678

Fenical, W. (1993). Chemical studies of marine bacteria: developing a new resource. *Chem. Rev.* 93, 1673–1683. doi: 10.1021/cr00021a001

Fenical, W., and Jensen, P. R. (2006). Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat. Chem. Biol.* 2, 666–673. doi: 10.1038/nchembio841

Fiedler, H.-P., Bruntner, C., Bull, A. T., Ward, A. C., Goodfellow, M., Potterat, O., et al. (2005). Marine actinomycetes as a source of novel secondary metabolites. *Antonie Van Leeuwenhoek* 87, 37–42. doi: 10.1007/s10482-004-6538-8

Fischbach, M. A., and Walsh, C. T. (2006). Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chem. Inform.* 37, 3468–3496. doi: 10.1002/chin.200644265

Fondi, M., Orlandini, V., Maida, I., Perrin, E., Papaleo, M. C., Emiliani, G., et al. (2012). Draft genome sequence of the volatile organic compound-producing Antarctic bacterium *Arthrobacter* sp. strain TB23, able to inhibit cystic fibrosis pathogens belonging to the *Burkholderia cepacia* complex. *J. Bacteriol.* 194, 6334–6335. doi: 10.1128/JB.01432-12

Fuentes, S., Ding, G.-C., Cárdenas, F., Smalla, K., and Seeger, M. (2015). Assessing environmental drivers of microbial communities in estuarine soils of the Aconcagua River in Central Chile. *FEMS Microbiol. Ecol.* 91:fiv110. doi: 10.1093/femsec/fiv110

Gao, B., and Gupta, R. S. (2005). Conserved indels in protein sequences that are characteristic of the phylum *Actinobacteria*. *Int. J. Syst. Evol. Microbiol.* 55, 2401–2412. doi: 10.1099/ijs.0.63785-0

Genilloud, O. (2014). The re-emerging role of microbial natural products in antibiotic discovery. *Antonie Van Leeuwenhoek* 106, 173–188. doi: 10.1007/s10482-014-0204-6

Gontang, E., Fenical, W., and Jensen, P. (2007). Phylogenetic diversity of gram-positive bacteria cultured from marine sediments. *Appl. Environ. Microbiol.* 73, 3272–3282. doi: 10.1128/AEM.02811-06

Gontang, E. A., Gaudêncio, S. P., Fenical, W., and Jensen, P. R. (2010). Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria. *Appl. Environ. Microbiol.* 76, 2487–2499. doi: 10.1128/AEM.02852-09

Goodfellow, M., and Fiedler, H.-P. (2010). A guide to successful bioprospecting: informed by actinobacterial systematics. *Antonie Van Leeuwenhoek* 98, 119–142. doi: 10.1007/s10482-010-9460-2

Gulder, T. A. M., and Moore, B. S. (2010). Chasing the treasures of the sea – bacterial marine natural products. *Curr. Opin. Microbiol.* 12, 252–260. doi: 10.1016/j.mib.2009.05.002

Haber, M., and Ilan, M. (2014). Diversity and antibacterial activity of bacteria cultured from Mediterranean *Axinella* spp. *sponges. J. Appl. Microbiol.* 116, 519–532. doi: 10.1111/jam.12401

Haefner, B. (2003). Drugs from the deep: marine natural products as drug candidates. *Res. Focus* 8, 536–544. doi: 10.1016/s1359-6446(03)02713-2

Hakvåg, S., Fjaervik, E., Josefsen, K. D., Ian, E., Ellingsen, T. E., and Zotchev, S. B. (2008). Characterization of *Streptomyces* spp. isolated from the sea surface microlayer in the Trondheim Fjord, Norway. *Mar. Drugs* 6, 620–635. doi: 10.3390/md6040620

Hodges, T. W., Slattery, M., and Olson, J. B. (2012). Unique actinomycetes from marine caves and coral reef sediments provide novel PKS and NRPS biosynthetic gene clusters. *Mar. Biotechnol.* 14, 270–280. doi: 10.1007/s10126-011-9410-7

Hong, J., Ru, L., Lujie, C., Hao, L., Ilei, N., and Yunyang, L. (2010). Actinobacterial diversity of marine sediments samples from Chile. *Acta Microbiol. Sin.* 50, 862–869.

Hong, K., Gao, A.-H., Xie, Q.-Y., Gao, H. G., Zhuang, L., Lin, H.-P., et al. (2009). Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Mar. Drugs* 7, 24–44. doi: 10.3390/md7010024

Jensen, P. R., Gontang, E., Mafnas, C., Mincer, T. J., and Fenical, W. (2005). Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. *Environ. Microbiol.* 7, 1039–1048. doi: 10.1111/j.1462-2920.2005.00785.x

Jiang, S., Sun, W., Chen, M., Dai, S., Zhang, L., Liu, Y., et al. (2007). Diversity of culturable actinobacteria isolated from marine sponge *Haliclona* sp. *Antonie Van Leeuwenhoek* 92, 405–416. doi: 10.1007/s10482-007-9169-z

Joint, I., Mühling, M., and Querellou, J. (2010). Culturing marine bacteria - an essential prerequisite for biodiscovery. *Microb. Biotechnol.* 3, 564–575. doi: 10.1111/j.1751-7915.2010.00188.x

Katz, L., and Baltz, R. H. (2016). Natural product discovery: past, present, and future. *J. Ind. Microbiol. Biotechnol.* 43, 155–176. doi: 10.1007/s10295-015-1723-5

Kester, D. R., Duedall, I. W., Connors, D. N., and Pytkowicz, R. M. (1967). Preparation of artificial seawater. *Limnol. Oceanogr.* 12, 176–179. doi: 10.4319/lo.1967.12.1.0176

Kim, B. K., Kim, O.-S., Moon, E. Y., and Chun, J. (2009). Proposal to transfer *Flavobacterium oceanosedimentum* Carty and Litchfield 1978 to the genus *Curtobacterium* as *Curtobacterium oceanosedimentum* comb. nov. *FEMS Microbiol. Lett.* 296, 137–141. doi: 10.1111/j.1574-6968.2009.01628.x

Kim, T. K., Garson, M. J., and Fuerst, J. A. (2005). Marine actinomycetes related to the "*Salinospora*" group from the Great Barrier Reef sponge *Pseudoceratina clavata*. *Environ. Microbiol.* 7, 509–518. doi: 10.1111/j.1462-2920.2005.00716.x

Knight, V., Sanglier, J.-J., DiTullio, D., Braccili, S., Bonner, P., Waters, J., et al. (2003). Diversifying microbial natural products for drug discovery. *Appl. Microbiol. Biotechnol.* 62, 446–458. doi: 10.1007/s00253-003-1381-9

Kuang, W., Li, J., Zhang, S., and Long, L. (2015). Diversity and distribution of Actinobacteria associated with reef coral *Porites lutea*. *Front. Microbiol.* 6:1094. doi: 10.3389/fmicb.2015.01094

Kurahashi, M., Fukunaga, Y., Sakiyama, Y., Harayama, S., and Yokota, A. (2010). Euzebya tangerina gen. nov., sp. nov., a deeply branching marine actinobacterium isolated from the sea cucumber Holothuria edulis, and proposal of Euzebyaceae fam. nov., *Euzebyales* ord. nov. and *Nitriliruptoridae* subclassis nov. *Int. J. Syst. Evol. Microbiol.* 60, 2314–2319. doi: 10.1099/ijs.0.016543-0

Kurtböke, D. İ., Grkovic, T., and Quinn, R. J. (2015). "Marine Actinomycetes in biodiscovery," in *Marine Microbiology and Biotechnology*, ed S. Kim (Berlin; Heidelberg: Springer), 663–676.

Lagger, C., Häussermann, V., Försterra, G., and Tatián, M. (2009). Ascidians from the southern Chilean Comau Fjord. *Spixiana* 32, 173–186.

Lam, K. S. (2006). Discovery of novel metabolites from marine actinomycetes. *Curr. Opin. Microbiol.* 9, 245–251. doi: 10.1016/j.mib.2006.03.004

Lane, D. J. (1991). "16S/23S rRNA Sequencing.pdf," in *Nucleic Acid Techniques in Bacterial Systematicsid*, eds E. Stackebrandt, and M. Goodfellow (New York, NY: John Wiley and *Sons)*, 115–175.

Lee, D. W., Lee, J. M., Seo, J. P., Schumann, P., Kim, S. J., and Lee, S. D. (2008). *Phycicola gilvus* gen. nov., sp. nov., an actinobacterium isolated from living seaweed. *Int. J. Syst. Evol. Microbiol.* 58, 1318–1323. doi: 10.1099/ijs.0.65283-0

Lee, H. L., Zainal, N., Azman, A. S., Eng, S. K., Ab Mutalib, N. S., Yin, W. F., et al. (2014b). Streptomyces pluripotens sp. nov., a bacteriocin-producing streptomycete that inhibits meticillin-resistant *Staphylococcus aureus*. *Int. J. Syst. Evol. Microbiol.* 64, 3297–3306. doi: 10.1099/ijs.0.065045-0

Lee, H. L., Zainal, N., Azman, A. S., Eng, S. K., Bog, B. H., Yin, W. F., et al. (2014a). Diversity and antimicrobial activities of actinobacterial isolated from tropical mangrove sediments in Malaysia. *Sci. World J.* 2014:698178. doi: 10.1155/2014/698178

Leiva, S., Alvarado, P., Huang, Y., Wang, J., and Garrido, I. (2015). Diversity of pigmented Gram-positive bacteria associated with marine macroalgae from Antarctica. *FEMS Microbiol. Lett.* 362:fnv206. doi: 10.1093/femsle/fnv206

León, J., Liza, L., Soto, I., Cuadra, D. L., and Patiño, L. (2007). Actinomycetes bioactivos de sedimento marino de la costa central del Perú. *Rev. Peru. Microbiol.* 14, 259–270. doi: 10.15381/rpb.v14i2.1796

Livermore, D. M. (2009). Has the era of untreatable infections arrived? *J. Antimicrob. Chemother.* 64, i29–i36. doi: 10.1093/jac/dkp255

Magarvey, N., Keller, J., Bernan, V., Dworkin, M., and Sherman, D. H. (2004). Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Appl. Environ. Microbiol.* 70, 7520–7529. doi: 10.1128/AEM.70.12.7520-7529.2004

Mahmoud, H. M., and Kalendar, A. A. (2016). Coral-associated actinobacteria: diversity, abundance, and biotechnological potentials. *Front. Microbiol.* 7:204. doi: 10.3389/fmicb.2016.00204

Maldonado, L., Fenical, W., Jensen, P., Kauffman, C., Mincer, T., Ward, A., et al. (2005). *Salinispora arenicola* gen. nov., sp. nov. and *Salinispora tropica* sp. nov., obligate marine actinomycetes belonging to the family *Micromonosporaceae*. *Int. J. Syst. Evol. Microbiol.* 55, 1759–1766. doi: 10.1099/ijs.0.63625-0

Maldonado, L. A., Stach, J. E. M., Ward, A. C., Bull, A. T., and Goodfellow, M. (2008). Characterisation of micromonosporae from aquatic environments using molecular taxonomic methods. *Antonie Van Leeuwenhoek* 94, 289–298. doi: 10.1007/s10482-008-9244-0

Mincer, T. J., Jensen, P. R., Kauffman, C. A., and Fenical, W. (2002). Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Appl. Environ. Microbiol.* 68, 5005–5011. doi: 10.1128/AEM.68.10.5005-5011.2002

Montalvo, N. F., Mohamed, N. M., Enticknap, J. J., and Hill, R. T. (2005). Novel actinobacteria from marine sponges. *Antonie Van Leeuwenhoek* 87, 29–36. doi: 10.1007/s10482-004-6536-x

Moore, E., Arnscheidt, A., Krüger, A., Strömpl, C., and Mau, M. (2004). Simplified protocols for the preparation of genomic DNA from bacterial cultures. *Molecular Microbial Ecology Manual, vol. 1* (Dodrecht: Kluwer Academic Publishers), 3–18.

Motta, A., and Brandelli, A. (2002). Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J. Appl. Microbiol.* 92, 63–70. doi: 10.1046/j.1365-2672.2002.01490.x

Newman, D. J., and Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* 75, 311–335. doi: 10.1021/np200906s

Orlandini, V., Maida, I., Fondi, M., Perrin, E., Papaleo, M. C., Bosi, E., et al. (2014). Genomic analysis of three sponge-associated *Arthrobacter* Antarctic strains, inhibiting the growth of *Burkholderia cepacia* complex bacteria by synthesizing volatile organic compounds. *Microbiol. Res.* 169, 593–601. doi: 10.1016/j.micres.2013.09.018

Palma-Fleming, H., Cornejo, C., González, M., Pérez, V., Gutierrez, E., Sericano, J. L., et al. (2008). Polycyclic aromatic hydrocarbons and polychorinated biphenyls in coastal environments of Valdivia and Valparaiso, Chile. *J. Chil. Chem. Soc.* 53, 1533–1538. doi: 10.4067/S0717-97072008000200020

Pathom-Aree, W., Stach, J. E. M., Ward, A. C., Horikoshi, K., Bull, A. T., and Goodfellow, M. (2006). Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. *Extremophiles* 10, 181–189. doi: 10.1007/s00792-005-0482-z

Penn, K., and Jensen, P. R. (2012). Comparative genomics reveals evidence of marine adaptation in *Salinispora* species. *BMC Genomics* 13:86. doi: 10.1186/1471-2164-13-86

Pham, T. M., Wiese, J., Wenzel-Storjohann, A., and Imhoff, J. F. (2016). Diversity and antimicrobial potential of bacterial isolates associated with the soft coral *Alcyonium digitatum* from the Baltic Sea. *Antonie van Leeuwenhoek* 109, 105–119. doi: 10.1007/s10482-015-0613-1

Qin, S., Chen, H.-H., Zhao, G.-Z., Li, J., Zhu, W.-Y., Xu, L.-H., et al. (2012). Abundant and diverse endophytic actinobacteria associated with medicinal plant *Maytenus austroyunnanensis* in Xishuangbanna tropical rainforest revealed by culture-dependent and culture-independent methods. *Environ. Microbiol. Rep.* 4, 522–531. doi: 10.1111/j.1758-2229.2012.00357.x

Rashid, M., Fakruddin, M., Mazumdar, R. M., Kaniz, F., and Chowdhury, A. (2014). Anti-bacterial activity of pigments isolated from pigment-forming soil bacteria. *Br. J. Pharm. Res.* 4, 880–894. doi: 10.9734/BJPR/2014/5148

Reddy, G. S. N., Prakash, J. S. S., Prabahar, V., Matsumoto, G. I., Stackebrandt, E., and Shivaji, S. (2003). Kocuria polaris sp. nov., an orange-pigmented psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. *Int. J. Syst. Evol. Microbiol.* 53, 183–187. doi: 10.1099/ijs.0.02336-0

Rokem, J. S., Vongsangnak, W., and Nielsen, J. (2011). Comparative metabolic capabilities for *Micrococcus luteus* NCTC 2665, the "Fleming" strain, and actinobacteria. *Biotechnol. Bioeng.* 108, 2770–2775. doi: 10.1002/bit.23212

Rosselló-Móra, R., and Amann, R. (2015). Past and future species definitions for Bacteria and Archaea. *Syst. Appl. Microbiol.* 38, 209–216. doi: 10.1016/j.syapm.2015.02.001

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Salomon, C. E., Magarvey, N. A., and Sherman, D. H. (2004). Merging the potential of microbial genetics with biological and chemical diversity: an even brighter future for marine natural product drug discovery. *Nat. Prod. Rep.* 21, 105–121. doi: 10.1039/b301384g

Sen, A., Daubin, V., Abrouk, D., Gifford, I., Berry, A. M., and Normand, P. (2014). Phylogeny of the class *Actinobacteria* revisited in the light of complete genomes. The orders "*Frankiales*" and *Micrococcales* should be split into coherent entities: proposal of *Frankiales* ord. nov., *Geodermatophilales* ord. nov., *Acidothermales* ord. nov. an. *Int. J. Syst. Evol. Microbiol.* 64, 3821–3832. doi: 10.1099/ijs.0.063966-0

Sepúlveda, J., Pantoja, S., and Hughen, K. A. (2011). Sources and distribution of organic matter in northern Patagonia fjords, Chile (~44–47°S): a multi-tracer approach for carbon cycling assessment. *Cont. Shelf Res.* 31, 315–329. doi: 10.1016/j.csr.2010.05.013

Ser, H. L., Palanisamy, U. D., Yin, W. F., Abd Malek, S. N., Chan, K. G., Goh, B. H., et al. (2015). Presence of antioxidative agent, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- in newly isolated *Streptomyces mangrovisoli* sp. nov. *Front. Microbiol.* 6:854. doi: 10.3389/fmicb.2015.00854

Ser, H. L., Tan, L. T. H., Palanisamy, U. D., Abd Malek, S. N., Yin, W. F., Chan, K. G., et al. (2016). *Streptomyces antioxidans* sp. nov., a novel mangrove soil actinobacterium with antioxidative and neuroprotective potentials. *Front. Microbiol.* 7:899. doi: 10.3389/fmicb.2016.00899

Silva, N. (2006). "Oxígeno disuelto, pH y nutrientes en canales y fiordos australes," in *Avances en el Conocimiento Oceanográfico de las Aguas Interiores Chilenas*, eds N. Silva and S. Palma (Valparaíso: Comité Oceanográfico Nacional). 37–43.

Sobarzo, M. (2009). "La Región de los fiordos de la zona sur de Chile," in *Aspectos Oceanográficos*, eds V. Häussermann and G. Försterra (Santiago: Nature in Focus), 6.

Stach, J. E. M., Maldonado, L. A., Ward, A. C., Goodfellow, M., and Bull, A. T. (2003). New primers for the class *Actinobacteria*: application to marine and terrestrial environments. *Environ. Microbiol.* 5, 828–841. doi: 10.1046/j.1462-2920.2003.00483.x

Staunton, J., and Wilkinson, B. (1997). Biosynthesis of Erythromycin and Rapamycin. *Chem. Rev.* 97, 2611–2630. doi: 10.1021/cr9600316

Subramani, R., and Aalbersberg, W. (2012). Marine actinomycetes: an ongoing source of novel bioactive metabolites. *Microbiol. Res.* 167, 571–580. doi: 10.1016/j.micres.2012.06.005

Sun, W., Zhang, F., He, L., Karthik, L., and Li, Z. (2015). Actinomycetes from the South China Sea sponges: isolation, diversity, and potential for aromatic polyketides discovery. *Front. Microbiol.* 6:148. doi: 10.3389/fmicb.2015.01048

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Ugalde, J. A., Gallardo, M. J., Belmar, C., Muñoz, P., Ruiz-Tagle, N., Ferrada-Fuentes, S., et al. (2013). Microbial life in a fjord: metagenomic analysis of a microbial mat in Chilean patagonia. *PLoS ONE* 8:e71952. doi: 10.1371/journal.pone.0071952

Vardhan Reddy, P. V., Shiva Nageswara Rao, S. S., Pratibha, M. S., Sailaja, B., Kavya, B., Manorama, R. R., et al. (2009). Bacterial diversity and bioprospecting for cold-active enzymes from culturable bacteria associated with sediment from a melt water stream of Midtre Lovenbreen glacier, an Arctic glacier. *Res. Microbiol.* 160, 538–546. doi: 10.1016/j.resmic.2009.08.008

Wang, Z., Xu, J., Li, Y., Wang, K., Wang, Y., Hong, Q., et al. (2010). *Rhodococcus jialingiae* sp. nov., an actinobacterium isolated from sludge of a carbendazim wastewater treatment facility. *Int. J. Syst. Evol. Microbiol.* 60, 378–381. doi: 10.1099/ijs.0.013219-0

Ward, A. C., and Bora, N. (2006). Diversity and biogeography of marine actinobacteria. *Curr. Opin. Microbiol.* 9, 279–286. doi: 10.1016/j.mib.2006.04.004

Wietz, M., Månsson, M., Bowman, J. S., Blom, N., Ng, Y., and Gram, L. (2012). Wide distribution of closely related, antibiotic-producing *Arthrobacter* strains

throughout the Arctic Ocean. *Appl. Environ. Microbiol.* 78, 2039–2042. doi: 10.1128/AEM.07096-11

Wu, Z., Xie, L., Xia, G., Zhang, J., Nie, Y., Hu, J., et al. (2005). A new tetrodotoxin-producing actinomycete, *Nocardiopsis dassonvillei*, isolated from the ovaries of puffer fish *Fugu rubripes*. *Toxicon* 45, 851–859. doi: 10.1016/j.toxicon.2005.02.005

Yuan, M., Yu, Y., Li, H.-R., Dong, N., and Zhang, X.-H. (2014). Phylogenetic diversity and biological activity of actinobacteria isolated from the Chukchi Shelf marine sediments in the Arctic Ocean. *Mar. Drugs* 12, 1281–1297. doi: 10.3390/md12031281

Zhang, H., Lee, Y. K., Zhang, W., and Lee, H. K. (2006). Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Antonie Van Leeuwenhoek* 90, 159–169. doi: 10.1007/s10482-006-9070-1

Zhang, X.-Y., He, F., Wang, G.-H., Bao, J., Xu, X.-Y., and Qi, S.-H. (2013). Diversity and antibacterial activity of culturable actinobacteria isolated from five species of the South China Sea gorgonian corals. *World J. Microbiol. Biotechnol.* 29, 1107–1116. doi: 10.1007/s11274-013-1279-3

Zotchev, S. B. (2012). Marine actinomycetes as an emerging resource for the drug development pipelines. *J. Biotechnol.* 158, 168–175. doi: 10.1016/j.jbiotec.2011.06.002

Zotchev, S. B. (2014). "Genomics-based insights into the evolution of secondary metabolite biosynthesis in actinomycete bacteria," in *Evolutionary Biology: Genome Evolution, Speciation, Coevolution and Origin of Life*, ed P. Pontarotti (Cham: Springer International Publishing), 35–46.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.1.1 Addendum Chapter I

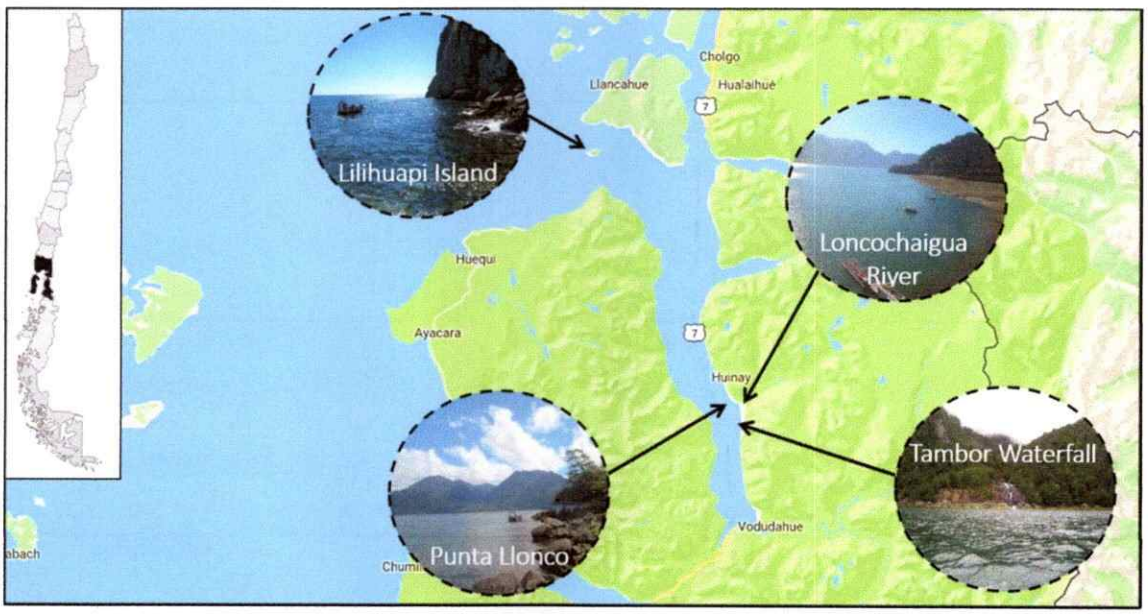


Figure 5: Sampling sites in the Comau fjord. Marine sediment samples were obtained from four different locations in the Comau fjord in Northern Chilean Patagonia (Inset: Chilean map; Los Lagos, X Region, depicted in black). Marine sediments were collected from Lilihuapi Island (42° 20,634'S; 72° 27,429'W) Lloncochaigua River (42° 22,37'S; 72° 27,25'W), Punta Llonco (42° 22,32'S; 72° 25,45'W) and Tambor Waterfall (42° 24,161'S; 72° 25,235'W).

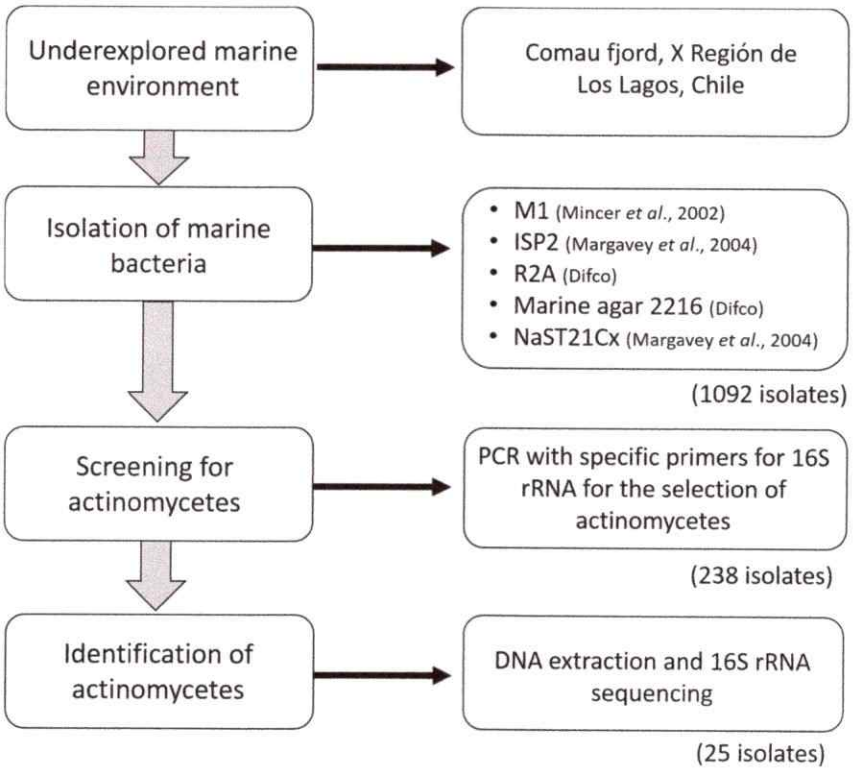


Figure 6: Strategy for culture-dependent identification of actinomycetes. Isolation of bacteria was performed with several different media using marine sediment samples obtained from Comau fjord. After 3 months-period incubation, a total of 1092 isolates were retrieved. Selection of actinomycetes was performed through PCR, and subsequent 16S rRNA gene sequencing led to the identification, at the genus level, of 25 marine actinomycetes strains. For culturable actinomycetes diversity obtained, please see **Figure 2** of **Chapter I**.

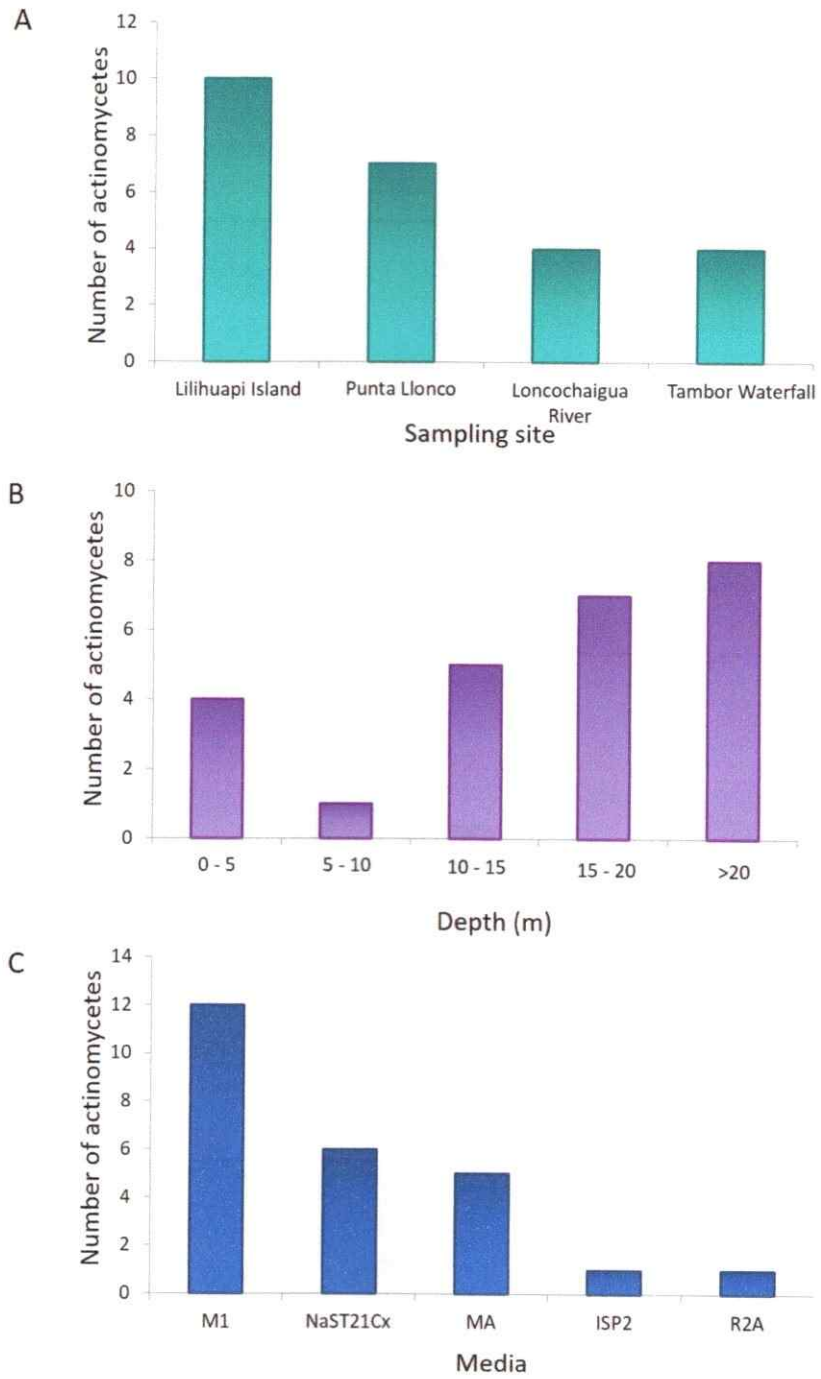


Figure 7: Actinomycetes obtained from environmental sampling. Number of actinomycetes strains according to: **A)** sampling site; **B)** marine sediment depth; **C)** selective media used for isolation of marine bacteria.

Table 1. Actinomycetes isolated in this study. The location of origin, depth of the marine sediment and media used for isolation is shown for each actinomycete strain.

STRAIN	GENUS IDENTIFICATION	SAMPLING SITE	DEPTH (m)	MEDIA
H-CA8B	*Arthrobacter* sp.	Lilihuapi Island	22.9	M1
H-JH1	*Arthrobacter* sp.	Lilihuapi Island	11.3	MA
H-JH3	*Arthrobacter* sp.	Lilihuapi Island	11.3	MA
H-CA4	*Brachybacterium* sp.	Lilihuapi Island	22.9	M1
H-CD1	*Brachybacterium* sp.	Tambor Waterfall	15.6	M1
H-CE9	*Brachybacterium* sp.	Lilihuapi Island	22.9	M1
H-CF1	*Brachybacterium* sp.	Lilihuapi Island	19.3	M1
H-CG1	*Brachybacterium* sp.	Tambor Waterfall	6.1	M1
H-BE7	*Brevibacterium* sp.	Punta Lloncó	25.1	M1
H-EH3	*Corynebacterium* sp.	Lloncochaigua River	0.25	R2A
H-KF5	*Corynebacterium* sp.	Lilihuapi Island	11.3	MA
H-BE10	*Curtobacterium* sp.	Punta Llonco	25.1	M1
H-CD9A	*Curtobacterium* sp.	Punta Llonco	14.5	M1
H-ED12	*Curtobacterium* sp.	Punta Llonco	25.1	ISP2
H-KA4	*Dietzia* sp.	Tambor Waterfall	15.6	NaST21Cx
H-KA9	*Kocuria* sp.	Lilihuapi Island	19.3	NaST21Cx
H-KB5	*Kocuria* sp.	Lloncochaigua River	0.25	NaST21Cx
H-KB6	*Kocuria* sp.	Lloncochaigua River	0.25	NaST21Cx
H-KA10	*Kocuria* sp.	Punta Llonco	25.1	NaST21Cx
H-KB1	*Kocuria* sp.	Lloncochaigua River	0.25	NaST21Cx
H-JH7	*Kocuria* sp.	Lilihuapi Island	11.3	MA
H-CD9B	*Micrococcus* sp.	Punta Llonco	14.5	M1
H-CA8F	*Rhodococcus* sp.	Lilihuapi Island	22.9	M1
H-KF8	*Streptomyces* sp.	Punta Llonco	14.5	MA
H-CB3	*Streptomyces* sp.	Tambor Waterfall	15.6	M1

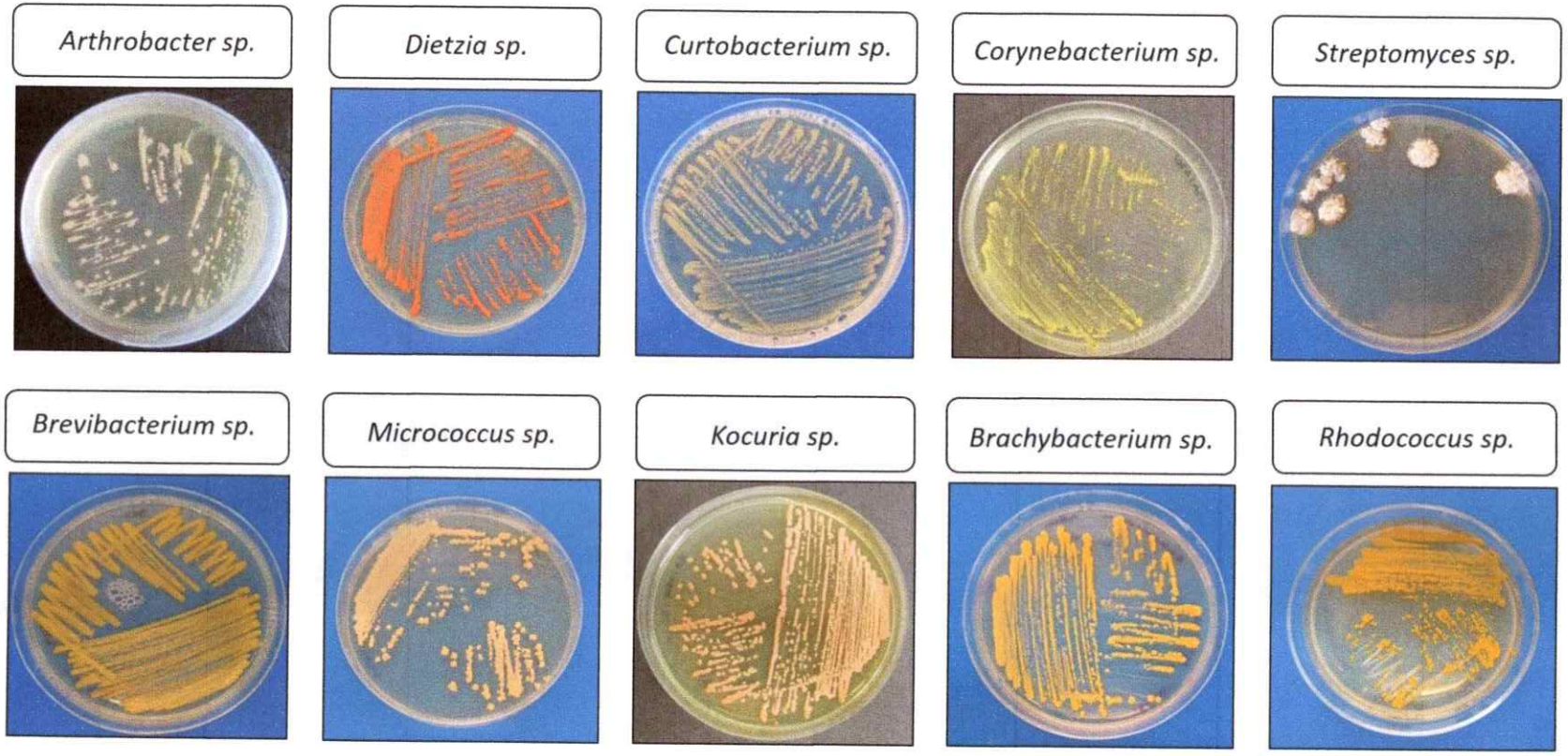


Figure 8: Morphological characterization of actinomycetes isolated in this study. Representative strains of actinomycetes that were isolated from marine sediments of Comau fjord. In total, 10 different genera were retrieved using a culture-dependent approach. Physiological characterization of colony pigmentation and morphology was studied in several nutrient-rich media and growth-conditions, such as incubation temperature, salinity tolerance and sea water requirement.

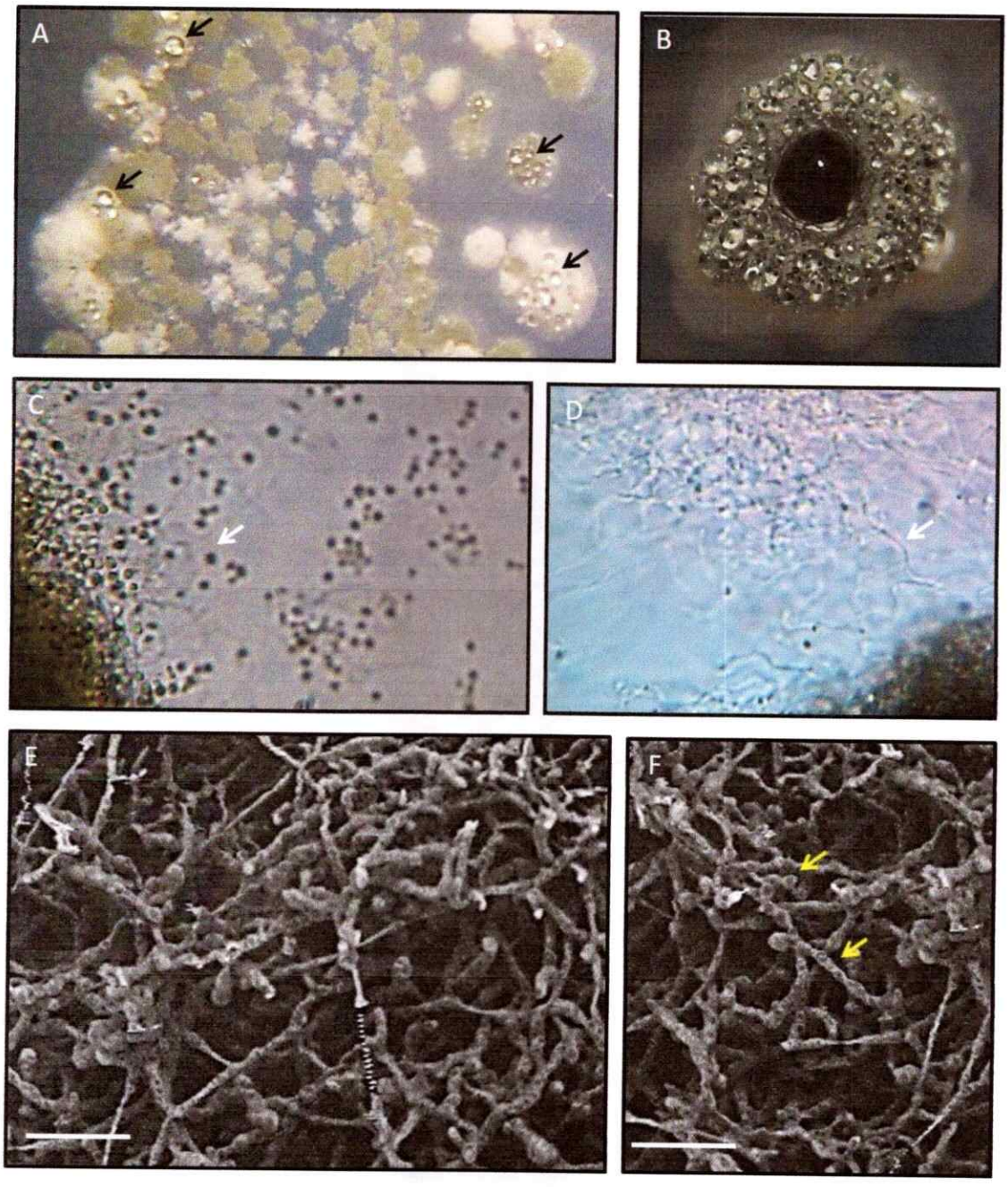


Figure 9: Microscopic characterization of *Streptomyces* sp. H-KF8. Black arrows show droplet exudates; White arrows show spore and hyphae, respectively; yellow arrows show spore chain assembly. **A)** Stereoscopic image of Colony growth in ISP2-ASW agar after 15 days of incubation **B)** Stereoscopic image of colony grown on Marine agar after 20 days of incubation. **C)** Optic microscopic image at 1000X of aerial mycelium from ISP2 medium. **D)** Optic microscopic image at 1000X of vegetative mycelium from ISP3 medium. **E)** Low Voltage Electron Microscopy (LVEM) of mycelium from ISP3 medium. Scale bar, 10 µm. **F)** LVEM of spore chain assembly from ISP3 medium. Scale bar, 10 µm. To see other related pictures, see **Figure 2**, **Chapter III**.

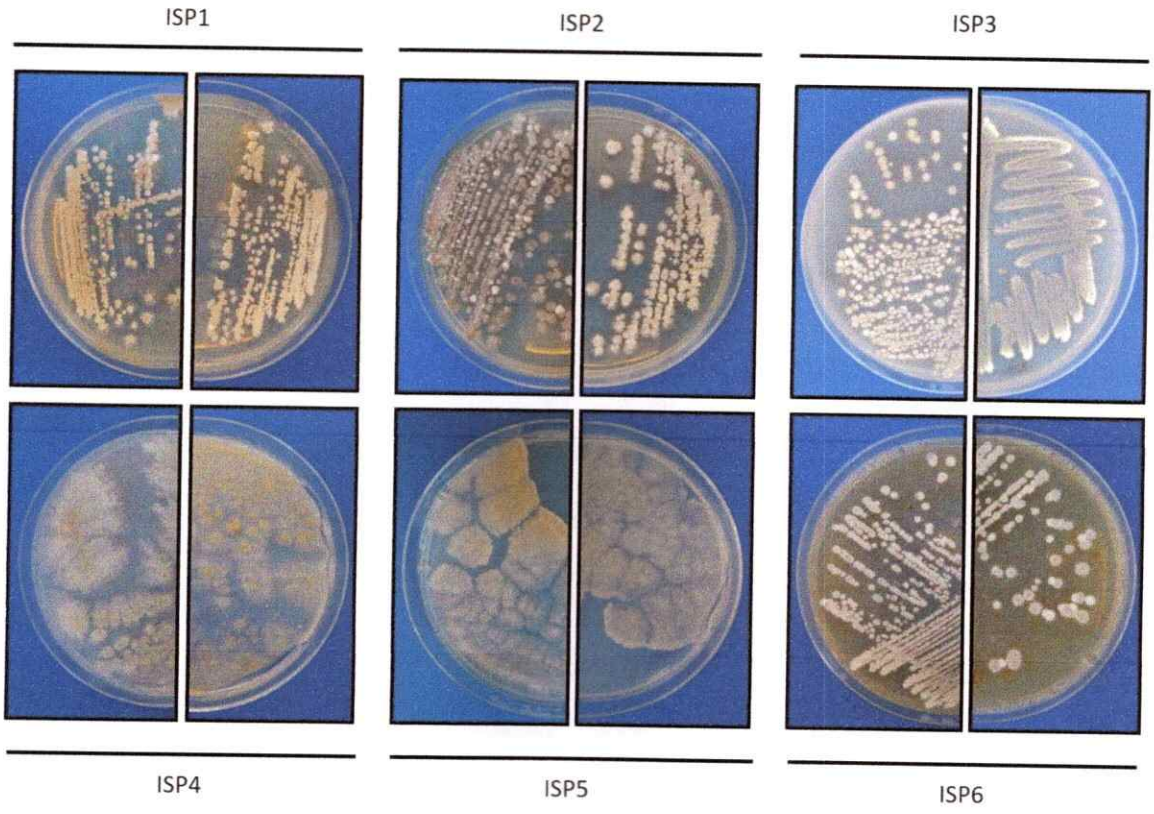


Figure 10: Physiological characterization of *Streptomyces* isolates in ISP media. Left panel, *Streptomyces* sp. H-CB3; right panel, *Streptomyces* sp. H-KF8. ISP media are prepared as a standard for *Streptomyces* isolates, as standard based on the **I**nternational ***S**treptomyces* **P**roject. Composition of media is as follows: ISP1, tryptone + yeast extract; ISP2, yeast extract + malt extract + dextrose; ISP3, oatmeal; ISP4, starch + inorganic salts; ISP5, L-asparragine + glycerol + K_2HPO_4; ISP6, peptone iron agar + yeast extract.

Table 2. Physiological characterization of actinomycetes: growth at different temperatures. Actinomycetes were grown on TSA-ASW medium and growth was monitored up to 15 days (until 3 months for 4°C).

STRAIN	GENERA	4 °C	20 °C	30 °C	37 °C	45 °C
H-JH3	*Arthrobacter* sp.	+	+	+	+	-
H-CG1	*Brachybacterium* sp.	+	+	+	+	-
H-BE7	*Brevibacterium* sp.	+	+	+	+	-
H-EH3	*Corynebacterium* sp.	-	+	+	+	-
H-ED12	*Curtobacterium* sp.	-	+	+	+	+
H-KB5	*Kocuria* sp.	+	+	+	+	-
H-KA4	*Dietzia* sp.	+	+	+	+	-
H-CD9B	*Micrococcus* sp.	-	+	+	+	-
H-CA8F	*Rhodococcus* sp.	+	+	+	-	-
K-HF8	*Streptomyces* sp.	+	+	+	+	-
H-CB3	*Streptomyces* sp.	+	+	+	+	-

Table 3. Physiological characterization of actinomycetes: tolerance to salinity (% NaCl). Actinomycetes were grown on LB-MQ medium and growth was monitored up to 15 days.

STRAIN	GENERA	0 %	1 %	3.5 %	5 %	7 %	10 %	20 %
H-JH3	*Arthrobacter* sp.	+	+	+	+	+	+	-
H-CG1	*Brachybacterium* sp.	+	+	+	+	+	+	-
H-BE7	*Brevibacterium* sp.	+	+	+	+	+	+	-
H-EH3	*Corynebacterium* sp.	-	-	-	-	+	-	-
H-ED12	*Curtobacterium* sp.	+	+	+	+	+	+	-
H-KB5	*Kocuria* sp.	+	+	+	+	+	+	-
H-KA4	*Dietzia* sp.	+	+	+	+	+	-	-
H-CD9B	*Micrococcus* sp.	+	+	+	+	+	-	-
H-CA8F	*Rhodococcus* sp.	+	-	-	-	-	-	-
K-HF8	*Streptomyces* sp.	+	+	+	+	+	-	-
H-CB3	*Streptomyces* sp.	+	+	+	+	+	-	-

Table 4. Physiological characterization of actiobacteria: Artificial sea water (ASW) requirement. Actinomycetes were grown on modified ISP2 medium and growth was monitored up to 15 days.

STRAIN	GENERA	ISP2-MQ	ISP2-ASW	ISP2-3.5 % NaCl
H-JH3	*Arthrobacter* sp.	-	+	-
H-CG1	*Brachybacterium* sp.	-	+	-
H-BE7	*Brevibacterium* sp.	+/-	+	+
H-EH3	*Corynebacterium* sp.	-	+	-
H-ED12	*Curtobacterium* sp.	+	+	+
H-KB5	*Kocuria* sp.	-	+	-
H-KA4	*Dietzia* sp.	-	+	-
H-CD9B	*Micrococcus* sp.	-	-	-
H-CA8F	*Rhodococcus* sp.	-	+	-
K-HF8	*Streptomyces* sp.	-	+	-
H-CB3	*Streptomyces* sp.	-	+	-

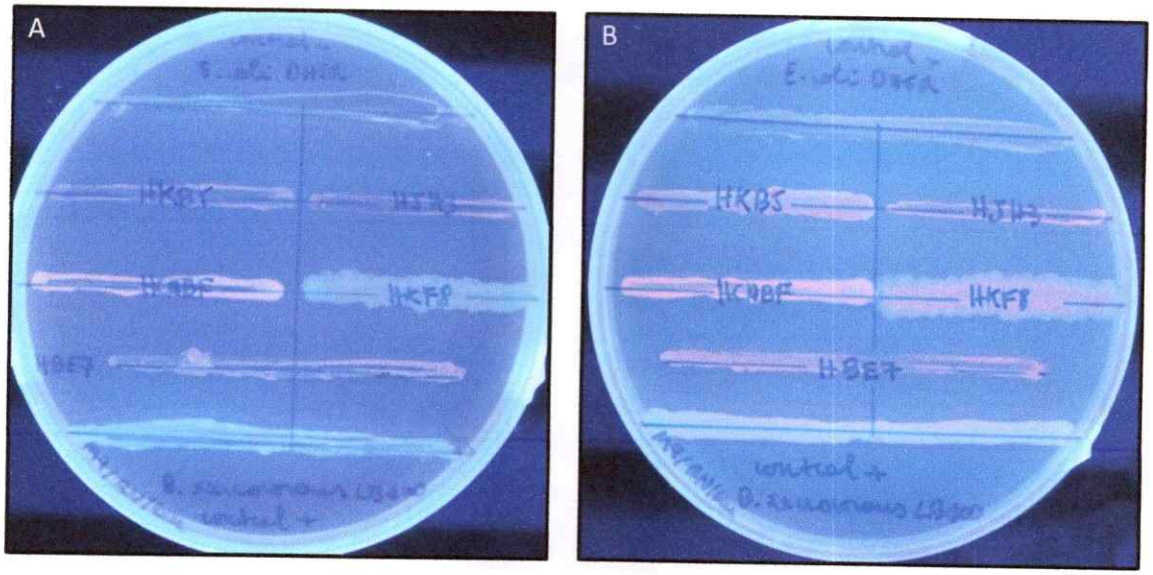


Figure 11: Biochemical characterization of PHAs production. Fluorescence of selected isolates in the polyhydroxyalkanoates (PHAs) assay. M9 medium was prepared with nile red and glucose as carbon source. A red fluorescence from the colonies is appreciated when the bacterium has the ability to produce polyhydroxyalkanoates (Spiekermann *et al.*, 1999). First line, *Escherichia coli* DH5α as a negative control (no red fluorescence). Second line, left: *Kocuria* sp. H-KB5; right: *Arthrobacter* sp. H-JH3. Third line, left: *Rhodococcus* sp. H-CA8F; right: *Streptomyces* sp. H-KF8. Fourth line, *Brevibacterium* sp. H-BE7. Fifth line, *Burkholderia xenovorans* LB400 as a positive control (red fluorescence). **A)** 24 h of incubation. **B)** 72 h of incubation.

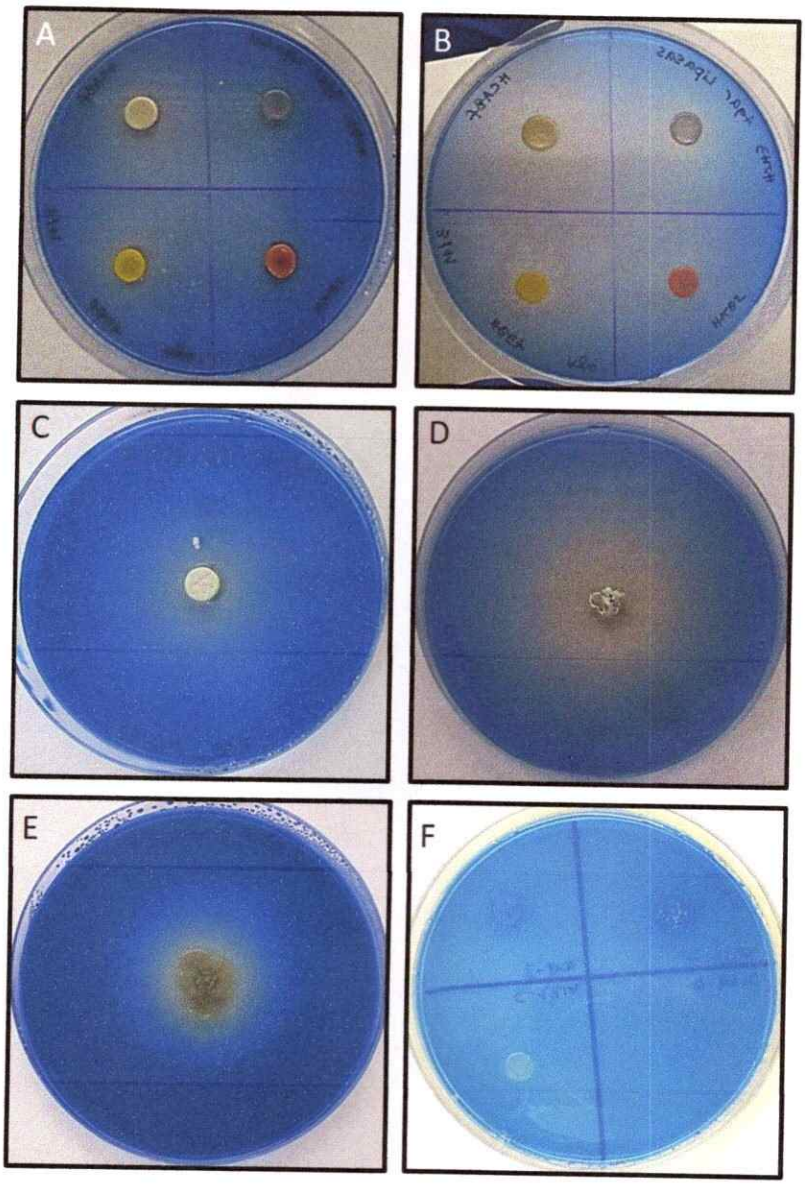
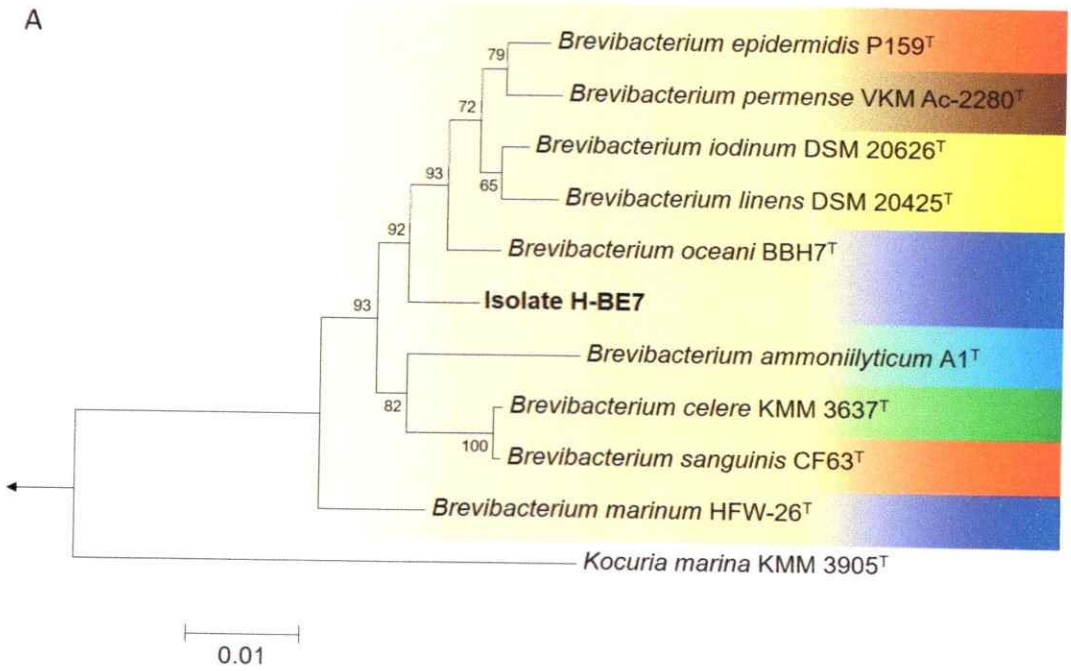
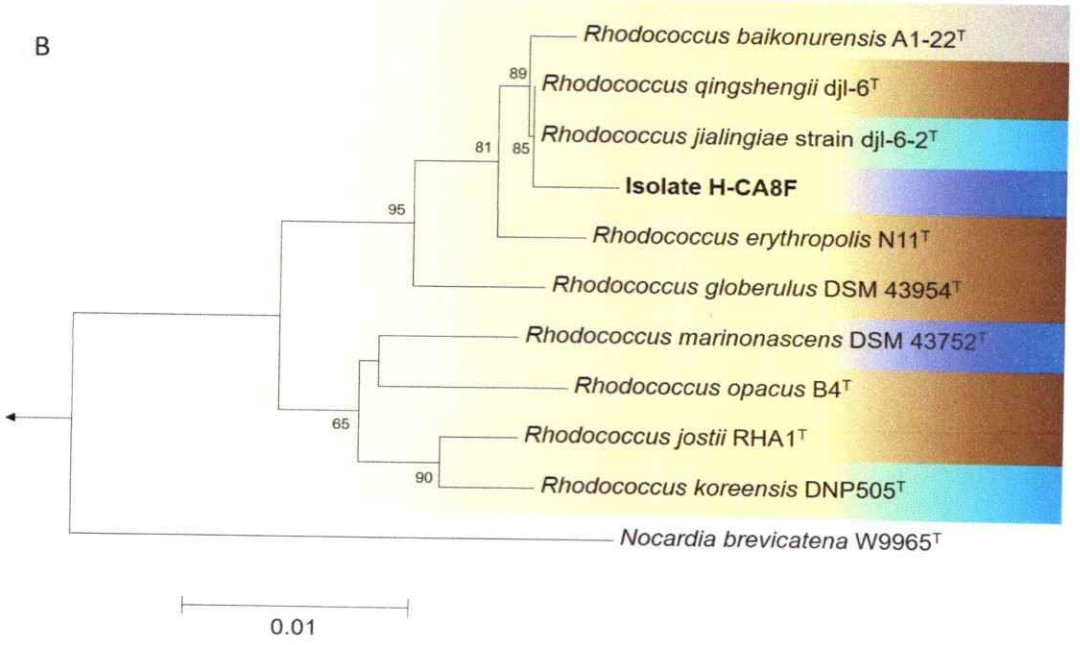


Figure 12: Biochemical characterization. Lipase activity assay of selected isolates using the Spirit Blue agar and tributyrin as substrate. A white halo surrounding the colony is visible when the assay is positive due to the utilization of the substrate and therefore, presence of active lipases can be suggested. On the contrary, agar maintains it blue colour if assay is negative. **A)** After 24 h of growth; First line: *Rhodococcus* sp. H-CA8F (left) and *Arthrobacter* sp. H-JH3 (right). Second line: *Brevibacterium* sp. H-BE7 (left) and *Kocuria* sp. H-KB5 (right). **B)** After 72 h of growth; First line: *Rhodococcus* sp. H-CA8F (left) and *Arthrobacter* sp. H-JH3 (right). Second line: *Brevibacterium* sp. H-BE7 (left) and *Kocuria* sp. H-KB5 (right). **C)** *Streptomyces* sp. H-KF8 after 24 h of growth. **D)** *Streptomyces* sp. H-KF8 after 72 h of growth. **D)** *Pseudomonas fluorescens* CHAO as positive control **E)** Actinomycetes from our culture collection obtained from Valparaiso as negative control.


A
Brevibacterium epidermidis P159T
Brevibacterium permense VKM Ac-2280T
Brevibacterium iodinum DSM 20626T
Brevibacterium linens DSM 20425T
Brevibacterium oceani BBH7T
Isolate H-BE7
Brevibacterium ammoniilyticum A1T
Brevibacterium celere KMM 3637T
Brevibacterium sanguinis CF63T
Brevibacterium marinum HFW-26T
Kocuria marina KMM 3905T
79
72
93
65
92
93
82
100
0.01


B
Rhodococcus baikonurensis A1-22T
Rhodococcus qingshengii djl-6T
Rhodococcus jialingiae strain djl-6-2T
Isolate H-CA8F
Rhodococcus erythropolis N11T
Rhodococcus globerulus DSM 43954T
Rhodococcus marinonascens DSM 43752T
Rhodococcus opacus B4T
Rhodococcus jostii RHA1T
Rhodococcus koreensis DNP505T
Nocardia brevicatena W9965T
89
81
85
95
65
90
0.01

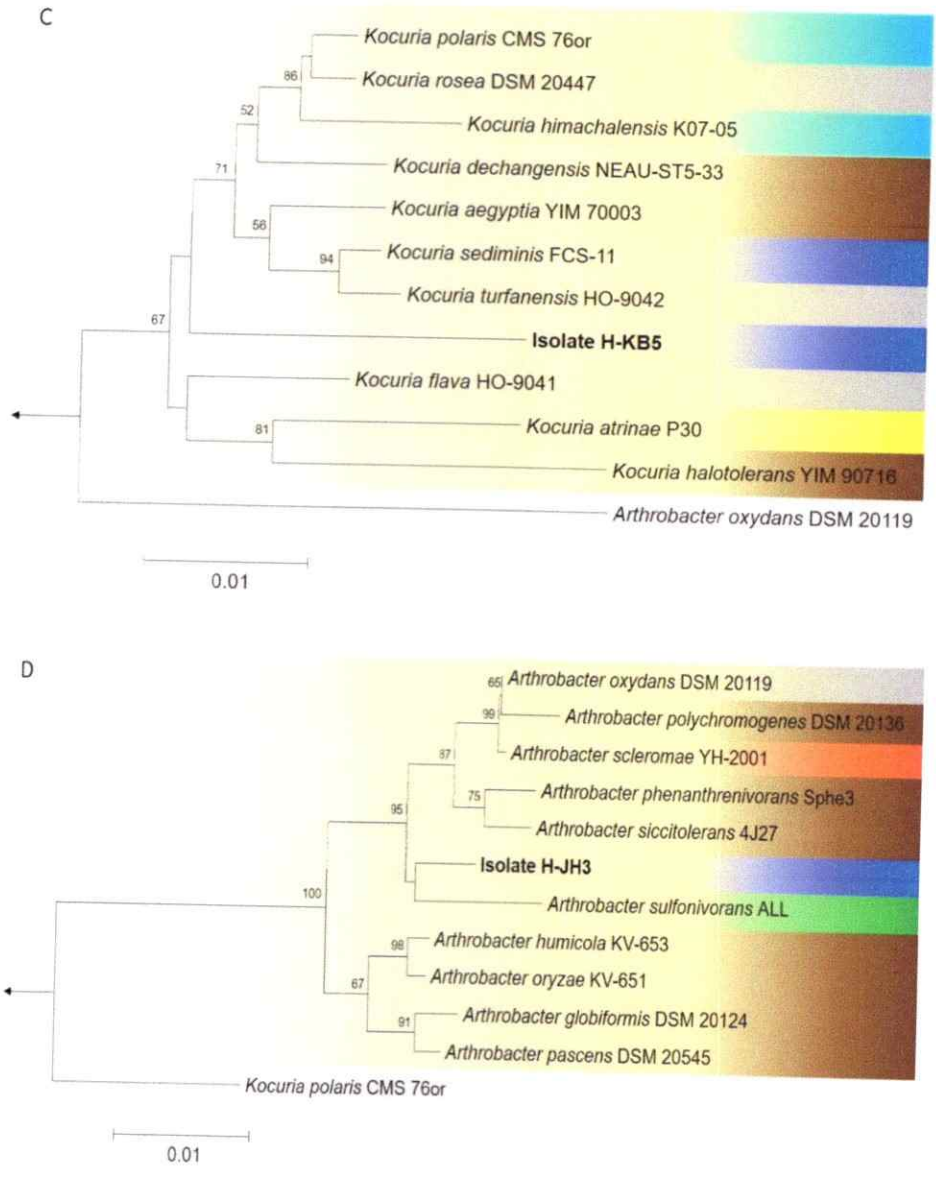


Figure 13: Phylogenetic relationships of non-myceliar actinomycetes. Neighbour-joining tree of 16S rRNA gene. Node numbers represent the percentage of bootstrap replicates (1000 resampling) which supported the proposed branching order shown at consistent nodes (values below 50% are not shown). Arrow points to the main outgroup *E. coli* K12 (AP012306) for comparison all four trees. A second outgroup was used for each genus, which is not coloured. Scale bar corresponds to 0.01 substitutions per nucleotide positions. Colours depict the source of isolation: blue, marine; light blue, fresh water; turquoise, polar; brown, soil; orange, clinical; green, rhizosphere; gray, air; yellow, food. **A)** *Brevibacterium* sp. strain H-BE7; **B)** *Rhodococcus* sp. strain H-CA8F; **C)** *Kocuria* sp. strain H-KB5; **D)** *Arthrobacter* sp. strain H-JH3.

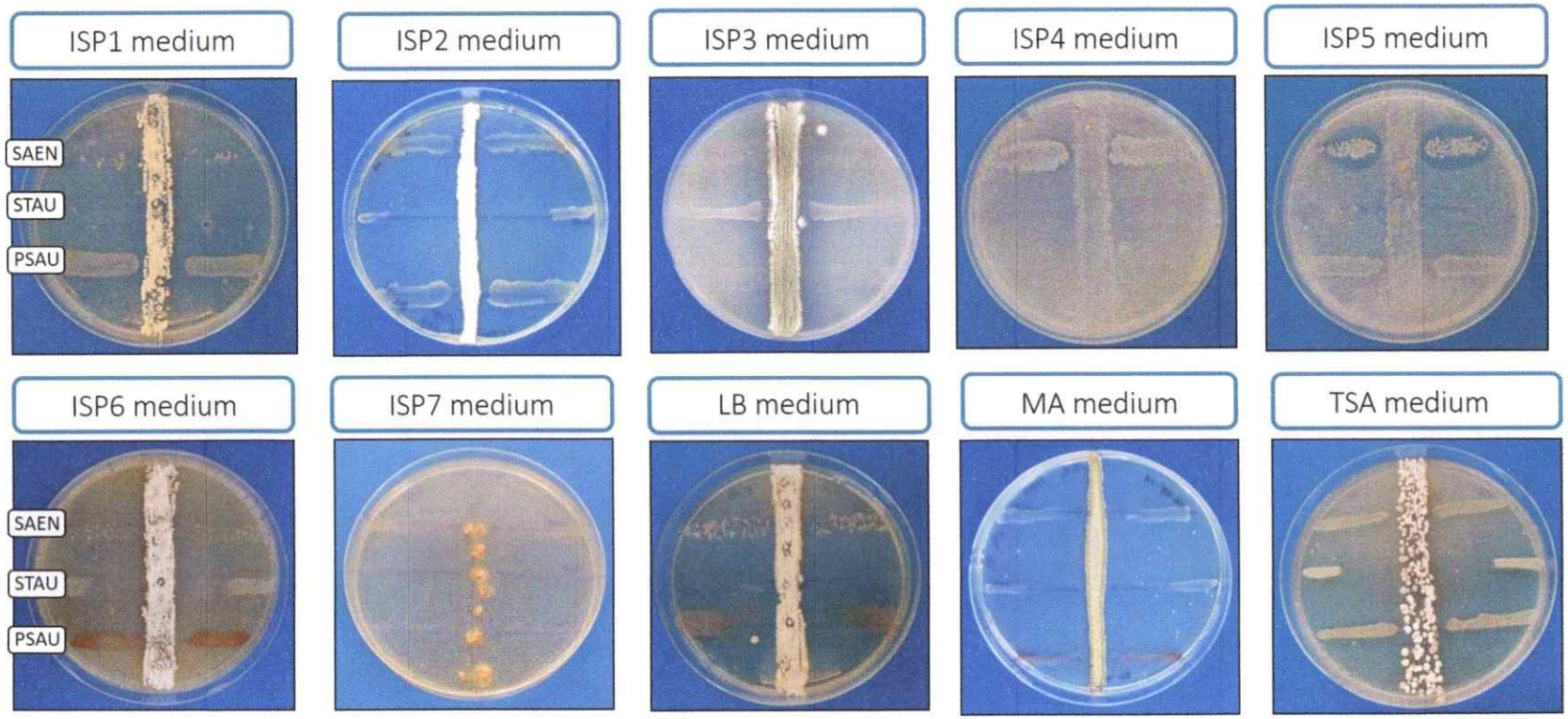


Figure 14: Antimicrobial activity assay for *Streptomyces* sp. H-KF8. Cross-streak method against reference bacterial strains in several media for *Streptomyces* sp. H-KF8 (see **Chapter I** for methods). For complete results of all Comau fjord strains, please see **Table 1** and **Figure 3** of **Chapter I**. For complete results of antimicrobial activity of *Streptomyces* sp. H-KF8 strain in all media, please see **Table 1** of **Chapter III**. For further antimicrobial activity analysis considering results in liquid media, please see **Table 2** of **Chapter I**. All media are supplemented with ASW, with exception of MA medium (Marine Agar). Bacterial reference strains are as follows: SAEN, *S. enterica* LT2^{T}; STAU, *S. aureus* NBRC 100910^{T}; PSAU, *P. aeruginosa* DSM 50071^{T}.

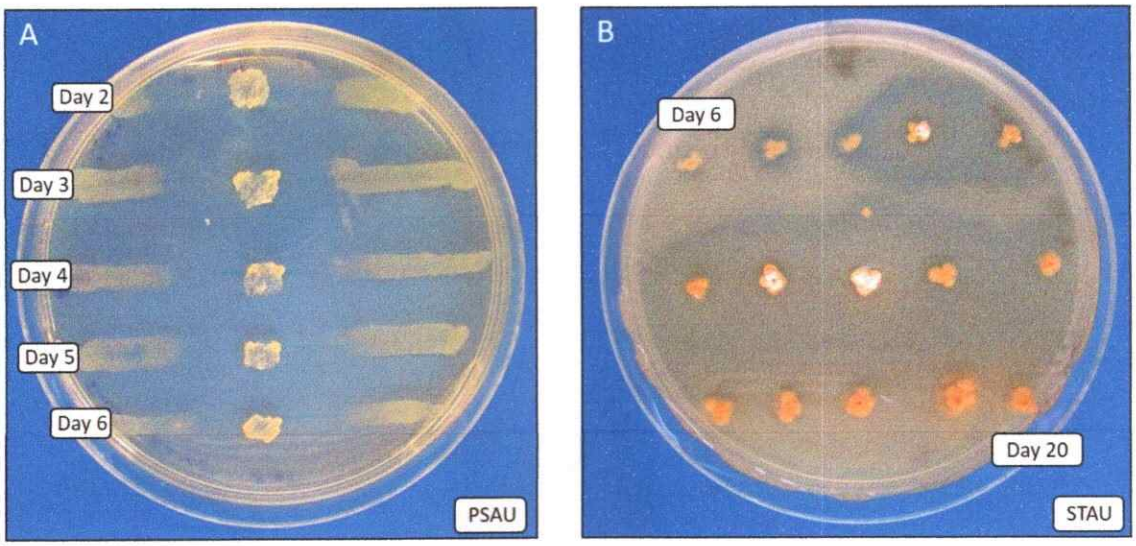


Figure 15: Antimicrobial activity using Time Course Assay. Time course was performed with the aim to elucidate the starting day of antimicrobial synthesis by the producing strain. Bioactive secondary metabolites are often produced after primary growth phase, which is subsequently dependent of the growth rate of each strain. **A)** For non-mycelial strains, macrocolonies were incubated for different days (from 2 to 6 days) at the middle of the plate, and the last day the bacterial reference strains were streak in a perpendicular way. As an example, activity of *Arthrobacter* sp. H-JH3 against *P. aeruginosa* DSM 50071^T in ISP2 medium is shown, where activity can be seen from day 2, onwards. **B)** For mycelial strains, macrocolonies were incubated several days (from 6 to 20 days) making three lines in the plate, and the last day the bacterial reference strain was placed above using the double-layer method. As an example, activity of *Streptomyces* sp. H-KF8 against *S. aureus* NBRC 100910^T in TSA-ASW medium is shown, where activity can be seen from day 9, onwards. For time course with one colony per plate, see **Figure 4, Chapter III**.

Chapter II: Genome sequence of *Streptomyces* sp. H-KF8, a marine actinobacteria isolated from a Northern Chilean Patagonian fjord

jga-genome/jga99917//jga4888d17z | xppws | S=3 | 1/3/17 | 23:16 | ArtID: | DOI:10.1128/genomeA.01645-16

Editor: | Section: **Prokaryotes** | Designation:

AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements™

PROKARYOTES

Genome Sequence of *Streptomyces* sp. H-KF8, a Marine actinobacteria Isolated from a Northern Chilean Patagonian Fjord

Agustina Undabarrena,[a] Juan Antonio Ugalde,[b] Eduardo Castro-Nallar,[c] Michael Seeger,[a] Beatriz Cámara[a]

Laboratorio de Microbiología Molecular y Biotecnología Ambiental, Departamento de Química & Centro de Biotecnología Daniel Alkalay Lowitt Universidad Técnica Federico Santa María, Valparaíso, Chile[a]; Centro de Genética y Genómica, Facultad de Medicina, Clínica Alemana Universidad del Desarrollo, Santiago, Chile[b]; Centro de Bioinformática y Biología Integrativa, Facultad de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile[c]

ABSTRACT *Streptomyces* sp. H-KF8 is a fjord-derived marine actinobacteria capable of producing antimicrobial activity. *Streptomyces* sp. H-KF8 was isolated from sediments of the Comau fjord located in the northern Chilean Patagonia. Here, we report the 7.7-Mb genome assembly, which represents the first genome of a Chilean marine actinobacteria.

Received 5 December 2016 **Accepted** 6 December 2016 **Published** 9 February 2017

Citation Undabarrena A, Ugalde JA, Castro-Nallar E, Seeger M, Cámara B. 2017. Genome sequence of *Streptomyces* sp. H-KF8, a marine actinobacteria isolated from a northern Chilean Patagonian fjord. Genome Announc 5:e01645-16. https://doi.org/10.1128/genomeA.01645-16.

Address correspondence to Beatriz Cámara, beatriz.camara@usm.cl.

Members of the genus *Streptomyces* are widely recognized for producing a plethora of bioactive secondary metabolites with antimicrobial, antifungal, and antitumoral properties (1). As marine environments are markedly different from terrestrial ones, it has been proposed that marine *Streptomyces* spp. may produce different types of bioactive compounds in comparison to their terrestrial counterparts (2).

The extensive coast of Chile is especially attractive for exploring marine actinobacterial communities. The remote Comau fjord, located in the northern Chilean Patagonia is a marine-protected area suitable for bioprospection due to its unique geologic nature. There are scarce reports within this area that involve the characterization of microbial communities from water samples (3), underwater microbial mats (4), and terrestrial hot spring mats (5, 6). Previously, we aimed to isolate actinobacteria from marine sediments obtained from various coastal locations at different depths and evaluate their antimicrobial potential (7). Among these, the marine actinobacteria *Streptomyces* sp. H-KF8, isolated from 15-m-deep marine sediments obtained from Punta Llonco, Comau fjord, was selected for whole-genome sequencing. A prominent activity against *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* was previously determined (7), and therefore, *Streptomyces* sp. H-KF8 is an interesting candidate to explore for drug discovery.

DNA extraction was performed with the Wizard Genomic DNA extraction kit (Promega). Next-generation sequencing data were provided by Macrogen and generated by Illumina HiSeq2000 (paired-end library of 2 × 100 bp) and PacBio (library construction of 5-kb average size and one SMRT cell with a P5-C3 chemistry) technologies. The whole genome was *de novo* assembled using Canu version 1.1 (8) and consisted of 11 scaffolds represented in one linear chromosome with a total of 7,684,888 bp, a GC content of 72.1%, and coverage of 500× (N_{50}, 4,115,122 bp; mean read length, 698,626 bp). The genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 3.1 (https://www.ncbi.nlm.nih.gov/genome/annotation_prok), leading to a total of 6,574 genes assigned as follows: 6,486 coding sequences, 67 tRNAs, 18 rRNAs, and three ncRNAs.

jga-genome/jga99917/jga4888d17z | xppws | S=3 | 1/3/17 | 23:16 | ArtID: | DOI:10.1128/genomeA.01645-16

An antiSMASH version 3.0 search (9) led to the identification of 26 biosynthetic gene clusters (BGCs) for secondary metabolites, including two polyketide synthases (PKSs), two nonribosomal peptides synthetases (NRPSs), and four hybrid PKS-NRPSs, which may be involved in the antimicrobial activity previously observed (7). Only 23% of the clusters have 100% similarity with other known BGCs. Further genomic analysis of these secondary metabolism routes may provide insights into the production of interesting candidates for natural product discovery.

To our knowledge, this is the first report of whole-genome sequencing of a marine *Streptomyces* strain in Chile, which may assist future comparative genomics studies. Next-generation sequencing techniques play a fundamental role in elucidating the biotechnological potential of environmental isolates, revealing their cryptic genetic features.

Accession number(s). The *Streptomyces* sp. H-KF8 genome sequence was deposited in GenBank under the accession number LWAB00000000.

ACKNOWLEDGMENTS

We thank the scuba divers and staff from the Huinay Scientific Field Station. This is publication number 147 of the Huinay Scientific Field Station.

A.U. acknowledges a Conicyt PhD fellowship and Conicyt Gastos Operacionales number 21120621. This work was supported by the Comisión Nacional de Investigación Científica y Tecnológica FONDECYT 11121571 (B.C.), and by CONICYT Fondecyt Iniciación grant 11140666 (J.A.U.) and grant 11160905 (E.C.N.); and Vetenskapsrådet (Swedish Research Council) number 2013-6713 (B.C.).

REFERENCES

1. Bérdy J. 2012. Thoughts and facts about antibiotics: where we are now and where we are heading. J Antibiot 65:441. https://doi.org/10.1038/ja.2012.54.
2. Manivasagan P, Venkatesan J, Sivakumar K, Kim SK. 2013. Marine actinobacterial metabolites: current status and future perspectives. Microbiol Res 168:311–332. https://doi.org/10.1016/j.micres.2013.02.002.
3. Elizondo-Patrone C, Hernández K, Yannicelli B, Olsen LM, Molina V. 2015. The response of nitrifying microbial assemblages to ammonium (NH_4^+) enrichment from salmon farm activities in a northern Chilean fjord. Estuar Coast Shelf Sci 166:131–142. https://doi.org/10.1016/j.ecss.2015.03.021.
4. Ugalde JA, Gallardo MJ, Belmar C, Muñoz P, Ruiz-Tagle N, Ferrada-Fuentes S, Espinoza C, Allen EE, Gallardo VA. 2013. Microbial life in a fjord: metagenomic analysis of a microbial mat in Chilean Patagonia. PLoS One 8:e71952. https://doi.org/10.1371/journal.pone.0071952.
5. Mackenzie R, Pedrós-Alió C, Díez B. 2013. Bacterial composition of microbial mats in hot springs in Northern Patagonia: variations with seasons and temperature. Extremophiles 17:123–136. https://doi.org/10.1007/s00792-012-0499-z.
6. Estrella Alcamán M, Fernandez C, Delgado A, Bergman B, Díez B. 2015. The cyanobacterium *Mastigocladus* fulfills the nitrogen demand of a terrestrial hot spring microbial mat. ISME J 9:2290–2303. https://doi.org/10.1038/ismej.2015.63.
7. Undabarrena A, Beltrametti F, Claverías FP, González M, Moore ER, Seeger M, Cámara B. 2016. Exploring the diversity and antimicrobial potential of marine *Actinobacteria* from the Comau fjord in northern Patagonia, Chile. Front Microbiol 7:1135. https://doi.org/10.3389/fmicb.2016.01135.
8. Berlin K, Koren S, Chin CS, Drake JP, Landolin JM, Phillippy AM. 2015. Assembling large genomes with single-molecule sequencing and locality-sensitive hashing. Nat Biotechnol 33:623–630. https://doi.org/10.1038/nbt.3238.
9. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243. https://doi.org/10.1093/nar/gkv437.

5.2.1 Addendum Chapter II

Table 5. Detection by PCR of PKS and NRPS biosynthetic genes in actinomycetes.

STRAIN	GENERA	PKS I	PKS II	PKS III	NRPS
H-JH3	*Arthrobacter* sp.	-	-	+	+
H-CG1	*Brachybacterium* sp.	+	+	-	+
H-BE7	*Brevibacterium* sp.	+	+	+	+
H-EH3	*Corynebacterium* sp.	-	+	-	+
H-ED12	*Curtobacterium* sp.	-	+	-	+
H-KB5	*Kocuria* sp.	-	+	+	+
H-KA4	*Dietzia* sp.	-	+	-	+
H-CD9B	*Micrococcus* sp.	-	-	-	-
H-CA8F	*Rhodococcus* sp.	-	+	-	+
K-HF8	*Streptomyces* sp.	-	+	+	+
H-CB3	*Streptomyces* sp.	-	+	+	+

*+, amplicon detected in the estimated size; -, amplicon detected in another size or absence of amplicon

Table 6. General features of whole genome from selected non-mycelial strains using Illumina sequencing.

STRAIN	GENERA	N° OF READS	LENGTH (bp avg)	ASSEMBLY LENGTH (bp)	CONTIGS	N50
H-KB5	*Kocuria* sp.	925702	232	3885308	521	14406
H-JH3	*Arthrobacter* sp.	1071832	279	2924959	58	109786
H-BE7	*Brevibacterium* sp.	902960	244	4125106	33	265313
H-CA8F	*Rhodococcus* sp.	725730	248	6468486	34	518650

*avg, average

Table 7. Number of biosynthethic genes (BGCs) detected in non-mycelial strains sequenced. A special focus in the number of NRPS metabolic routes is shown for each strain.

STRAIN	GENERA	TOTAL BGCs (N°)	NRPS (%)
H-KB5	*Kocuria* sp.	9	0
H-JH3	*Arthrobacter* sp.	1	0
H-BE7	*Brevibacterium* sp.	6	1 (16%)
H-CA8F	*Rhodococcus* sp.	21	9 (43%)

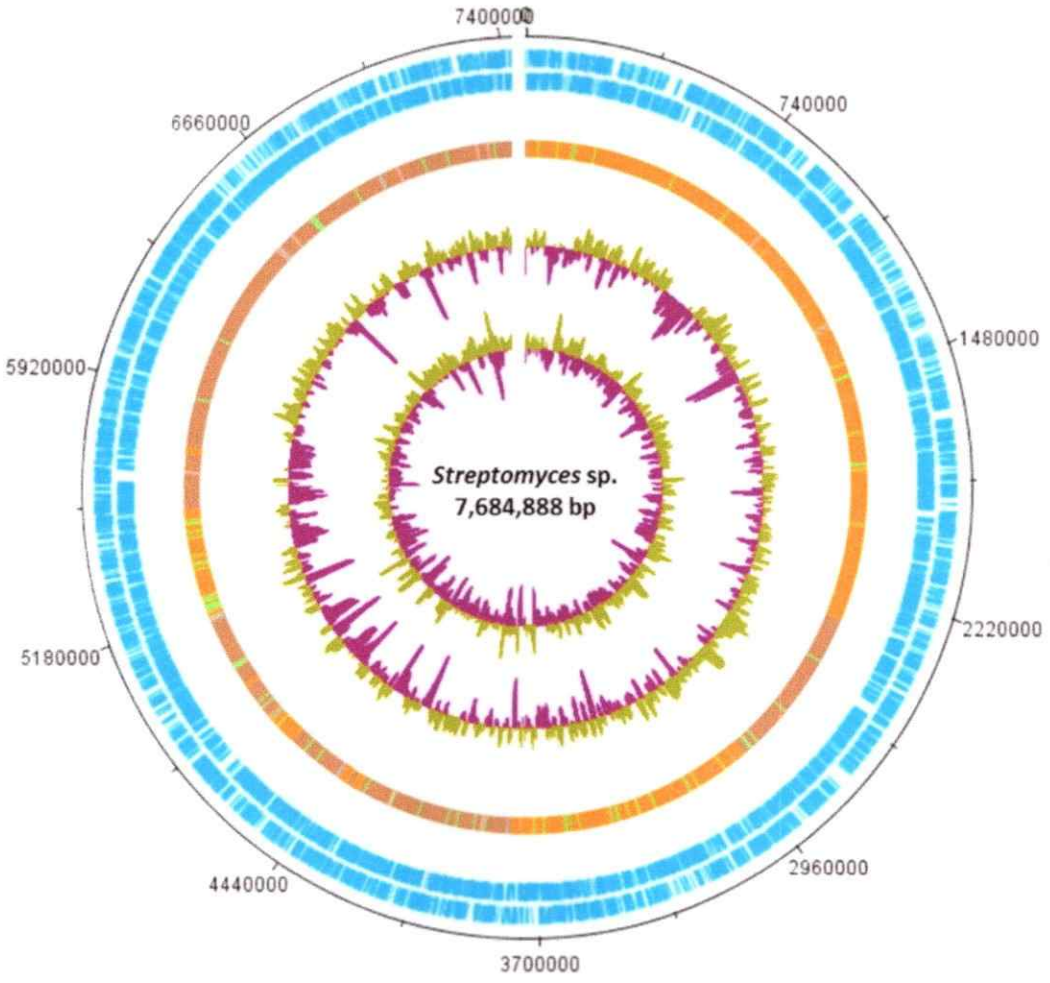


Figure 16: Whole genome sequencing of *Streptomyces* sp. H-KF8. Circular representation of the linear chromosome consisting of 11 contigs, covering the 7.7 Mbp genome. From outside inward: DNA strands reverse and forward; contigs, GC content, GC skew. Complete genome properties and general features are described in **Chapter II**. For BGCs location around the genome, see **Figure 6**, **Chapter III**.

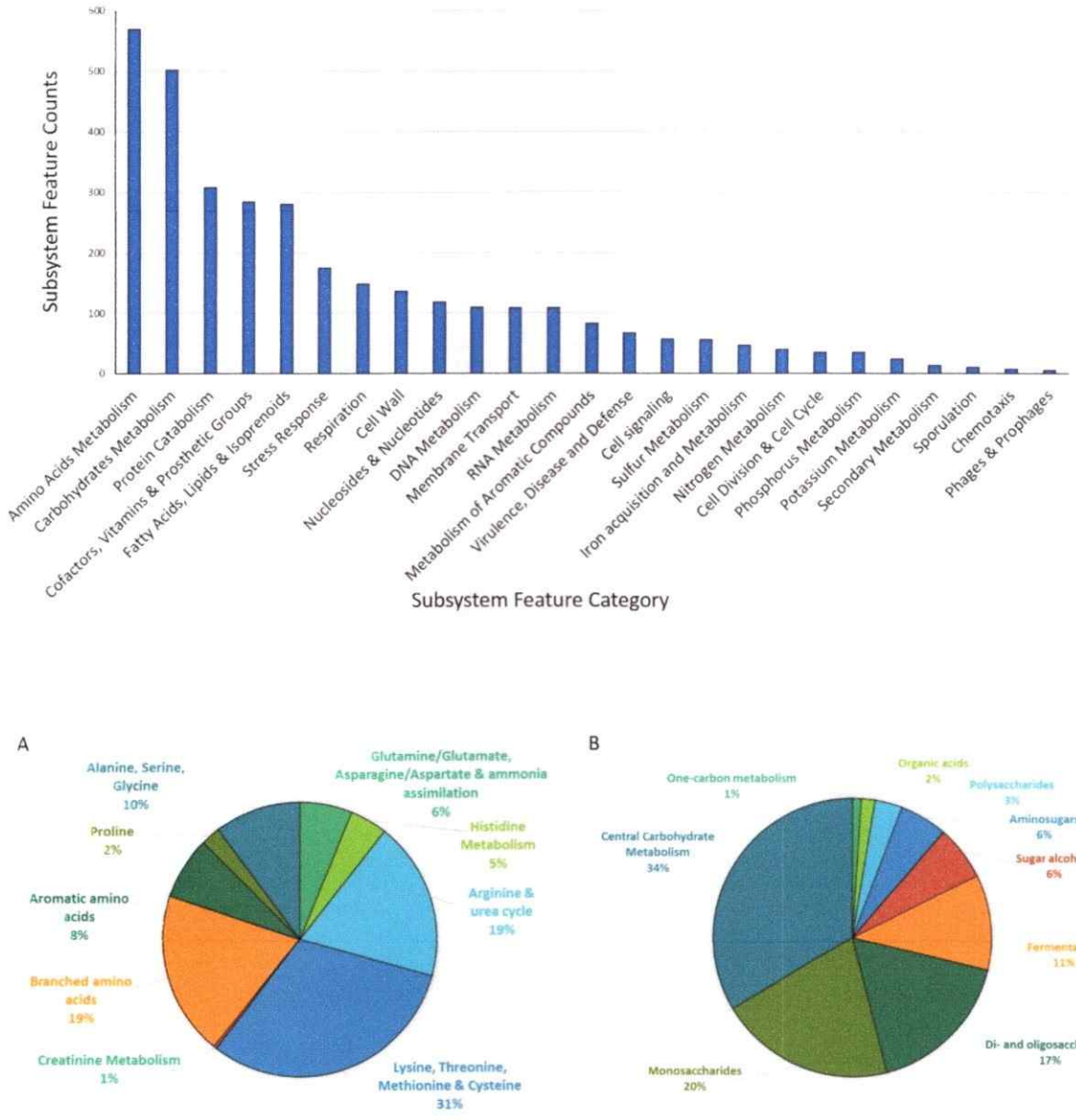


Figure 17: Categories of coding genes among *Streptomyces* sp. H-KF8 genome sequence, grouped in RAST subsystem categories. Coding DNA sequences (CDS) were also grouped by Cluster of Orthologous Groups (COGs) categories, which are shown in **Table 2** from **Chapter III**. In addition, comparison of COGs with two other *Streptomyces* strains, one from terrestrial- and the other from marine-derived sources is presented in **Figure 4**, **Chapter III**. Upper panel: Most abundant subsystem categories are represented by the amino acid metabolism and carbohydrates metabolism, which detail is present in the bottom panel. **A)** Amino acid metabolism. **B)** Carbohydrate metabolism.

Chapter III: Genomic data mining of the marine actinobacteria *Streptomyces* sp. H-KF8 unveils insights into multi-stress related genes and metabolic pathways involved in antimicrobial synthesis

PeerJ

Genomic data mining of the marine actinobacteria *Streptomyces* sp. H-KF8 unveils insights into multi-stress related genes and metabolic pathways involved in antimicrobial synthesis

Agustina Undabarrena[1], Juan A. Ugalde[2,3], Michael Seeger[1] and Beatriz Cámara[1]

[1] Departmento de Química y Centro de Biotecnología, Universidad Técnica Federico Santa María, Valparaiso, Chile

[2] Centro de Genética y Genómica, Facultad de Medicina Clinica Alemana, Universidad del Desarrollo, Santiago, Chile

[3] Programa de Genómica Microbiana, Facultad de Medicina Clinica Alemana, Universidad del Desarrollo, Santiago, Chile

Submitted 13 September 2016
Accepted 14 December 2016
Published 25 January 2017

Corresponding author
Beatriz Cámara,
beatriz.camara@usm.cl

Academic editor
Valeria Souza

Additional Information and Declarations can be found on page 22

DOI 10.7717/peerj.2912

ABSTRACT

Streptomyces sp. H-KF8 is an actinobacterial strain isolated from marine sediments of a Chilean Patagonian fjord. Morphological characterization together with antibacterial activity was assessed in various culture media, revealing a carbon-source dependent activity mainly against Gram-positive bacteria (*S. aureus* and *L. monocytogenes*). Genome mining of this antibacterial-producing bacterium revealed the presence of 26 biosynthetic gene clusters (BGCs) for secondary metabolites, where among them, 81% have low similarities with known BGCs. In addition, a genomic search in *Streptomyces* sp. H-KF8 unveiled the presence of a wide variety of genetic determinants related to heavy metal resistance (49 genes), oxidative stress (69 genes) and antibiotic resistance (97 genes). This study revealed that the marine-derived *Streptomyces* sp. H-KF8 bacterium has the capability to tolerate a diverse set of heavy metals such as copper, cobalt, mercury, chromate and nickel; as well as the highly toxic tellurite, a feature first time described for *Streptomyces*. In addition, *Streptomyces* sp. H-KF8 possesses a major resistance towards oxidative stress, in comparison to the soil reference strain *Streptomyces violaceoruber* A3(2). Moreover, *Streptomyces* sp. H-KF8 showed resistance to 88% of the antibiotics tested, indicating overall, a strong response to several abiotic stressors. The combination of these biological traits confirms the metabolic versatility of *Streptomyces* sp. H-KF8, a genetically well-prepared microorganism with the ability to confront the dynamics of the fiord-unique marine environment.

Subjects Genomics, Microbiology

Keywords Marine actinomycete, Genome mining, *Streptomyces*, Biosynthethic gene clusters, Antimicrobial activity, Heavy metal tolerance, Abiotic stressors, Chilean Patagonian fjord

INTRODUCTION

There has been a burst of genomic data in recent years due to the advances in various technologies such as next-generation sequencing. Whole genome sequencing is providing

information-rich data that can hugely contribute and orientate the discovery of natural products in microorganisms. Indeed genome mining has been positioned as a fundamental bioinformatics-approach in the natural product field (*McAlpine et al., 2005*; *Van Lanen & Shen, 2006*; *Challis, 2008*; *Doroghazi & Metcalf, 2013*; *Jensen et al., 2014*; *Antoraz et al., 2015*; *Tang et al., 2015a*; *Tang et al., 2015b*; *Katz & Baltz, 2016*). Natural products have clearly demonstrated to play a significant role in drug discovery, in fact 78% of antibiotics marketed during 1982–2002 originated from natural products (*Peláez, 2006*). Considering the year 2014, 25% of the approved new chemical entities were from natural or natural-derived products (*Newman & Cragg, 2016*). In natural environments, these metabolites also play important roles as signal molecules, facilitating intra- or inter-species interactions within microbial communities related to virulence, colonization, motility, stress response and biofilm formation (*Romero et al., 2012*).

Streptomyces are mycelium-forming bacteria with a complex developmental life cycle that includes sporulation and programmed cell death processes (*Flärdh & Buttner, 2009*; *Yagüe et al., 2013*). Their unsurpassed richness and diversity concerning secondary metabolism pathways has made them valuable providers for bioactive molecules, being responsible for two-thirds of all known antibiotics (*Bérdy, 2012*). Genome mining has become a powerful tool to unveil the biotechnological potential of *Streptomyces* species, where biosynthetic gene clusters (BGCs) can be identified (*Weber et al., 2015*) and even predict the chemical core structure of the molecules. Unlike other bacteria, *Streptomyces* have linear chromosomes (*Chen et al., 2002*) and their genome sizes are within the largest in the bacterial world (*Weber et al., 2003*), ranging from 6.2 Mb for *Streptomyces cattleya* NRRL 8057 (*Barbe et al., 2011*) to 12.7 Mb for *Streptomyces rapamycinicus* NRRL 5491 (*Baranasic et al., 2013*), considering complete sequenced genomes to date (*Kim et al., 2015*). Up to 5% of their genomes are devoted to the synthesis of secondary metabolites (*Ikeda et al., 2003*). The ability to produce a wide variety of bioactive molecules is based on the fact that they contain the largest numbers of BGCs such as polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS), or even PKS-NRPS hybrids (*Challis, 2008*). The genes required for secondary metabolites biosynthesis are typically clustered together (*Zazopoulos et al., 2003*) and are tightly regulated both by specific regulation of each product (*Bibb & Hesketh, 2009*) or by pleiotropic mechanisms of regulation that can control several pathways at the same time (*Martin & Liras, 2012*). Due to these interesting properties, nearly 600 species and 30,000 strains of *Streptomyces* have been identified (*Euzéby, 2011*). To date, 653 *Streptomyces* genome assemblies are available in GenBank database (*Studholme, 2016*) and this number is likely to keep increasing.

Although soil microorganisms from the *Streptomyces* genus have generated vast interest due to their exceptional role as antibiotic producers (*Bérdy, 2012*), their marine counterpart has been less explored. The marine ecosystem is highly diverse, with extreme abiotic selective pressures and immense biological diversity (*Lam, 2006*). In addition, many marine organisms have a sessile life style, needing chemical weapons for defense and survival (*Haefner, 2003*). Thus, research in natural products has been focusing on the isolation of microorganisms from corals (*Hodges, Slattery & Olson, 2012*; *Kuang et al., 2015*; *Mahmoud & Kalendar, 2016*; *Pham et al., 2016*), sponges (*Kim, Garson & Fuerst,*

2005; Montalvo et al., 2005; Zhang et al., 2006; Jiang et al., 2007; Vicente et al., 2013; Sun et al., 2015), as well as marine sediments (Mincer & Jensen, 2002; Magarvey et al., 2004; Jensen et al., 2005; León et al., 2007; Gontang, Fenical & Jensen, 2007; Duncan et al., 2014; Yuan et al., 2014). In spite of all the isolation studies associated to marine actinobacteria, relatively little is known about the molecular mechanisms behind bacterial adaptation to marine environments. It is supposed that marine actinobacteria have adapted through the development of specific biological traits (Tian et al., 2016), which has led to hypothesize that novel species from unexplored habitats may contain unique bioactive compounds (Axenov-Gribanov et al., 2016). In addition, marine habitats are under a dramatic pollution increase, where heavy metals have demonstrated to be one of the most negative causing impacts in living beings. While many metals (iron, zinc, manganese, copper, cobalt, nickel, vanadium, molybdenum) are essential micronutrients for enzymes and cofactors, they still are toxic when available in high concentrations, causing adversary effects mainly by oxidative stress damage to fundamental macromolecules (Schmidt et al., 2005). In this context, marine microorganisms have developed mechanisms through molecular adaptations in order to thrive in these adverse conditions. Moreover, secondary metabolites biosynthesis are strongly influenced by the presence and concentration of certain heavy metals in *Streptomyces* genus (Locatelli, Goo & Ulanova, 2016), and also oxidative stress can regulate antibiotic production (Kim et al., 2012; Beites et al., 2014) providing evidence of a molecular crosstalk response between these stressors.

In the South Pacific region, Chile has an extensive marine coast that remains mostly unexplored. Bioprospecting of actinobacteria for the discovery of novel marine-derived natural products, specifically antibiotics, has been carried out in Valparaíso Central Bay (Claverías et al., 2015) and in the Comau fjord in Northern Patagonia (Undabarrena et al., 2016a). Both sites proved to be a rich source for novel species of actinobacteria with antimicrobial properties. In this context, the genome of a selected antimicrobial-producer marine *Streptomyces* strain from Comau fjord was sequenced (Undabarrena et al., in press). In this study, we aimed to conduct a combined genomic, metabolic and physiological analysis of the marine *Streptomyces* sp. H-KF8 bacterium, through the further exploration of its antimicrobial activity and the genome mining of the BGCs encoded in its genome. In addition, the genetic and functional response to abiotic stressors such as oxidative stress, heavy metals and antibiotics, which may play an important role in the evolution of secondary metabolism genes, was evaluated in *Streptomyces* sp. H-KF8.

METHODS AND MATERIALS

Bacterium selection

Underwater samples were previously collected from marine sediments from the Marine Protected Area of the Comau fjord, in the Northern Chilean Patagonia (Undabarrena et al., 2016a). Fjords are especially attractive due to its unique biogeographic characteristics, being a deep narrow inlet with significantly eroded bottom and communication with open sea (Bredhold et al., 2007). Comau fjord is one of the deepest; it has a high precipitation rate crucial for fresh water input; where water surface temperatures ranges between 5

and >20 °C, sustaining a thermohaline circulation (*Bustamante, 2009*; *Sobarzo, 2009*). As microorganisms of these ecosystems may display remarkable genetic features of tolerance to the dynamics of these abiotic stressors, marine actinobacteria were isolated with several culture media and identified through 16S rRNA gene sequence (*Undabarrena et al., 2016a*). Antimicrobial potential was screened using two strategies, including assessing the antimicrobial activity of crude extracts derived from liquid cultures (*Undabarrena et al., 2016a*). *Streptomyces* sp. H-KF8 was selected due to its antimicrobial activities against *S. aureus, L. monocytogenes* and *E. coli* for whole genome sequencing, representing the first genome of Chilean marine actinobacteria (*Undabarrena et al., in press*).

Phenotypic characterization

Streptomyces sp. H-KF8 was characterized morphologically in several media agar plates: ISP1-ISP9 (Shirling & Gottlieb, 1966), Marine Agar (MA) 2216 (Difco) and Triptic Soy Agar (TSA) (Difco NO 236950). All media, with exception of MA, were prepared with artificial sea water (ASW) (*Kester et al., 1967*) as the strain has a specific ASW requirement for growth (*Undabarrena et al., 2016a*; *Undabarrena et al., in press*). Plates were incubated at 30 °C and visible colonies appeared after 5–7 days. Microscopic images were obtained with a Leica Zoom2000 stereoscope (Arquimed), Gram-staining was performed with an optical microscope L2000A (Arquimed) with 1,000× magnification, and unstained low voltage electron microscopy (LVEM) was used for high contrast images (Delong LVEM5 microscope, Universidad Andrés Bello, Chile) after 21 days of *Streptomyces* sp. H-KF8 growth in ISP3-ASW media (*Vilos et al., 2013*).

Antimicrobial activity

Antimicrobial activity was evaluated previously in ISP2 and TSA-ASW agar plates, and activity was corroborated by liquid culture derived crude extracts (*Undabarrena et al., 2016a*). In this study, a further evaluation of antimicrobial activity was assessed in 15 different media agar plates, to explore the relation between *Streptomyces* sp. H.KF8 morphology and antimicrobial activity. Various media were employed: ISP1-ISP9; MA; King B; Medium V (*Marcone et al., 2010*); LB-ASW; Actino Agar (Difco) and NaST21Cx (*Magarvey et al., 2004*), using cross-streak method as previously described (*Haber & Ilan, 2014*). The assay was slightly modified for marine actinobacteria by our group (*Claverías et al., 2015*; *Undabarrena et al., 2016a*). Antimicrobial activity was measured against five reference bacteria: *Staphylococcus aureus* NBRC 100910^T; *Listeria monocytogenes* 07PF0776; *Salmonella enterica* subsp enterica LT2^T; *Escherichia coli* FAP1 and *Pseudomonas aeruginosa* DSM50071^T. Briefly, inhibition zones were seen as part of the bacterial line where the reference bacteria did not grow, and ranked qualitatively as: −, no inhibition; ±, attenuated growth of target bacterium; +, <50% growth inhibition of target bacterium (1–5 mm of the line); ++, 50% growth inhibition of target bacterium (6–10 mm of the line); +++, >50% growth inhibition of target bacterium (≥11 mm of the line). All experiments were performed in duplicate, using as internal control one of the reference bacteria.

Additionally, the double-layer method (*Westerdahl et al., 1991*) was employed, in order to perform a time-course assay to ascertain the days of incubation where most activity was

being produced. *Streptomyces* sp. H-KF8 macrocolonies were incubated on ISP2-ASW, ISP3-ASW, TSA-ASW and MA agar plates. Macrocolonies were grown individually from five to 20 days on the same agar plate, and subsequently, 7 mL of modified-LB (7 g/L of agar instead of 15 g/L) with an aliquot of 100 μL of an overnight pre-grown *S. aureus* bacterial culture with an OD = 0.3 was added above the macrocolonies of *Streptomyces* sp. H-KF8. Inhibition zones were observed after incubation of plates for 24 h at 37 °C. If inhibition zones overlapped, the experiment was repeated on separate agar plates, where only one macrocolony in the center of the plate was incubated.

Genome Mining and Bioinformatic analysis

Streptomyces sp. H-KF8 whole genome sequencing was performed by Illumina and PacBio (*Undabarrena et al., in press*). Genome reads were *de novo* assembled using Canu (version 1.1) (*Berlin et al., 2015*) into 11 contigs, representing one linear chromosome of 7,684,888 bp genome. Full genome sequencing details can be found elsewhere (*Undabarrena et al., in press*). Gene calling an annotation was performed using the Prokaryotic Genome Annotation Pipeline (PGAP) at NCBI (version 3.1) (*Tatusova et al., 2016*). Genes were assigned to EggNOG categories (*Huerta-Cepas et al., 2016*) via an HMM search with HMMER3 (http://hmmer.org). Genetic determinants involved in biological traits analyzed in this report were manually established and the amino acidic signatures were validated based on domain hits through Basic Local Alignment Search Tool (BLAST) from NCBI. Also, BGCs were identified through AntiSMASH (version 3.0) online platform. Snapgene software (version 2.3.2) was used to visualize ORFs related to functional biological traits from each linear contig. Artemis software (version 16.0.0) was used to construct the graphic representation of the circular chromosome, and to assign by colors manually all the different categories of BGCs on it.

Functional response to Heavy Metal(loid)s

For metal-resistance experiments, agar plates containing filtered salts of several metal(loid) solutions were prepared. Metals were diluted to obtain the following final concentration in media plates: $CuSO_4$ (0.25 mM, 0.5 mM and 0.75 mM); $CoCl_2$ (2 mM, 4 mM and 6 mM); $ZnSO_4$ (50 mM and 100 mM); $CdCl_2$ (0.75 mM and 1.5 mM); $HgCl_2$ (20 μM, 40 μM and 60 μM); K_2TeO_3 (10 μM, 20 μM and 40 μM); K_2CrO_4 (10 μM, 17 μM and 20 μM); Na_2HAsO_4 (50 mM and 100 mM); $NaAsO_3$ (2.5 mM and 5 mM) and $NiSO_4$ (5 mM, 10 mM and 15 mM). *Streptomyces* sp. H-KF8 was evaluated after 5, 10 and 20 days of growth in TSA-ASW plates. Additionally, a special Minimal Medium (MM) used to evaluate metal resistance in *Streptomyces* spp. was prepared (*Schmidt et al., 2009*), modified with the addition of ASW. Experiment was performed with two biological replicates. Reference values for metal concentrations were decided based on metal-tolerance *Streptomyces* obtained from literature (*Schmidt et al., 2005*; *Schmidt et al., 2009*; *Wang et al., 2006*; *Polti, Amoroso & Abate, 2007*). Agar plates without addition of any metals were prepared as negative controls.

Functional response to oxidative stress

For oxidative stress experiments, tolerance to hydrogen peroxide (H_2O_2) at various concentrations (0.2 M, 0.5 M, 1 M, 2 M, and 4 M) was evaluated by directly adding 10 μL of the H_2O_2 solution to a sterile paper disk positioned on a TSA-ASW agar plate where *Streptomyces* sp. H-KF8 was streaked out to grow as a thin lawn (*Dela Cruz et al., 2010*). The model strain *Streptomyces violaceoruber* A3(2) (DSM 40783) was used to test the tolerance response. Inhibitions areas (cm^2) were observed after 5 days of growth at 30 °C. Experiment was performed with three biological replicates, and standard deviation was calculated. A statistical analysis by Student's *t*-Test was carried out considering a *p*-value <0.01.

Functional response Antibiotics

Susceptibility to model antibiotics of *Streptomyces* sp. H-KF8 was explored previously (*Undabarrena et al., 2016a*). However, in this report a further characterization was pursued. *Streptomyces* sp. H-KF8 was grown on Mueller-Hinton agar plates prepared with ASW (MH-ASW) and commercial standard disks of model antibiotics were placed above. The following antibiotics were tested: Amoxicillin 25 μM, Bacitracin 0.09 IU, Novobiocin 5 μ g and Erythromycin 15 μg (LabClín); Optochin 5 μg (BritaniaLab); Clindamycin 2 μg, Oxacillin 1 μg, Ciprofloxacin 5 μg, Ceftriaxone 30 μg, Chloramphenicol 30 μg, Penicillin 10 UOF, Cefotaxime 30 μg, Gentamicin 10 μg and Ampicillin 10 μg (Valtek). After 5 days of incubation at 30 °C, radios of the inhibition halos were measured, and inhibition areas (cm^2) were calculated. Data was compared with standarized cut off values from Clinical and Laboratory Standards Institute (CLSI) from year 2016, to determine susceptibility or resistance against each antibiotic tested. Experiments were performed using three biological replicates, and standard deviation was calculated for each antibiotic.

RESULTS

Phenotypic characterization

Morphological analysis of *Streptomyces* sp. H-KF8 was carried out by strain growth in several media, containing different carbon sources (Fig. 1; inset colony morphology). Growth of *Streptomyces* sp. H-KF8 was observed in the standard ISP1-ISP9 agar plates, although differences in growth rates and pigmentation were noticed (Figs. 1A–1F). On ISP1 (yeast extract, pancreatic digest of casein), ISP2 (yeast extract, malt extract, dextrose) and ISP6 (peptone, yeast extract and iron) media, white mycelia was observed, with appearance of grayish-spores after 14 days of growth. In contrast, when *Streptomyces* sp. H-KF8 was grown on ISP3 (outmeal), ISP4 (soluble starch and inorganic salts), ISP5 (glycerol and asparagine) and ISP9 (glucose) media, creamy mycelia was observed, with appearance of white spores at the periphery of the colonies. In contrast, poor growth was observed in ISP7 (tyrosine) medium. A different morphology was perceived when *Streptomyces* sp. H-KF8 was grown on MA medium (Fig. 1G). Colony size was comparatively smaller (5.06 ± 1.1 mm in ISP2 vs 3.12 ± 0.78mm in MA; $p < 0.01$), and a dark-grey turning into black pigmentation was noticed within 5 days of growth. On TSA-ASW plates, a white mycelium was observed with no change in pigmentation over time, but with presence of

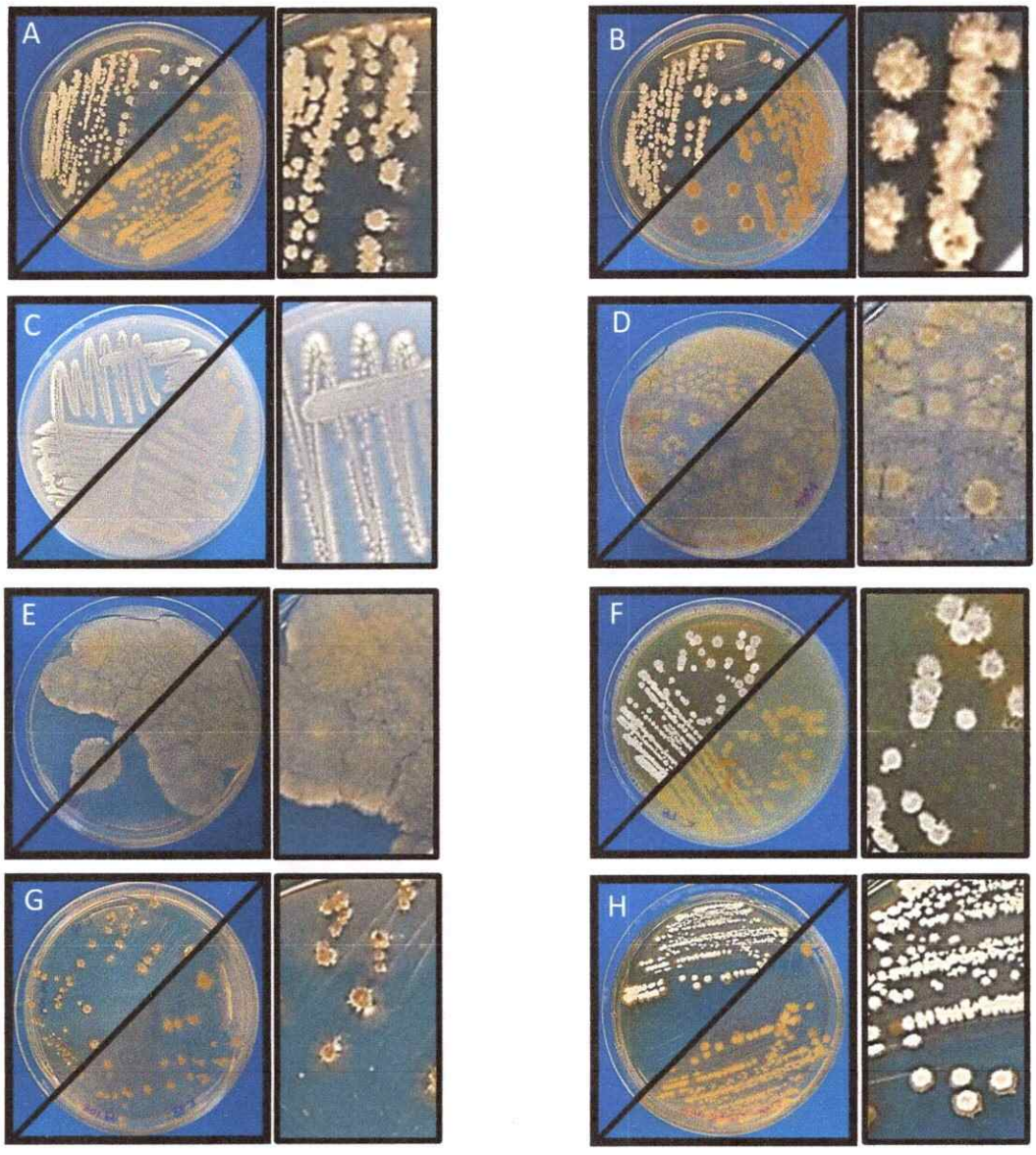


Figure 1 Morphology of *Streptomyces* sp. H-KF8. Macrocolony showing anverse and reverse growth in several media. Inset shows a zoom of colony morphology. (A) ISP1-ASW; (B) ISP2-ASW; (C) ISP3-ASW; (D) ISP4-ASW; (E) ISP5-ASW; (F) ISP6-ASW; (G) Marine Agar (MA); (H) TSA-ASW.

exudate drops in the colony surface (Fig. 1H). Additionally, morphology was visualized microscopically, and typical *Streptomyces* structures of development such as hyphae and spores were observed (Fig. 2). Exudate drops were appreciated in ISP2 medium during late growth phase (Fig. 2A), spores were identified with optical microscopy (Fig. 2B) and hyphae with Gram staining (Fig. 2B). Moreover, the complex network of intertwined hyphae and early spore chain assemblies was observed by LVEM microscopy, which is a distinctive feature of *Streptomyces* genus (Fig. 2D).

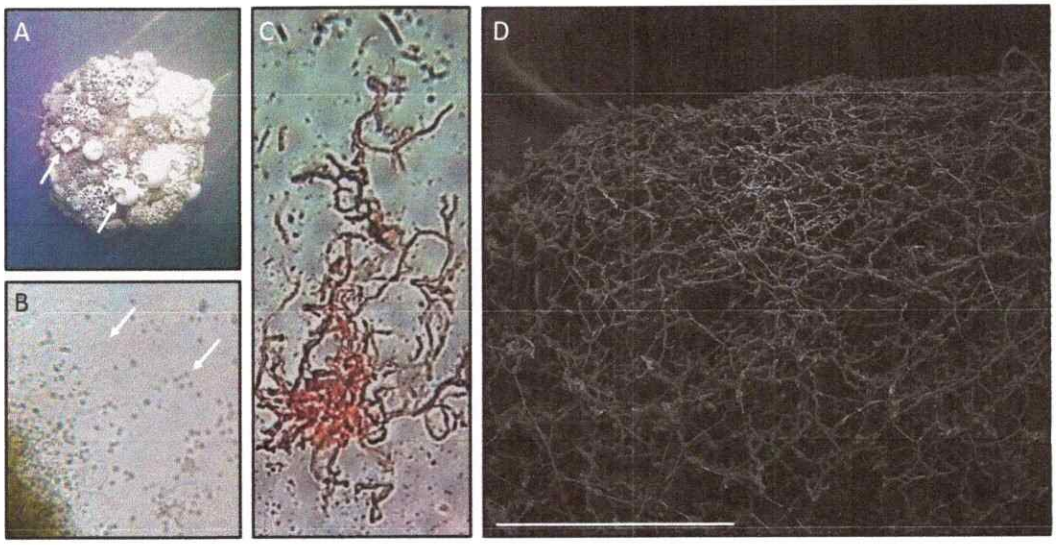


Figure 2 Microscopy of *Streptomyces* sp. H-KF8. (A) Stereoscope zoom of a macrocolony grown in ISP2-ASW agar plate. Arrows shows exudates. (B) Optic Microscopy image at 1,000×. Arrows indicate hyphae and spores, respectively. (C) *Streptomyces* sp. H-KF8 gram staining, showing hyphae. (D) Scanning Electron Microscopy (LVEM) image of *Streptomyces* sp. H-KF8 grown on ISP3-ASW agar plates for 21 days. Bar represent 100 μm.

Antimicrobial activity

Antimicrobial activity of *Streptomyces* sp. H-KF8 was further characterized using agar media with different carbon sources (Table 1). In general, antimicrobial activity was more evident against Gram-positive reference bacteria (*S. aureus* and *L. monocytogenes*), although inhibition against *E. coli* was also observed in most media, which is consistent with results obtained from *Streptomyces* sp. H-KF8 crude extracts (*Undabarrena et al., 2016a*). *P. aeruginosa* was the reference bacterium less inhibited. Among the 15 different media used, inhibition of at least one reference bacterium was noted in 87% of the media. Best media for antimicrobial activity were ISP1, ISP2, ISP6, and V media, where inhibition of four of the five reference bacteria was observed. Notably, in ISP2 medium a unique attenuation of *P. aeruginosa* growth was observed. Alternatively, a time-course assay using the double-layer method was performed to visualize the starting day of the antimicrobial activity, in four media that presented inhibition. Even though at day 5 a relatively scarce colony growth of *Streptomyces* sp. H-KF8 was observed in ISP2 medium, at day 6 it was possible to visualize a modest inhibition against *S. aureus* (Fig. 3A). Yet, inhibition zone increased as incubation time for *Streptomyces* sp. H-KF8 extended, as shown in Fig. 3B, showing a maximum halo size at day 15 (Fig. 3C), suggesting a tight relation between growth and antimicrobial activity which is also correlated to the carbon source of the media tested.

Bioinformatic analysis and Genome mining for BGCs

Whole genome sequencing and genome features were previously described (*Undabarrena et al., in press*). Briefly, *Streptomyces* sp. H-KF8 genome was assembled into 11 contigs, with a total genome length of 7,684,888 bp, and a G + C content of 72.1%. A total

Table 1 Antibacterial activity of *Streptomyces* sp. H-KF8 in several culture media.

Medium	Bacterial strains[a]				
	STAU	LIMO	PSAU	SAEN	ESCO
ISP1	+++	+++	−	+++	+++
ISP2	+++	+/−	+/−	−	+
ISP3	+	+	−	−	+
ISP4	+	−	−	−	−
ISP5	+	−	−	−	−
ISP6	++	+++	−	+	+
ISP7	+++	−	−	+/−	++
ISP9	+++	+++	−	−	−
TSA-ASW	+++	+/−	−	−	+
MA	+++	+++	−	−	++
King B	−	+/−	−	−	−
Medium V	++	++	−	+/−	+++
LB-ASW	+++	++	−	+/−	−
Actino Agar	−	−	−	−	−
NaST21Cx	−	−	−	−	−

Notes.

−, no inhibition; +/−, attenuated growth; +, <50% growth inhibition (1–5 mm); ++, 50% growth inhibition (6–10 mm); +++, >50% growth inhibition (≥11 mm).

[a]STAU, *S. aureus*; LIMO, *L. monocytogenes*; PSAU, P. *aeruginosa*; SAEN, *S. enterica*; ESCO, *E. coli*.

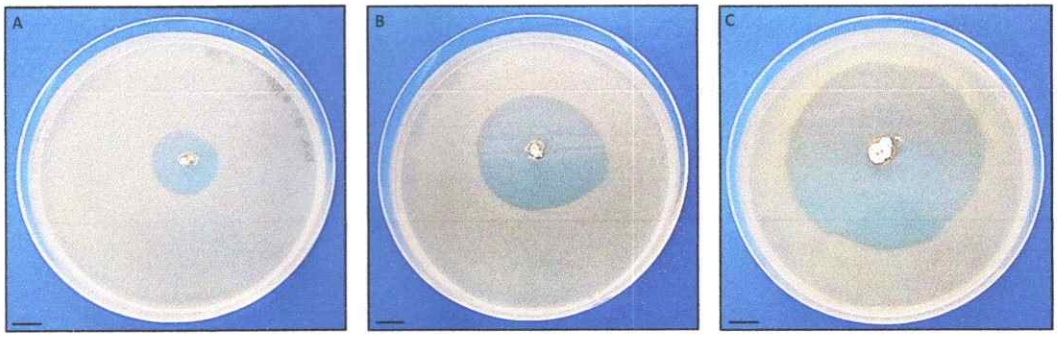


Figure 3 **Antibacterial activity of *Streptomyces* sp. H-KF8.** Photographs depict inhibition zone against *Staphylococcus aureus*. Bar represents 1 mm. Time course was performed using the double-layer method, at various incubation days: (A) 6 days; (B) 9 days; (C) 15 days.

of 6,574 genes are represented among 6,486 CDS, 67 tRNAs and 6 16S rRNAs. Genes with coding sequences were grouped into COGs categories, although 808 genes remain ungrouped. Description and gene percentage of each category is depicted in Table 2. For *Streptomyces* sp. H-KF8, the most abundant categories were transcription (522 genes), carbohydrate transport and metabolism (362 genes), and amino acid transport and metabolism (362 genes). The *Streptomyces* sp. H-KF8 categorized genes were compared to the model *Streptomyces violaceoruber* A3(2) isolated from soil (*Bentley et al., 2002*) and the marine *Streptomyces* sp. TP-A0598 (*Komaki et al., 2015*), in order to observe if these features could be considered as source-derived traits (Fig. 4). As there are scarce

Table 2 COGs distribution of genes with coding sequences in *Streptomyces* sp. H-KF8.

COG functional categories	Abbreviation	No of genes	Percentage (%)
Energy production and conversion	C	275	4.18
Cell division and chromosome partitioning	D	41	0.62
Amino acid transport and metabolism	E	322	4.90
Nucleotide transport and metabolism	F	89	1.35
Carbohydrate transport and metabolism	G	362	5.51
Coenzyme transport and metabolism	H	136	2.07
Lipid metabolism	I	142	2.16
Translation	J	168	2.56
Transcription	K	522	7.94
DNA replication and repair	L	217	3.30
Cell envelope biogenesis, outer membrane	M	169	2.57
Cell motility	N	0	0.00
Post-translational modification, protein turnover, chaperones	O	135	2.05
Inorganic ion transport and metabolism	P	223	3.39
Secondary metabolism	Q	148	2.25
General function prediction only	R	238	3.62
Function unknown	S	2,111	32.11
Signal transduction	T	283	4.30
Defense mechanisms	V	185	2.81
Not in COGs	–	808	12.29

reports available on marine *Streptomyces* genomes that include COGs detailed annotation, *Streptomyces* sp. TP-A0598 is one of the few that have these characteristics, and therefore selected for comparison. While all three strains showed the same tendency in the categories previously named in terms of abundancy, differences were observed in terms of percentage in transcription and carbohydrate metabolism categories, where *S. violaceoruber* A3(2) strain was slightly higher. On the other hand, both marine strains (*Streptomyces* sp. H-KF8 and *Streptomyces* sp. TP-A0598) showed higher number of genes related to categories of post-translational modification, protein turnover and chaperone functions, as well as in secondary metabolism and translation categories.

Secondary metabolism category comprises 2.3% of the *Streptomyces* sp. H-KF8 genome, being slightly higher when compared to both strains, the soil-derived *S. violaceoruber* A3(2), and the marine-derived *Streptomyces sp.* TP-A0598, accounting for 1.9% and 2.0% of their genomes, respectively. A bioinformatics analysis was performed using the antiSMASH tool to detect biosynthetic gene clusters (BGCs) present in *Streptomyces* sp. H-KF8 that may explain the antimicrobial activity observed, and a total of 26 BGCs were detected (*Undabarrena et al., in press*). In this report, we show that the spatial distribution of the 26 BGCs are evenly allocated throughout the contigs of *Streptomyces* sp. H-KF8 genome (Fig. 5), which were grouped into 11 different types (NRPS, PKS, hybrids, terpenes, RiPP, ectoine, melanine, siderophores, lantipeptides and butyrolatones). Furthermore, a comparison of the BGCs present in *Streptomyces* sp. H-KF8 with other known BGCs

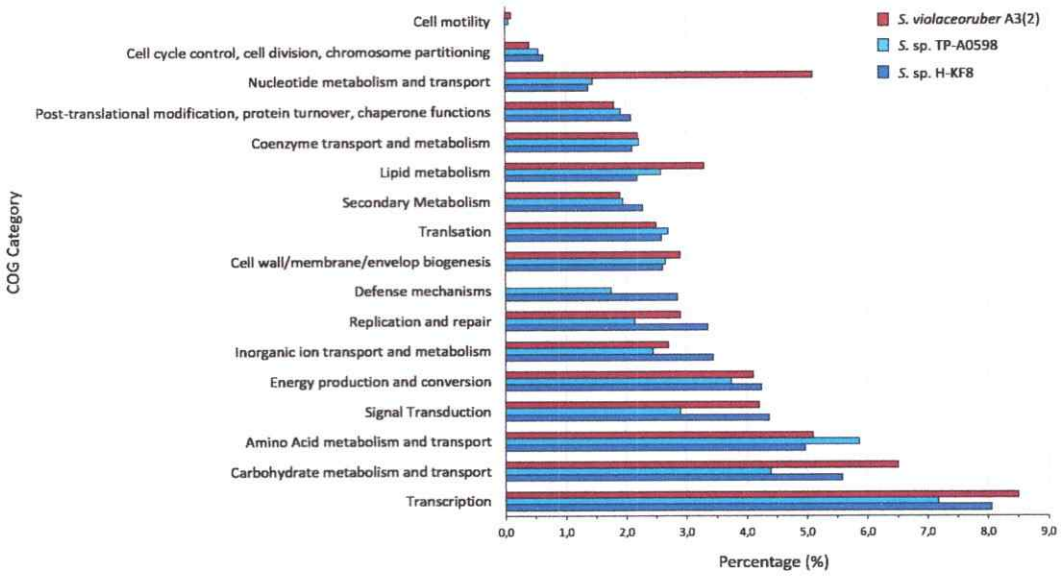


Figure 4 Comparative genomics of COGs categories. Percentage of each COG category is shown for the different *Streptomyces* species, where blue is *Streptomyces* sp. H-KF8; light blue is the marine-derived *Streptomyces* sp. TP-A0598; and red is the soil-derived *Streptomyces violaceoruber* A3(2).

deposited into the MIBiG database, was performed (Table 3). In this line, *Streptomyces* sp. H-KF8 bears two NRPSs BGCs with very low similarity to BGCs involved in the synthesis of the lipoglycopeptide antibiotic mannopeptimycin, produced by *S. hygroscopicus* (*Magarvey et al., 2006*); and the streptolydigin antibiotic, which interferes with the RNA elongation by inhibition of the bacterial RNA polymerase (*Olano et al., 2009*), with 7% and 13% of gene similarity, respectively (Table 3). The two PKSs predicted in *Streptomyces* sp. H-KF8 genome corresponds to the type II spore pigment BGC showing 83% of gene similarity, and also another BGC where only 6% of gene similarity to the antibacterial kirromycin BGC from *S. collinus* Tü 365 was found (*Weber et al., 2008*) (Table 3). A total of eight hybrid clusters, where four of them are PKS-NRPS hybrids were also predicted, which presented low gene similarities with other known BGCs, except for one NRPS-PKS type I cluster (Table 3). In addition, other BGCs found in *Streptomyces* sp. KF8 included five terpenes BGCs, two lantipeptides and two ribosomally synthesized and post-translationally modified peptides (RiPPs) such as the lassopeptide and bacteriocin BGCs. In general, only six BGCs from *Streptomyces* sp. H-KF8 genome displayed 100% gene similarity to their most related known cluster. Examples of these consists on the BGC for the previously mentioned antibiotics moenomycin (*Ostash, Saghatelian & Walker, 2007*) and albaflavenone (*Zhao et al., 2008*) (Table 3). Additionally, BGCs for the aromatic carotene isorenieratene, involved in anoxigenic photosynthesis in *S. griseus* (*Krügel et al., 1999*), the conserved osmolite ectoine, that may provide protection from osmotic stress (*Prabhu et al., 2004*; *Graf et al., 2008*) and the melanin pigment clusters (*Guo et al., 2014*; *Sivaperumal, Kamala & Rajaram, 2015*) were observed with 100% similarity. Most of the BGCs (65%) presented low similarity to BGCs of known compounds, evidencing the potential of *Streptomyces* sp. H-KF8 strain to produce novel bioactive molecules.

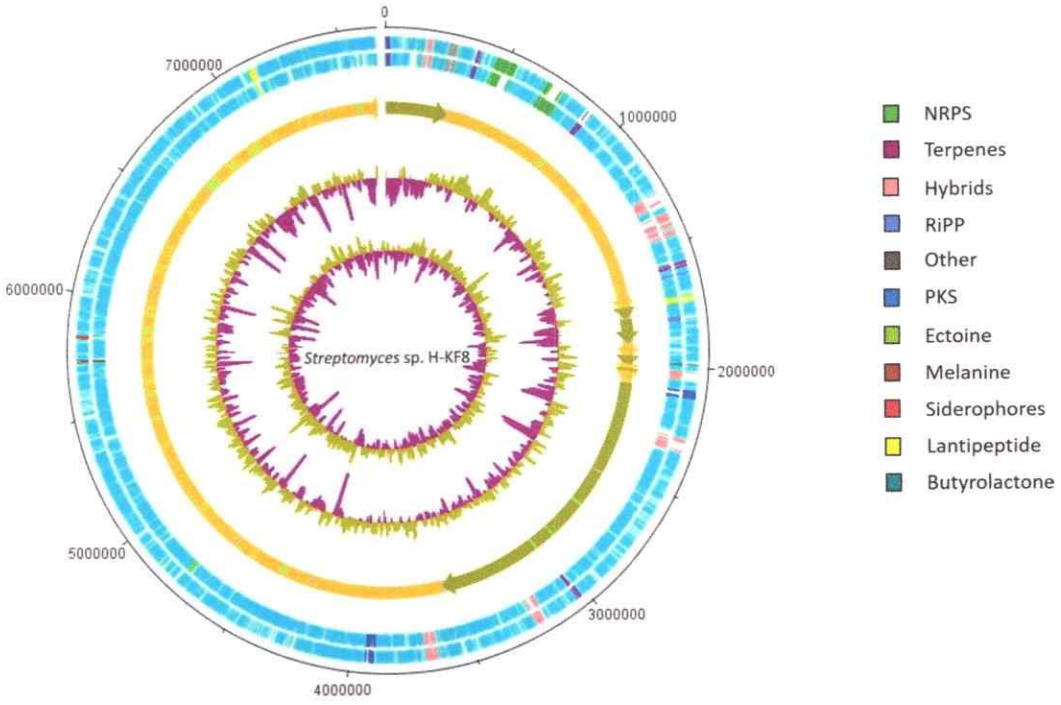


Figure 5 Representation of chromosome features and BGCs of *Streptomyces* sp. H-KF8. Colors depict the different classification types of secondary metabolism gene clusters along the sequenced genome. NRPS, Non-ribosomal peptide synthetase; PKS, polyketide synthase; RiPP, ribosomally synthesized and post-translationally modified peptides. From outside inward: DNA strands reverse and forward; contigs; GC content; GC skew.

Due to the dynamics of environmental parameters from the unique isolation site of *Streptomyces* sp. H-KF8, genome mining of pathways involved in response to abiotic stressors such as heavy metals, oxidative stress and antibiotics were also analyzed in this study, in order to unveil genetic determinants that may explain tolerance to these stressful environmental conditions.

Functional response to heavy metals and metalloids

Genetic determinants involved in heavy metal-resistance in *Streptomyces* sp. H-KF8 were analyzed by genome mining, and at least 49 predicted genes may be playing a role in such tolerance (Fig. 6A). Amongst these, the most abundant genes were related to tellurite, followed by arsenate, copper and mercury, and, to a lesser extent, chromate, nickel and cobalt tolerance (Fig. 6A). Tellurite resistance genetic determinants involved seven *terD* genes, four *terB* genes, two *yceC* genes, one *terC* gene and one *tehB* gene that encodes a tellurite methyltransferase. In addition, 11 genetic determinants for arsenic tolerance were found, involving three *arsC* genes encoding arsenate reductases, two genes *arsA* encoding arsenical pump-driving ATPases, five genes *arsR* encoding arsenical transcriptional regulators, and the arsenical resistance protein encoding gene *acr3*. Genetic determinants encoding for copper resistance genes, included *copA* and *mco* genes encoding multicopper oxidases, *copD* encoding a copper resistance protein, two genes *ycnJ* encoding for copper transport proteins, and two genes for the copper-sensing transcriptional

Table 3 Biosynthetic gene clusters (BGCs) for secondary metabolites in *Streptomyces* sp. H-KF8.

AntiSMASH type descriptor	Scaffold	Length (bp)	Predicted product (%[a])	MIBiG-ID
NRPS	tig_02	81,285	Streptolydigin (13%)	BGC0001046
NRPS	tig_02	79,174	Mannopeptimycin (7%)	BGC0000388
PKS T1	tig_138	33,925	Kirromycin (6%)	BGC0001070
PKS T2	tig_139	42,512	Spore Pigment (83%)	BGC0000271
NRPS-PKS T1	tig_138	50,808	SGR PTMS (100%)	BGC0001043
NRPS-PKS T1	tig_139	52,764	Neomycin (5%)	BGC0000710
NRPS-PKS T1	tig_02	56,103	Himastatin (12%)	BGC0001117
NRPS-PKS T3	tig_02	54,318	Furaquinocin A (21%)	BGC0001078
Terpene-Siderophore	tig_02	50,603	Isorenieratene (100%)	BGC0000664
Nucleoside-Phosphoglycolipid	tig_00	35,469	Moenomycin (100%)	BGC0000805
Oligosaccharide-PKS T1	tig_16	42,574	Stambomycin (52%)	BGC0000151
Lantipeptide-PKS T1	tig_138	61,004	Unknown	–
Terpene	tig_02	26,858	Hopene (76%)	BGC0000663
Terpene	tig_00	20,992	Unknown	–
Terpene	tig_02	21,253	Unknown	–
Terpene	tig_02	22,162	Unknown	–
Terpene	tig_138	21,220	Albaflavenone (100%)	BGC0000660
Lantipeptide	tig_02	21,819	Unknown	–
Lantipeptide	tig_139	24,585	Unknown	–
Bacteriocin	tig_02	11,412	Unknown	–
Lassopeptide	tig_10	22,692	Unknown	–
Siderophore	tig_139	11,808	Desferrioxiamine B (83%)	BGC0000940
Butyrolactone	tig_14	11,073	Griseoviridin/Viridogrisein (11%)	BGC0000459
Ectoine	tig_139	10,398	Ectoine (100%)	BGC0000853
Melanin	tig_139	10,509	Melanin (100%)	BGC0000910
Other	tig_00	43,290	Stenothricin (13%)	BGC0000431

Notes.

[a]Percentage of genes from known BGCs that show similarity to genes predicted for BGCs from *Streptomyces* sp. H-KF8.

regulator, *csoR*. Mercury resistance genes consisted in the mercury reductase encoding gene *merA*, and the mercury transcriptional regulator *merR*. In addition, the *czcD* and *rcnA* genes coding for efflux pumps for cadmium, zinc, cobalt and nickel, respectively, together with the *chrR* gene encoding a chromate reductase, and general heavy metal tolerance such as the *hmt1* gene and seven genes encoding for *merR*-family transcriptional regulators, were also found. Considering all the genetic determinants listed above, we attempted to determine if *Streptomyces* sp. H-KF8 was able to grow on various metal-containing media. *Streptomyces* sp. H-KF8 was able to tolerate copper-, cobalt-, mercury-, tellurite-, chromate- and nickel-containing media, as shown in Fig. 6B for the maximum concentrations tested. Despite the arsenic tolerance-related genes present in *Streptomyces* sp. H-KF8 genome, comprising 27% of the total number of metal-related genes, no evident growth of *Streptomyces* sp. H-KF8 was perceived in this metalloid-containing medium, even in the two different toxic forms of arsenic tested: arsenate and arsenite. Also, no growth was observed in media containing cadmium or zinc.

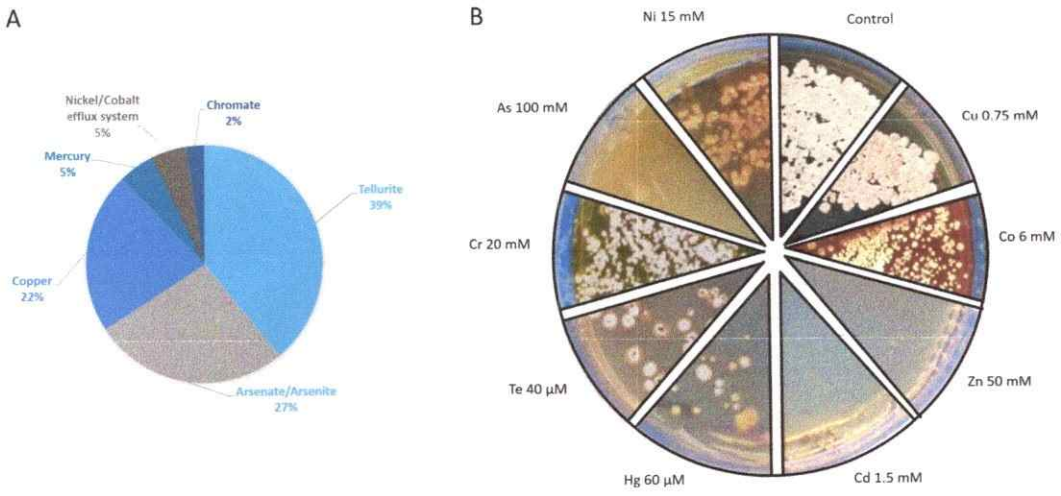


Figure 6 **Metal-tolerance response in *Streptomyces* sp. H-KF8.** (A) Genetic determinants involved in metal-resistance observed by genome mining. (B) Functional response of metal-resistance in TSA-ASW agar plates. Images show maximum concentration of metal(loids) where growth of *Streptomyces* sp. H-KF8 was observed. Concentrations below these values also presented growth. Control, agar plate without any metal.

Functional response to oxidative stress

A significant amount of genes (69 genes) that may participate in the detoxification of reactive oxygen species (ROS) were found within the *Streptomyces* sp. H-KF8 genome (Fig. 7A). Genes for mycothiol biosynthesis (20 genes), thioredoxin and thioredoxin reductases system (11 genes), alkyl hydroperoxide reductases (nine genes), glutaredoxin and glutathione peroxidase system (four genes), catalases (three genes), and superoxide dismutases (three genes), among others, were identified (Fig. 7A). Interestingly, genes involved in osmotic stress detoxification of chlorinated and brominated compounds such as three *bpo* genes encoding for bromoperoxidases, one *cpo* gene encoding for a chloroperoxidase and one gene encoding for a chlorite dismutase were also present in *Streptomyces* sp. H-KF8 genome (Fig. 7A). Concerning transcriptional regulators controlling the redox balance, transcriptional factors from *perR*, *rex*, *lysR* and *soxR* families, were also present. Due to an important genetic content of oxidative stress related genes, response of *Streptomyces* sp. H-KF8 to the toxic H_2O_2 was tested, and compared to the model streptomycete *S. violaceoruber* A3(2). At various H_2O_2 concentrations, *Streptomyces* sp. H-KF8 displayed smaller susceptibility areas against the toxic, in comparison with *S. violaceoruber* A3(2) (Figs. 7B and 7C, respectively). A significant difference of the suceptibility areas among the two strains was observed at concentrations of 1 M, 2 M and 4 M of H_2O_2, indicating a major resistance response of *Streptomyces* sp. H-KF8 towards H_2O_2 toxicity (Fig. 7D).

Functional response to antibiotics

Antibiotic-producing *Streptomyces* strains usually encode resistance genes within their BGCs to protect themselves against the noxious action of the synthetized compound (*Zotchev, 2014*). In this line, resistance of *Streptomyces* sp. H-KF8 to commercial antibiotics

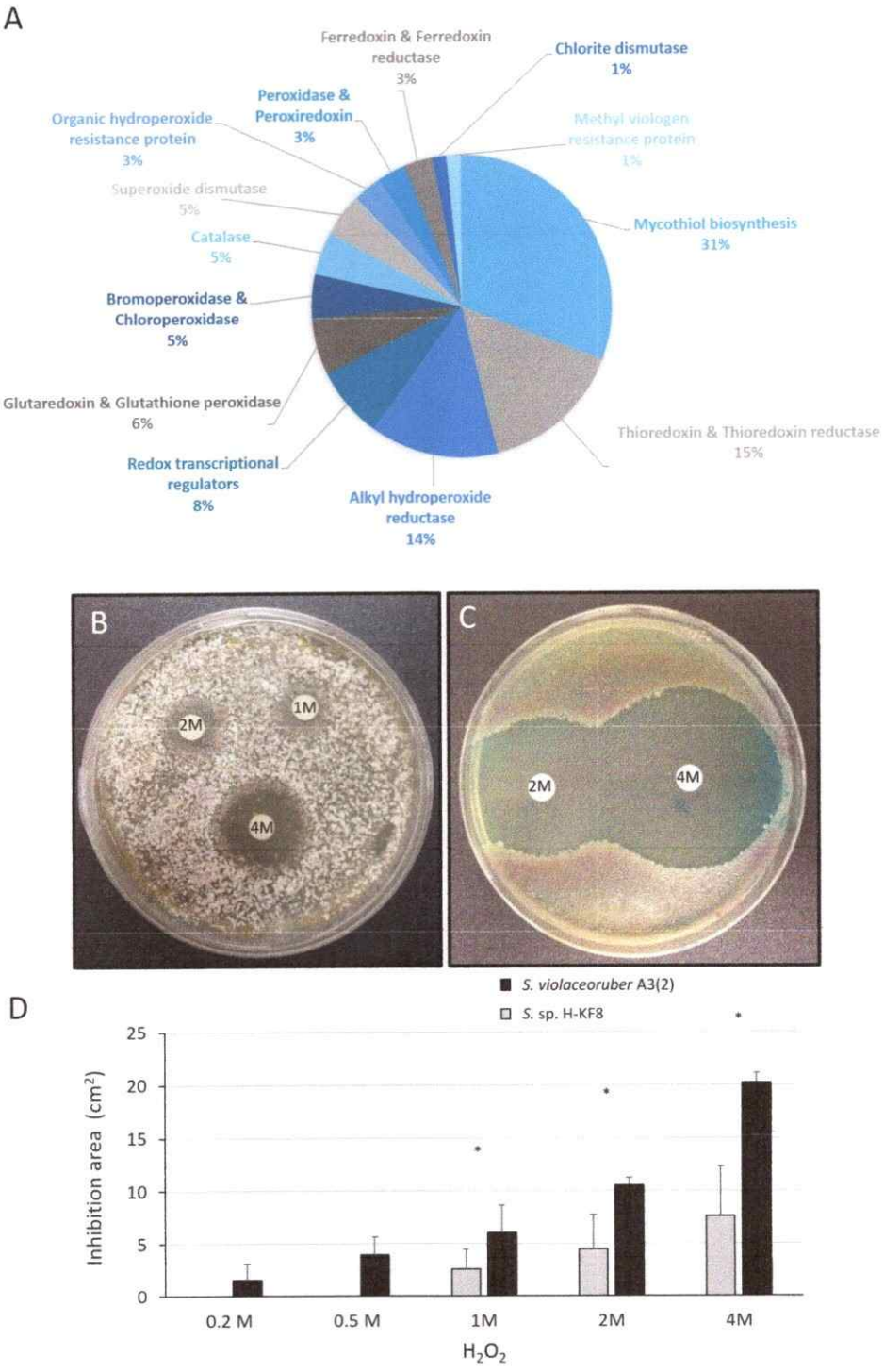


Figure 7 Oxidative stress response of *Streptomyces* sp. H-KF8. (A) Genetic determinants involved in oxidative stress-resistance observed by genome mining. Functional response of (B) *Streptomyces* sp. H-KF8 and (C) *Streptomyces violaceoruber* A3(2) respectively, showing comparative inhibition zones with hydrogen peroxide where the concentration of hydrogen peroxide used in each disk is shown. (D) Quantitative assay of inhibition area of both *Streptomyces* strains facing several concentrations of hydrogen peroxide. Asterisks indicate significant differences between strains (*t*-Test considering a p-value <0.01).

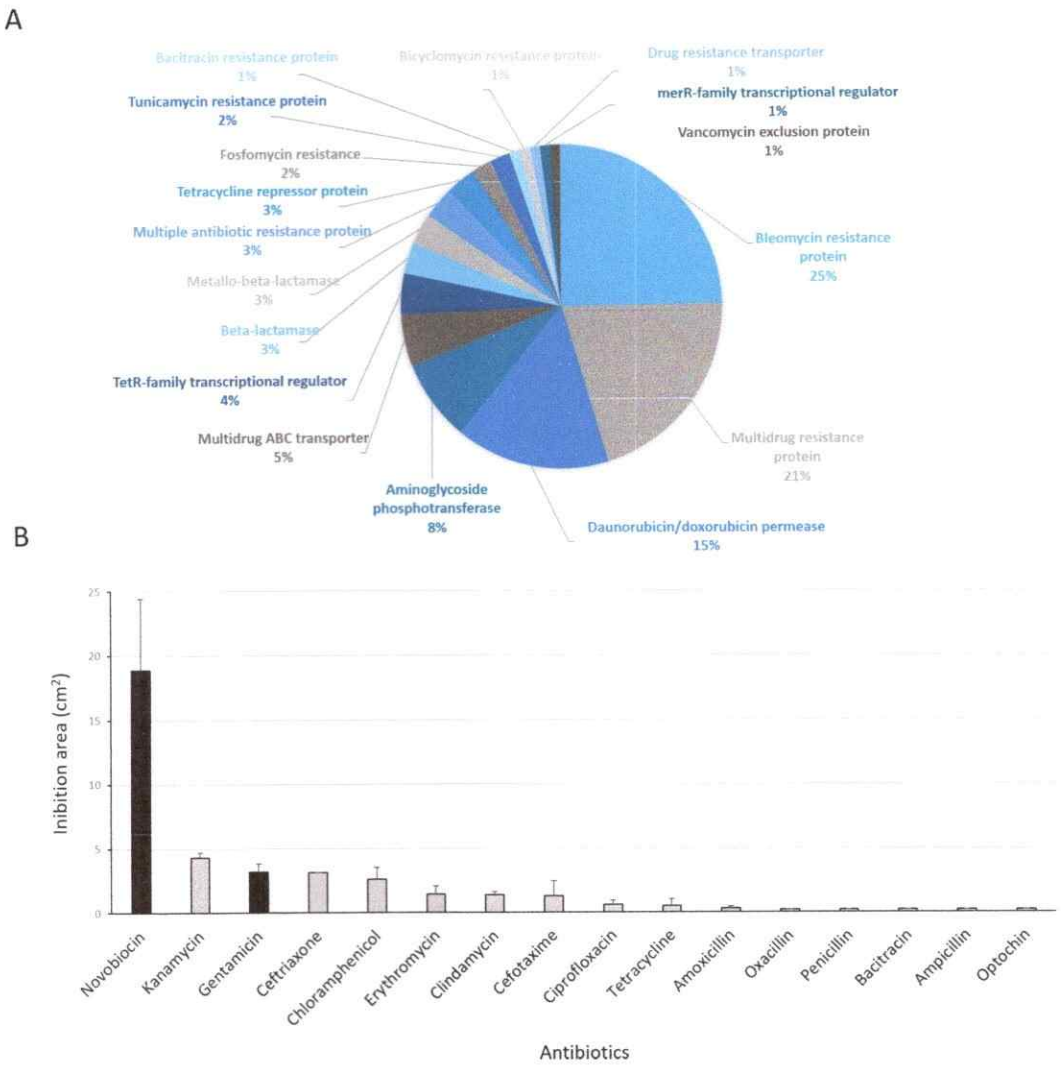


Figure 8 Antibiotic-resistance response in *Streptomyces* sp. H-KF8. (A) Genetic determinants involved in antibiotic-resistance observed by genome mining. (B) Functional response of antibiotic-resistance in MH-ASW agar plates. Black columns indicate susceptibility to the antibiotic tested and grey columns indicate resistance to the antibiotic tested.

with different biological targets was explored. Genome mining revealed more than 90 genes that could be involved in antibiotic resistance. The most abundant genes encode for bleomycin resistance proteins (24 genes). Specific resistance genes related to modification and inactivation of antibiotics such as aminoglycoside phosphotransferases (eight genes), β-lactamases (three genes), metallo-β-lactamases (three genes), and one gene for erythromycin esterase and penicillin amidase, respectively, were identified (Fig. 8A). In addition, genes for efflux of toxic compounds including multidrug resistance proteins (20 genes), daunorubicin/doxorubicin ABC transporter permeases (15 genes), multidrug ABC transporters (seven genes) and one gene encoding for a multidrug MFS transporter, were detected (Fig. 8A). Among the transcriptional regulators, the TetR-family transcriptional regulators were the most abundant, with 10 genes. Also, the marR-family

transcriptional regulator and three *marR* genes encoding for multiple antibiotic resistance proteins were identified (Fig. 8A). In the functional assay against 16 different antibiotics tested, *Streptomyces* sp. H-KF8 exhibited an 88 % of resistance-response, being susceptible to only two antibiotics: novobiocin, which targets the DNA gyrase, and gentamicin, which inhibits protein synthesis by irreversibly binding to the 30S subunit of the bacterial ribosome (Fig. 8B).

DISCUSSION

In this report, phenotypic analysis of *Streptomyces* sp. H-KF8 in several agar media was assessed, revealing in general one week of incubation time to obtain colonies and two weeks for sporulation; although growth rates, sporulation rates and pigmentation differs throughout the different media used. Antimicrobial production in *Streptomyces* sp. H-KF8 was enhanced in late growth phase (>10 days) and favoured in media where sporulation was observed. *Streptomyces* genus is characterized for slow growth and a complex developmental life cycle (*Flärdh & Buttner, 2009*). Physiological differentiation is tightly linked to secondary metabolism and hence, sporulation capacities of *Streptomyces* might enhance the discovery of new compounds (*Chater, 2013*; *Kalan et al., 2013*; *Zhu et al., 2015*). In addition, antibiotics synthesis is regulated by environmental nutrients, such as carbon sources. Media carbon source has an important effect on antibiotic production, being demonstrated that when bacteria are grown with a preferred carbon source, secondary metabolism seems repressed (*Sánchez et al., 2010*). This may explain the differences in inhibition patterns observed for the *Streptomyces* sp. H-KF8 antagonistic assays displayed in the various media tested, showing a maximum inhibition halo against *S. aureus* after 15 days of incubation. Due to the interesting antibacterial activity of *Streptomyces* sp. H-KF8, its whole-genome was sequenced and previously reported (*Undabarrena et al., in press*). Thus, in this study an extended genome analysis for *Streptomyces* sp. H-KF8 was performed, in order to gain insights into the mechanisms by which it displays antibiotic biosynthesis and resistance to multiple stressors.

Genome mining has been used in various fields to describe the exploitation of genomic information for the discovery of new processes, targets and products (*Challis, 2008*). Through genome sequencing and bioinformatic analysis using antiSMASH platform (*Medema et al., 2011*; *Blin et al., 2013*; *Weber et al., 2015*), it is possible to address the secondary metabolic potential of a strain by identification of its biosynthesis gene clusters (BGCs) (*Iftime et al., 2016*). A total of 26 BGCs were previously detected in *Streptomyces* sp. H-KF8 genome (*Undabarrena et al., in press*). In this report, an extended genetic analysis including the distribution of these BGCs along *Streptomyces* sp. H-KF8 genome was determined and comparison with known BGCs from the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database, which compiles a total of 1,170 experimentally characterized known gene clusters (*Medema et al., 2015*) was aimed. *Streptomyces* sp. H-KF8 BGCs include two PKSs, two NRPSs and four hybrid PKS-NRPS, four other hybrids, five terpenes, two lantipeptides, one bacteriocin, lassopeptide, siderophore, butyrolactone, ectoine, melanin, and one with unknown classification. Notably, *Streptomyces* sp. H-KF8

presented only six BGCs with 100% similarity to a known cluster; suggesting that most secondary metabolites produced by *Streptomyces* sp. H-KF8 are yet to be elucidated, and can contribute to the discovery of novel natural products. In this context, genome mining has proven to be a fundamental tool for genome-based natural product discovery (*Jensen et al., 2014*), and has guided the discovery of novel natural products from several marine actinobacteria (*Gulder & Moore, 2010*; *Tang et al., 2015b*). Among these are the aromatic polyketide angucyclinone antibiotic (*Zhang et al., 2012*) and polyene macrolides with antifungal activity (*Tang et al., 2015a*). Moreover, marine *Streptomyces* metabolites are produced by different metabolic pathways in comparison to their terrestrial counterparts (*Li et al., 2011*; *Lee et al., 2014*; *Barakat & Beltagy, 2015*). These metabolites emerge as a result of the unique and dynamic conditions of the ocean, such as high hydrostatic pressure, low temperature, variation in salinity, and depletion of micronutrients proper of the marine environment (*Das, Lyla & Khan, 2006*; *Lam, 2006*; *De Carvalho & Fernandes, 2010*). Despite that marine adaptations are scarcely studied, recent comparative genomics of marine-derived *Streptomyces* unveiled an enrichment in TrK and BCCT transporters, along with the observation that their genomes are generally smaller in size and have a slightly higher GC content in comparison to *Streptomyces* from other environmental sources (*Tian et al., 2016*). *Streptomyces* sp. H-KF8 genome is consistent with these findings, holding distinctive biological and genomic signatures acknowledged for marine *Streptomyces* strains. Therefore, its metabolite biosynthesis may be under marine abiotic selective pressures, hence modulating secondary metabolism production.

Comparative genomics encompassing completely sequenced *Streptomyces* obtained from several isolation sources revealed that the most abundant COG categories were transcription (K), followed by carbohydrate metabolism (G) and amino acid metabolism (E) (*Kim et al., 2015*). This is in agreement with the most abundant categories found in the *Streptomyces* sp. H-KF8 genome, which also could explain the versatility of *Streptomyces* sp. H-KF8 to grow in several media with different carbon sources. Furthermore, in marine-derived *Streptomyces*, a higher proportion of genes belonging to the COG categories of translation (J) and post-translational modification, protein turnover and chaperones (O) was observed (*Tian et al., 2016*). Accordingly, the (J) and (O) COGs categories were also overrepresented in both marine strains analyzed, *Streptomyces* sp. H-KF8 and *Streptomyces* sp. TP-A0598 (*Komaki et al., 2015*), in comparison to the terrestrial *Streptomyces violaceoruber* A3(2) (*Bentley et al., 2002*). This may indicate an important role of protein metabolism in marine environments, probably due to the active responses against abiotic stressors and the dynamics that microorganisms have to overcome to survive in these extreme ecosystems. In addition, our analysis showed an increase in the categories of cell cycle control, cell division, chromosome portioning (D), secondary metabolism (Q) and defense mechanisms (V), for both marine strains in comparison to *Streptomyces violaceoruber* A3(2). Percentage of the COG category for defense mechanisms (V) in *Streptomyces* sp. H-KF8 was interestingly higher (2,81%) than in *Streptomyces* sp. TP-A0598 (1,8%), and comparatively similar with what was observed for deep-sea bacteria (3,0%) (*Qin et al., 2011*). As the defense mechanism category includes genes for resistance to heavy metals, osmotic and oxidative stress as well as antibiotics, the functionality of these biological traits was evaluated for

Streptomyces sp. H-KF8, and notably, an important resistance to these multiple stressors was evidenced.

Environmental pollution by heavy metals can arise due to anthropogenic and/or geogenic sources. Although metal-resistant strains isolated from contaminated areas have been described (*Amoroso et al., 2001*; *Schmidt et al., 2005*; *Schmidt et al., 2009*; *Polti, Amoroso & Abate, 2007*; *Albarracin et al., 2008*; *Haferburg et al., 2008*; *Siñeriz, Kothe & Abate, 2009*; *Lin et al., 2011*; *El Baz et al., 2015*), there is limited information about the physiology of *Streptomyces* in presence of environmental metal pollutants. Due to the naturally high concentrations of certain heavy metals in Chilean northern Patagonia (*Guevara et al., 2004*; *Revenga et al., 2012*; *Hermanns & Biester, 2013*) product of the highly active seismic and volcanic activity (*Pantoja, Luis Iriarte & Daneri, 2011*), the ability of *Streptomyces* sp. H-KF8 to grow in several metal(loid)s supplemented media was evaluated. Surprisingly, resistance to copper, cobalt, mercury, tellurite, chromate and nickel was revealed.

Interestingly, the most abundant genes in *Streptomyces* sp. H-KF8 were related to tellurite resistance, involving the tellurite methyltransferase (encoded by *tehB*) and several tellurite resistance genes (*terB*, *terC*, *terD*, *yceC*). Although the *ter* operon has been described previously (*Taylor, 1999*), specification of its mechanism of action remains obscure (*Chasteen et al., 2009*). Mainly, it has been shown that tellurite detoxification is via enzymatic reduction by several flavoprotein-mediated non-specific metabolic enzymes (*Arenas-Salinas et al., 2016*), or by non-enzymatic mechanisms mediated by intracellular thiols like glutathione (*Turner et al., 2001*). Either way, tellurite reduction generates oxygen reactive species (ROS), especially superoxide anion (O_2^-), which is deleterious to fundamental cell macromolecules producing protein oxidation, lipid peroxidation and DNA damage (*Pérez et al., 2007*; *Tremaroli, Fedi & Zannoni, 2007*). Surprisingly, *Streptomyces* sp. H-KF8 did not show black pigmentation after tellurite exposure, which is a distinctive phenotype that indicates tellurite reduction to elemental tellurium (*Taylor, 1999*), suggesting that other mechanisms of resistance could be involved in *Streptomyces* sp. H-KF8. To our knowledge, this is the first tellurite-resistant *Streptomyces* strain described so far.

Additionally, resistance to mercury at a concentration of 60 μM was observed for *Streptomyces* sp. H-KF8. In general, bacteria capable of resisting mercury above 20 μM, should possess specific detoxification systems, as mercury is one of the most toxic elements on earth and produces several health concerns for macroorganisms (*Das, Dash & Chakraborty, 2016*). In bacteria, two different resistance operons are known, the basic narrow-spectrum *mer* operon *merRTPA* for inorganic mercury, and the broad-spectrum operon that additionally contains *merB*, which provides protection against organo-mercurial compounds (*Barkay, Miller & Summers, 2003*). In addition, it was recently demonstrated that mercury resistance mechanisms could also be involved in tellurite cross-resistance (*Rodriguez-Rojas et al., 2015*). Studies in *Streptomyces* includes *S. lividans* 132, that carries two divergently transcribed operons named *merAB* and *merRTP* in the chromosome (*Sedlmeier & Altenbuchner, 1992*; *Brünker et al., 1996*; *Rother, Mattes & Altenbuchner, 1999*), and two *Streptomyces* spp. strains isolated from estuarine sediments

where these genes were also observed in giant linear plasmids (*Ravel, Schrempf & Hill, 1998*; *Ravel et al., 2000*). Interestingly, the genetic operons mentioned above were not detected in *Streptomyces* sp. H-KF8, despite the fact that a mercury-resistance phenotype was evidenced. Instead, the presence of two mercury-related genes, the transcriptional regulator *merR* and the mercuric reductase *merA*, may be playing a role in such resistance. MerA is a flavoprotein NADPH-dependent enzyme responsible for the reduction of mercury(II) to the elemental and less toxic volatile mercury(0) (*Barkay, Miller & Summers, 2003*). Similarly, evidence of functional operons conformed either by *merA* or *merRA* have been previously reported in archaea (*Boyd & Barkay, 2012*).

However, no evident growth was observed in the presence of arsenate or arsenite, although *Streptomyces* sp. H-KF8 bears at least 11 genetic determinants that could involved in its detoxification. In general, the arsenic resistance operon consists of *arsRABCD* genes, where *arsC* encodes for an arsenate reductase that converts arsenate to arsenite, which is then exported through the ArsAB ATPase-efflux pump. In *Streptomyces* sp. H-KF8, *arsA*, *arsC* and *arsR* genes are present, but lack the *arsB* gene, which encodes an arsenite antiporter, crucial for anchoring ArsA to the inner membrane with concomitant detoxification of arsenite. Absence of the *arsB* gene may explain the sensitivity of *Streptomyces* sp. H-KF8 towards these toxics. Arsenic resistance genes are generally widespread amongst both Gram-positive and Gram-negative bacteria, reflecting its broad distribution in the environment (*Silver & Phung, 2005*). In fact, these genes were also conserved in several marine streptomycetes from the South China Sea (*Tian et al., 2016*).

Streptomyces sp. H-KF8 displayed a notorious copper-resistant phenotype, concordant with the detection of three *copA* genes encoding for multicopper oxidases that may be responsible for the oxidation of Cu(I) to its less toxic form Cu(II) (*Hobman & Crossman, 2014*). Copper is an essential metal for living beings, but is extremely toxic at higher concentrations (*Gaetke & Chow, 2003*). Moreover, Chile is the major copper-producing country in the world, due its geological nature (*Wacaster, 2015*). Hence, the widespread of copper resistant genetic determinants that has been demonstrated in Chilean marine sediments (*Besaury et al., 2013*) is expected.

Resistance to nickel and cobalt in *Streptomyces* sp. H-KF8 might be given by the *rcnA* gene that participates in the efflux system of these metals. Highly nickel- and cobalt-resistant *Streptomyces* were found in an acid mine drainage, where growth in media containing up to 10 mM nickel(II) or 3 mM cobalt(II) was observed (*Schmidt et al., 2005*). In this report, *Streptomyces* sp. H-KF8 was able to grow even at higher concentrations: 15 mM nickel(II) and 6 mM cobalt(II), respectively. Furthermore, chromate toxicity (20 mM) might be overcome in *Streptomyces* sp. H-KF8 due to the presence of the *chrR* gene encoding a chromate reductase involved in the enzymatic reduction of chromate to the less harmful chromite cation (*Das, Dash & Chakraborty, 2016*). Previously reported *Streptomyces* chromate-resistant strains isolated from sugar cane plant were able to grow in 17 mM, where also chromate-removing activity was demonstrated (*Polti, Amoroso & Abate, 2007*).

Metal exposure and adverse abiotic environmental factors produces a general condition of oxidative stress in microorganisms. As oxidative stress is hazardous for fundamental

macromolecules, bacteria have evolved several mechanisms to protect themselves from these environmental stresses. In *Streptomyces* sp. H-KF8, an exceptional response to several concentrations of H_2O_2 was observed, compared to the model *Streptomyces violaceoruber* A3(2) which was more susceptible towards the toxic. Consequently, a wide number of genetic determinants related to ROS response were present in the *Streptomyces* sp. H-KF8 genome. Remarkably, a high number of thioredoxins (*trx*) and alkyl hydroperoxide reductases (*ahp*) genes (nine of each) were found in *Streptomyces* sp. H-KF8, in comparison with *Streptomyces violaceoruber* A3(2) where five and one genes were described, respectively. The *ahp* and *trx* are fundamental H_2O_2-inducible genes that encodes for enzymes known to participate in the bacterial response to oxidative stress, which are regulated by *oxyR* in *E. coli* (*Storz & Imlay, 1999*; *Seaver & Imlay, 2001*; *Chiang & Schellhorn, 2012*). The *oxyR* regulon is not present in *Streptomyces* sp. H-KF8, but instead two copies of the *perR* regulator fulfill its role in Gram-positive bacteria (*Ricci et al., 2002*; *Dubbs & Mongkolsuk, 2012*). Also, the *ohrR* transcriptional regulator that senses organic peroxide (ROOH) and sodium hypochlorite (NaOCl) (*Dubbs & Mongkolsuk, 2012*) was found in *Streptomyces* sp. H-KF8. In addition, several genes regulated by the *soxR* transcriptional regulatory system such as glutaredoxin and glutathione peroxidase, superoxide dismutases (*sod*), catalases (*kat*) and thioredoxin reductases were recognized in *Streptomyces* sp. H-KF8 genome, which overall may be accounting for it resistance through H_2O_2 exposure. Even more, the chromate reductase (*chrR*) previously mentioned, could also provide additional protection against H_2O_2 (*Das, Dash & Chakraborty, 2016*). Interestingly, unusual genes encoding for bromoperoxidases, chloroperoxidases and chlorite dismutases, involved in osmotic stress detoxification of brominated and chlorinated toxic compounds which are abundant in the marine environments (*Sander et al., 2003*; *Bouwman et al., 2012*), were also present in *Streptomyces* sp. H-KF8 genome. On the other hand, *Streptomyces violaceoruber* A3(2) possess only one chloroperoxidase, suggesting that this might represent another marine adaptation trait for *Streptomyces* sp. H-KF8. Osmotic and oxidative stress response seems to be regulated via a network of sigma factors in *Streptomyces violaceoruber* A3(2), that controls the activation of several oxidative defense proteins, chaperones and systems that provide osmolytes and mycothiol (*Lee et al., 2005*). Consistently, a high amount of genes for mycothiol biosynthesis was identified in *Streptomyces* sp. H-KF8. Mycothiol is the major low-molecular-weight thiol present in actinobacteria, and serves as a buffer to advert disulfide stress, in complement of the enzymatic system presented above (*Buchmeier & Fahey, 2006*; *Den Hengst & Buttner, 2008*).

Recently, evidence of heavy metal driving co-selection of antibiotic resistance in both natural environments (*Seiler & Berendonk, 2012*) and contaminated ones (*Li, Li & Zhang, 2015*; *Henriques et al., 2016*) have been reported. In this line, isolation of *Streptomyces* with both metal and antibiotic co-resistances have been described (*Van Nostrand et al., 2007*). In addition, co-evolution of resistance within closely related antibiotic-producing bacteria has been demonstrated for *Streptomyces* (*Laskaris et al., 2010*). Hence, the antibiotic response against pharmaceutical compounds was investigated in *Streptomyces* sp. H-KF8, and resistance was observed to all antibiotics tested, with exception of gentamicin and novobiocin. Resistance to almost all antibiotics tested, could be due to the presence of

multiple BGCs with different mode of action. A typical BGC cluster that produces a bioactive compound is generally coupled to its corresponding resistance gene (*Zotchev, 2014*). The phenomena of widespread distribution antibiotic resistance genes in natural environments is consequence of improper use of antibiotics in medical treatment, as well as by an indiscriminate use in agriculture, livestock and aquaculture (*Brown et al., 2006*). Phenomena such as the grasshopper effect may also contribute to the rapid transport of toxics around the globe through atmospheric and oceanic currents (*Sadler & Connell, 2012*).

Overall, our study shows the response of a marine *Streptomyces* sp. H-KF8 against several abiotic stressors such as heavy metals, oxidative stress and antibiotics, along with the genome mining of the biosynthetic gene clusters that could be involved in the antimicrobial activity observed. Altogether, these biological features may enable *Streptomyces* sp. H-KF8 to thrive in the complex fjord marine environment.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the "Comisión Nacional de Investigación Científica y Tecnológica" (FONDECYT 11121571 to BC and Swedish Research Council No 2013-6713. MS was supported by FONDECYT 1110992. AU was supported by Conicyt PhD fellowship and Conicyt Gastos Operacionales (No 21120621). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Comisión Nacional de Investigación Científica y Tecnológica.
FONDECYT: 11121571, 1110992.
Swedish Research Council: No 2013-6713.
Conicyt Gastos Operacionales: 21120621.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Agustina Undabarrena conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Juan A. Ugalde analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Michael Seeger analyzed the data, reviewed drafts of the paper.
- Beatriz Cámara conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

NCBI GenBank accession number of the whole-genome shotgun project LWAB00000000 http://www.ncbi.nlm.nih.gov/bioproject/317393.

REFERENCES

Albarracin VH, Avila AL, Amoroso MJ, Abate CM. 2008. Copper removal ability by *Streptomyces* strains with dissimilar growth patterns and endowed with cupric reductase activity. *FEMS Microbiology Letters* **288**:141–148 DOI 10.1111/j.1574-6968.2008.01335.x.

Amoroso MJ, Castro GR, Durán A, Peraud O, Oliver G, Hill RT. 2001. Chromium accumulation by two *Streptomyces* spp. isolated from riverine sediments. *Journal of Industrial Microbiology & Biotechnology* **26**:210–215 DOI 10.1038/sj.jim.7000112.

Antoraz S, Santamaria RI, Diaz M, Sanz D, Rodriguez H. 2015. Toward a new focus in antibiotic and drug discovery from the *Streptomyces* arsenal. *Frontiers in Microbiology* **6**:1–8 DOI 10.3389/fmicb.2015.00461.

Arenas-Salinas M, Vargas JI, Morales W, Pinto C, Muñoz P, Cornejo FA, Pugin B, Sandoval J, Diaz W, Muñoz-Villagrán C, Rodríguez FJ, Morales E, Vásquez CC, Arenas F. 2016. Flavoprotein-mediated tellurite reduction: structural basis and applications to the synthesis of tellurium-containing nanostructures. *Frontiers in Microbiology* **7**:1160 DOI 10.3389/FMICB.2016.01160.

Axenov-Gribanov DV, Voytsekhovskaya IV, Tokovenko BT, Protasov ES, Gamaiunov SV, Rebets YV, Luzhetskyy AN, Timofeyev MA. 2016. Actinobacteria isolated from an underground lake and moonmilk speleothem from the biggest conglomeratic karstic cave in Siberia as sources of novel biologically active compounds. *PLOS ONE* **11**:1–12 DOI 10.1371/journal.pone.0149216.

Barakat KM, Beltagy EA. 2015. Bioactive phthalate from marine *Streptomyces* ruber EKH2 against virulent fish pathogens. *Egyptian Journal of Aquatic Research* **41**:49–56 DOI 10.1016/j.ejar.2015.03.006.

Baranasic D, Gacesa R, Starcevic A, Zucko J, Blazic M, Horvat M, Gjuracic K, Fujs S, Hranueli D, Kosec G, Cullum J, Petkovic H. 2013. Draft genome sequence of *Streptomyces* rapamycinus strain NRRL 5491, the producer of the immunosuppressant rapamycin. *Genome Announcement* **1**: e00581–13 DOI 10.1099/ijs.

Barbe V, Bouzon M, Mangenot S, Badet B, Poulain J, Segurens B, Vallenet D, Marliere P, Weissenbach J. 2011. Complete genome sequence of *Streptomyces cattleya* NRRL 8057, a producer of antibiotics and fluorometabolites. *Journal of Bacteriology* **193**:5055–5056 DOI 10.1128/JB.05583-11.

Barkay T, Miller SM, Summers AO. 2003. Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiology Reviews* **27**:355–384 DOI 10.1016/S0168-6445(03)00046-9.

Beites T, Rodríguez-García A, Moradas-Ferreira P, Aparicio JF, Mendes MV. 2014. Genome-wide analysis of the regulation of pimaricin production in *Streptomyces*

natalensis by reactive oxygen species. *Applied Microbiology and Biotechnology* **98**:2231–2241 DOI 10.1007/s00253-013-5455-z.

Bentley S, Chater K, Cerdeño-Tárraga A-M, Challis GL, Thomson NR, James KD, Harris DE. 2002. Complete genome sequence of the model actinomycete *Streptomyces* coelicolor A3(2). *Nature* **417**:141–147 DOI 10.1038/417141a.

Bérdy J. 2012. Thoughts and facts about antibiotics: where we are now and where we are heading. *The Journal of Antibiotics* **65**:441–441 DOI 10.1038/ja.2012.54.

Berlin K, Koren S, Chin C-S, Drake JP, Landolin JM, Phillippy AM. 2015. Assembling large genomes with single-molecule sequencing and locality-sensitive hashing. *Nature Biotechnology* **33**:623–630 DOI 10.1038/nbt.3238.

Besaury L, Bodilis J, Delgas F, Andrade S, De la Iglesia R, Ouddane B, Quillet L. 2013. Abundance and diversity of copper resistance genes cusA and copA in microbial communities in relation to the impact of copper on Chilean marine sediments. *Marine Pollution Bulletin* **67**:16–25 DOI 10.1016/j.marpolbul.2012.12.007.

Bibb M, Hesketh A. 2009. *Analyzing the regulation of antibiotic production in streptomycetes.* Elsevier Inc.

Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, Weber T. 2013. antiSMASH 2.0—a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Research* **41**:204–212 DOI 10.1093/nar/gkt449.

Bouwman H, Kylin H, Choong kwet Yive NS, Tatayah V, Løken K, Utne Skaare J, Polder A. 2012. First report of chlorinated and brominated hydrocarbon pollutants in marine bird eggs from an oceanic Indian Ocean island. *Environmental Research* **118**:53–64 DOI 10.1016/j.envres.2012.05.009.

Boyd ES, Barkay T. 2012. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine. *Frontiers in Microbiology* **3**:1–13 DOI 10.3389/fmicb.2012.00349.

Bustamante MS. 2009. The southern Chilean fjord region: oceanographic aspects. In: *Marine benthic fauna of chilean patagonia.* 53–60.

Bredholdt H, Galatenko OA, Engelhardt K, Fjaervik E, Terekhova LP, Zotchev SB. 2007. Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. *Environmental Microbiology* **9**:2756–2764 DOI 10.1111/j.1462-2920.2007.01387.x.

Brown KD, Kulis J, Thomson B, Chapman TH, Mawhinney DB. 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Science of the Total Environment* **366**:772–783 DOI 10.1016/j.scitotenv.2005.10.007.

Brünker P, Rother D, Sedlmeier R, Klein J, Mattes R, Altenbuchner J. 1996. Regulation of the operon responsible for broad-spectrum mercury resistance in *Streptomyces lividans* 1326. *Molecular and General Genetics* **251**:307–315 DOI 10.1007/s004380050171.

Buchmeier N, Fahey RC. 2006. The mshA gene encoding the glycosyltransferase of mycothiol biosynthesis is essential in Mycobacterium tuberculosis Erdman. *FEMS Microbiology Letters* **264**:74–79 DOI 10.1111/j.1574-6968.2006.00441.x.

Challis GL. 2008. Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology* **154**:1555–1569 DOI 10.1099/mic.0.2008/018523-0.

Chasteen TG, Fuentes DE, Tantalean JC, Vasquez CC. 2009. Tellurite: history, oxitative stress, and molecular mechanisms of resistance. *FEMS Microbiology Reviews* **33**:820–832 DOI 10.1111/j.1574-6976.2009.00177.x.

Chater KF. 2013. Curing baldness activates antibiotic production. *Chemistry and Biology* **20**:1199–1200 DOI 10.1016/j.chembiol.2013.10.001.

Chen CW, Huang CH, Lee HH, Tsai HH, Kirby R. 2002. Once the circle has been broken: dynamics and evolution of *Streptomyces* chromosomes. *Trends in Genetics* **18**:522–529 DOI 10.1016/S0168-9525(02)02752-X.

Chiang SM, Schellhorn HE. 2012. Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. *Archives of Biochemistry and Biophysics* **525**:161–169 DOI 10.1016/j.abb.2012.02.007.

Claverías FP, Undabarrena A, González M, Seeger M, Cámara B. 2015. Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaíso bay, Chile. *Frontiers in Microbiology* **6**:1–11 DOI 10.3389/fmicb.2015.00737.

Das S, Dash HR, Chakraborty J. 2016. Genetic basis and importance of metal resistant genes in bacteria for bioremediation of contaminated environments with toxic metal pollutants. *Applied Microbiology and Biotechnology* **100**:2967–2984 DOI 10.1007/s00253-016-7364-4.

Das S, Lyla PS, Khan SA. 2006. Marine microbial diversity and ecology: importance and future perspectives. *Current Science* **90**:1325–1335.

De Carvalho CCCR, Fernandes P. 2010. Production of metabolites as bacterial responses to the marine environment. *Marine Drugs* **8**:705–727 DOI 10.3390/md8030705.

Dela Cruz R, Gao Y, Penumetcha S, Sheplock R, Weng K, Chander M. 2010. Expression of the *Streptomyces* coelicolor SoxR regulon is intimately linked with actinorhodin production. *Journal of Bacteriology* **192**:6428–6438 DOI 10.1128/JB.00916-10.

Den Hengst CD, Buttner MJ. 2008. Redox control in actinobacteria. *Biochimica et Biophysica Acta—General Subjects* **1780**:1201–1216 DOI 10.1016/j.bbagen.2008.01.008.

Doroghazi JR, Metcalf WW. 2013. Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *BMC Genomics* **14**:611 DOI 10.1186/1471-2164-14-611.

Dubbs JM, Mongkolsuk S. 2012. Peroxide-sensing transcriptional regulators in bacteria. *Journal of Bacteriology* **194**:5495–5503 DOI 10.1128/JB.00304-12.

Duncan KR, Haltli B, Gill KA, Correa H, Berrué F, Kerr RG. 2014. Exploring the diversity and metabolic potential of actinomycetes from temperate marine sediments from Newfoundland, Canada. *Journal of Industrial Microbiology and Biotechnology* **42**:57–72 DOI 10.1007/s10295-014-1529-x.

El Baz S, Baz M, Barakate M, Hassani L, El Gharmali A, Imziln B. 2015. Resistance to and accumulation of heavy metals by actinobacteria isolated from abandoned mining areas. *Scientific World Journal* **2015**: Article ID 761834 DOI 10.1155/2015/761834.

Ettlinger L, Corbaz R, Hütter R. 1958. Zur Systematik der Actinomyceten 4. Eine Arteneinteilung der Gattung *Streptomyces* Waksman and Henrici. *Archiv für Mikrobiologie* **31**:326–358 DOI 10.1017/CBO9781107415324.004.

Euzéby J. 2011. Notification that new names and new combinations have appeared in volume 59, part 8, of the IJSEM. *International Journal of Systematic and Evolutionary Microbiology* **61**:477–478 DOI 10.1099/ijs.0.019158-0.

Felsenstein J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17**:368–376 DOI 10.1007/BF01734359.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783–791.

Flärdh K, Buttner MJ. 2009. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nature Reviews Microbiology* **7**:36–49 DOI 10.1038/nrmicro1968.

Gaetke LM, Chow CK. 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* **189**:147–163 DOI 10.1016/S0300-483X(03)00159-8.

Gontang E, Fenical W, Jensen P. 2007. Phylogenetic diversity of gram-positive bacteria cultured from marine sediments. *Applied and Environmental Microbiology* **73**:3272–3282 DOI 10.1128/AEM.02811-06.

Graf R, Anzali S, Buenger J, Pfluecker F, Driller H. 2008. The multifunctional role of ectoine as a natural cell protectant. *Clinics in Dermatology* **26**:326–333 DOI 10.1016/j.clindermatol.2008.01.002.

Guevara SR, Bubach D, Vigliano P, Lippolt G, Arribére M. 2004. Heavy metal and other trace elements in native mussel Diplodon chilensis from Northern Patagonia Lakes, Argentina. *Biological Trace Element Research* **102**:245–263 DOI 10.1385/BTER:102:1-3:245.

Gulder TAM, Moore BS. 2010. Chasing the treasures of the sea—bacterial marine natural products. *Current Opinion in Microbiology* **12**:252–260 DOI 10.1016/j.mib.2009.05.002.Chasing.

Guo J, Rao Z, Yang T, Man Z, Xu M, Zhang X. 2014. High-level production of melanin by a novel isolate of *Streptomyces* kathirae. *FEMS Microbiology Letters* **357**:85–91 DOI 10.1111/1574-6968.12497.

Haber M, Ilan M. 2014. Diversity and antibacterial activity of bacteria cultured from Mediterranean Axinella spp. sponges. *Journal of Applied Microbiology* **116**:519–532 DOI 10.1111/jam.12401.

Haefner B. 2003. Drugs from the deep: marine natural products as drug candidates. *Research Focus* **8**:536–544.

Haferburg G, Kloess G, Schmitz W, Kothe E. 2008. "Ni-struvite"—a new biomineral formed by a nickel resistant *Streptomyces* acidiscabies. *Chemosphere* **72**:517–523 DOI 10.1016/j.chemosphere.2008.02.050.

Henriques I, Tacão M, Leite L, Fidalgo C, Araújo S, Oliveira C, Alves A. 2016. Co-selection of antibiotic and metal (loid) resistance in gram-negative epiphytic bacteria from contaminated salt marshes. *Marine Pollution Bulletin* **109(1)**:427–434 DOI 10.1016/j.marpolbul.2016.05.031.

Hermanns YM, Biester H. 2013. Anthropogenic mercury signals in lake sediments from southernmost Patagonia, Chile. *Science of the Total Environment* **445–446**:126–135 DOI 10.1016/j.scitotenv.2012.12.034.

Hobman JL, Crossman LC. 2014. Bacterial antimicrobial metal ion resistance. *Journal of Medical Microbiology* **64**:471–497 DOI 10.1099/jmm.0.023036-0.

Hodges TW, Slattery M, Olson JB. 2012. Unique actinomycetes from marine caves and coral reef sediments provide novel PKS and NRPS biosynthetic gene clusters. *Marine Biotechnology* **14**:270–280 DOI 10.1007/s10126-011-9410-7.

Huerta-Cepas J, Szklarczyk D, Forslund K, Cook H, Heller D, Walter MC, Rattei T, Mende DR, Sunagawa S, Kuhn M, Jensen LJ, Von Mering C, Bork P. 2016. eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Research* **44**:D286–D293 DOI 10.1093/nar/gkv1248.

Iftime D, Kulik A, Härtner T, Rohrer S, Niedermeyer THJ, Stegmann E, Weber T, Wohlleben W. 2016. Identification and activation of novel biosynthetic gene clusters by genome mining in the kirromycin producer *Streptomyces collinus* Tü 365. *Journal of Industrial Microbiology and Biotechnology* **43**:277–291 DOI 10.1007/s10295-015-1685-7.

Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, Sakaki Y, Hattori M, Omura S. 2003. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces* avermitilis. *Nature Biotechnology* **21**:526–531 DOI 10.1038/nbt820.

Jensen PR, Chavarria KL, Fenical W, Moore BS, Ziemert N. 2014. Challenges and triumphs to genomics-based natural product discovery. *Journal of Industrial Microbiology and Biotechnology* **41**:203–209 DOI 10.1007/s10295-013-1353-8.

Jensen PR, Gontang E, Mafnas C, Mincer TJ, Fenical W. 2005. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. *Environmental Microbiology* **7**:1039–1048 DOI 10.1111/j.1462-2920.2005.00785.x.

Jiang S, Sun W, Chen M, Dai S, Zhang L, Liu Y, Lee KJ, Li X. 2007. Diversity of culturable actinobacteria isolated from marine sponge Haliclona sp. *Antonie van Leeuwenhoek* **92**:405–416 DOI 10.1007/s10482-007-9169-z.

Kalan L, Gessner A, Thaker MN, Waglechner N, Zhu X, Szawiola A, Bechthold A, Wright GD, Zechel DL. 2013. A cryptic polyene biosynthetic gene cluster in *Streptomyces* calvus is expressed upon complementation with a functional bldA gene. *Chemistry and Biology* **20**:1214–1224 DOI 10.1016/j.chembiol.2013.09.006.

Katz L, Baltz RH. 2016. Natural product discovery: past, present, and future. *Journal of Industrial Microbiology and Biotechnology* **43**:155–176 DOI 10.1007/s10295-015-1723-5.

Kester DR, Duedall IW, Connors DN, Pytkowicz RM. 1967. Preparation of Artificial Seawater. *Limnology and Oceanography* **12**:176–179 DOI 10.4319/lo.1967.12.1.0176.

Kim JN, Kim Y, Jeong Y, Roe JH, Kim BG, Cho3 BK. 2015. Comparative genomics reveals the core and accessory genomes of *Streptomyces* species. *Journal of Microbiology and Biotechnology* **25**:1599–1605 DOI 10.4014/jmb.1504.04008.

Kim J-S, Lee H-N, Kim P, Lee H-S, Eung Soo K. 2012. Negative role of wblA in response to oxidative stress in *Streptomyces* coelicolor. *Journal of Microbiology and Biotechnology* **22**:736–741 DOI 10.4014/jmb.1112.12032.

Kim TK, Garson MJ, Fuerst JA. 2005. Marine actinomycetes related to the "Salinospora" group from the Great Barrier Reef sponge Pseudoceratina clavata. *Environmental Microbiology* **7**:509–518 DOI 10.1111/j.1462-2920.2004.00716.x.

Komaki H, Ichikawa N, Hosoyama A, Fujita N, Igarashi Y. 2015. Draft Genome Sequence of *Streptomyces* sp. TP-A0871, a Producer of Heronamide C. *Genome Announcements* **3**: e01429–15 DOI 10.1128/genomeA.01429-15.

Krügel H, Krubasik P, Weber K, Saluz HP, Sandmann G. 1999. Functional analysis of genes from *Streptomyces griseus* involved in the synthesis of isorenieratene, a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids* **1439**:57–64 DOI 10.1016/S1388-1981(99)00075-X.

Kuang W, Li J, Zhang S, Long L. 2015. Diversity and distribution of Actinobacteria associated with reef coral Porites lutea. *Frontiers in Microbiology* **6**:1–13 DOI 10.3389/fmicb.2015.01094.

Lam KS. 2006. Discovery of novel metabolites from marine actinomycetes. *Current Opinion in Microbiology* **9**:245–251 DOI 10.1016/j.mib.2006.03.004.

Laskaris P, Tolba S, Calvo-Bado L, Wellington L. 2010. Coevolution of antibiotic production and counter-resistance in soil bacteria. *Environmental Microbiology* **12**:783–796 DOI 10.1111/j.1462-2920.2009.02125.x.

Lee EJ, Karoonuthaisiri N, Kim HS, Park JH, Cha CJ, Kao CM, Roe JH. 2005. A master regulator σB governs osmotic and oxidative response as well as differentiation via a network of sigma factors in *Streptomyces* coelicolor. *Molecular Microbiology* **57**:1252–1264 DOI 10.1111/j.1365-2958.2005.04761.x.

Lee SH, Moon K, Kim H, Shin J, Oh DC, Oh KB. 2014. Bahamaolide A from the marine-derived *Streptomyces* sp. CNQ343 inhibits isocitrate lyase in Candida albicans. *Bioorganic and Medicinal Chemistry Letters* **24**:4291–4293 DOI 10.1016/j.bmcl.2014.07.021.

León J, Liza L, Soto I, Cuadra DL, Patiño L. 2007. Actinomycetes bioactivos de sedimento marino de la costa central del Perú. *Revista Peruana de Microbiologia* **14**:259–270.

Li A-D, Li L-G, Zhang T. 2015. Exploring antibiotic resistance genes and metal resistance genes in plasmid metagenomes from wastewater treatment plants. *Frontiers in Microbiology* **6**:1025 DOI 10.3389/fmicb.2015.01025.

Li F, Jiang P, Zheng H, Wang S, Zhao G, Qin S, Liu Z. 2011. Draft genome sequence of the marine bacterium *Streptomyces* griseoaurantiacus M045, which produces novel manumycin-type antibiotics with a pABA core component. *Journal of Bacteriology* **193**:3417–3418 DOI 10.1128/JB.05053-11.

Lin Y, Hao X, Johnstone L, Miller SJ, Baltrus DA, Rensing C, Wei G. 2011. Draft genome of *Streptomyces* zinciresistens K42, a novel metal-resistant species isolated from copper-zinc mine tailings. *Journal of Bacteriology* **193**:6408–6409 DOI 10.1128/JB.06165-11.

Locatelli FM, Goo K-S, Ulanova D. 2016. Effects of trace metal ions on secondary metabolism and the morphological development of streptomycetes. *Metallomics* DOI 10.1039/C5MT00324E.

Magarvey N, Keller J, Bernan V, Dworkin M, Sherman DH. 2004. Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Applied and Environmental Microbiology* **70**:7520–7529 DOI 10.1128/AEM.70.12.7520-7529.2004.

Magarvey NA, Haltli B, He M, Greenstein M, Hucul JA. 2006. Biosynthetic pathway for mannopeptimycins, lipoglycopeptide antibiotics active against drug-resistant gram-positive pathogens. *Antimicrobial Agents and Chemotherapy* **50**:2167–2177 DOI 10.1128/AAC.01545-05.

Mahmoud HM, Kalendar AA. 2016. Coral-associated Actinobacteria: diversity, abundance, and biotechnological potentials. *Frontiers in Microbiology* **7**:1–13 DOI 10.3389/fmicb.2016.00204.

Marcone GL, Carrano L, Marinelli F, Beltrametti F. 2010. Protoplast preparation and reversion to the normal filamentous growth in antibiotic-producing uncommon actinomycetes. *The Journal of Antibiotics* **63**:83–88 DOI 10.1038/ja.2009.127.

Martin JF, Liras P. 2012. Cascades and networks of regulatory genes that control antibiotic biosynthesis. In: *Reprogrammin microbial metabolic pathways.* 159–179.

McAlpine JB, Bachmann BO, Piraee M, Tremblay S, Alarco AM, Zazopoulos E, Farnet CM. 2005. Microbial genomics as a guide to drug discovery and structural elucidation: ECO-02301, a novel antifungal agent, as an example. *Journal of Natural Products* **68**:493–496 DOI 10.1021/np0401664.

Medema MH, Blin K, Cimermancic P, De Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R. 2011. AntiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic Acids Research* **39**:339–346 DOI 10.1093/nar/gkr466.

Medema MH, Kottmann R, Yilmaz P, Cummings M, Biggins JB, Blin K, De Bruijn I, Chooi YH, Claesen J, Coates RC, Cruz-Morales P, Duddela S, Düsterhus S, Edwards DJ, Fewer DP, Garg N, Geiger C, Gomez-Escribano JP, Greule A, Hadjithomas M, Haines AS, Helfrich EJN, Hillwig ML, Ishida K, Jones AC, Jones CS, Jungmann K, Kegler C, Kim HU, Kötter P, Krug D, Masschelein J, Melnik AV, Mantovani SM, Monroe EA, Moore M, Moss N, Nützmann H-W, Pan G, Pati A, Petras D, Reen FJ, Rosconi F, Rui Z, Tian Z, Tobias NJ, Tsunematsu Y, Wiemann P, Wyckoff E, Yan X, Yim G, Yu F, Xie Y, Aigle B, Apel AK, Balibar CJ, Balskus EP, Barona-Gómez F, Bechthold A, Bode HB, Borriss R, Brady SF, Brakhage AA, Caffrey P, Cheng Y-Q, Clardy J, Cox RJ, De Mot R, Donadio S, Donia MS, Van der Donk WA, Dorrestein PC, Doyle S, Driessen AJM, Ehling-Schulz M, Entian K-D, Fischbach MA, Gerwick L, Gerwick WH, Gross H, Gust

B, Hertweck C, Höfte M, Jensen SE, Ju J, Katz L, Kaysser L, Klassen JL, Keller NP, Kormanec J, Kuipers OP, Kuzuyama T, Kyrpides NC, Kwon H-J, Lautru S, Lavigne R, Lee CY, Linquan B, Liu X, Liu W, Luzhetskyy A, Mahmud T, Mast Y, Méndez C, Metsä-Ketelä M, Micklefield J, Mitchell DA, Moore BS, Moreira LM, Müller R, Neilan BA, Nett M, Nielsen J, O'Gara F, Oikawa H, Osbourn A, Osburne MS, Ostash B, Payne SM, Pernodet J-L, Petricek M, Piel J, Ploux O, Raaijmakers JM, Salas JA, Schmitt EK, Scott B, Seipke RF, Shen B, Sherman DH, Sivonen K, Smanski MJ, Sosio M, Stegmann E, Süssmuth RD, Tahlan K, Thomas CM, Tang Y, Truman AW, Viaud M, Walton JD, Walsh CT, Weber T, Van Wezel GP, Wilkinson B, Willey JM, Wohlleben W, Wright GD, Ziemert N, Zhang C, Zotchev SB, Breitling R, Takano E, Glöckner FO. 2015. The Minimum Information about a Biosynthetic Gene cluster (MIBiG) specification. *Nature Chemical Biology* **11**:625–631 DOI 10.1038/nchembio.1890.

Mincer TJ, Jensen PR. 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Applied and Environmental Microbiology* **68**:5005–5011 DOI 10.1128/AEM.68.10.5005-5011.2002.

Montalvo NF, Mohamed NM, Enticknap JJ, Hill RT. 2005. Novel actinobacteria from marine sponges. *Antonie van Leeuwenhoek* **87**:29–36 DOI 10.1007/s10482-004-6536-x.

Newman DJ, Cragg GM. 2016. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products* **75**:311–335 DOI 10.1021/np200906s.

Olano C, Gómez C, Pérez M, Palomino M, Pineda-Lucena A, Carbajo RJ, Braña AF, Méndez C, Salas JA. 2009. Deciphering biosynthesis of the RNA polymerase inhibitor streptolydigin and generation of glycosylated derivatives. *Chemistry and Biology* **16**:1031–1044 DOI 10.1016/j.chembiol.2009.09.015.

Ostash B, Saghatelian A, Walker S. 2007. A streamlined metabolic pathway for the biosynthesis of moenomycin A. *Chemistry and Biology* **14**:257–267 DOI 10.1016/j.chembiol.2007.01.008.

Pantoja S, Luis Iriarte J, Daneri G. 2011. Oceanography of the Chilean Patagonia. *Continental Shelf Research* **31**:149–153 DOI 10.1016/j.csr.2010.10.013.

Peláez F. 2006. The historical delivery of antibiotics from microbial natural products—Can history repeat? *Biochemical Pharmacology* **71**:981–990 DOI 10.1016/j.bcp.2005.10.010.

Pérez JM, Calderón IL, Arenas FA, Fuentes DE, Pradenas GA, Fuentes EL, Sandoval JM, Castro ME, Elías AO, Vásquez CC. 2007. Bacterial toxicity of potassium tellurite: unveiling an ancient enigma. *PLOS ONE* **2**:e211 DOI 10.1371/journal.pone.0000211.

Pham TM, Wiese J, Wenzel-Storjohann A, Imhoff JF. 2016. Diversity and antimicrobial potential of bacterial isolates associated with the soft coral Alcyonium digitatum from the Baltic Sea. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* **109**:105–119 DOI 10.1007/s10482-015-0613-1.

Polti MA, Amoroso MJ, Abate CM. 2007. Chromium(VI) resistance and removal by actinomycete strains isolated from sediments. *Chemosphere* **67**:660–667 DOI 10.1016/j.chemosphere.2006.11.008.

Prabhu J, Schauwecker F, Grammel N, Keller U, Bernhard M. 2004. Functional expression of the ectoine hydroxylase gene (*thpD*) from *Streptomyces chrysomallus* in *Halomonas elongata*. *Applied and Environmental Microbiology* **70**:3130–3132 DOI 10.1128/AEM.70.5.3130.

Qin Q-L, Li Y, Zhang Y-J, Zhou Z-M, Zhang W-X, Chen X-L, Zhang X-Y, Zhou B-C, Wang L, Zhang Y-Z. 2011. Comparative genomics reveals a deep-sea sediment-adapted life style of Pseudoalteromonas sp. SM9913. *The ISME Journal* **5**:274–284 DOI 10.1038/ismej.2010.103.

Ravel J, DiRuggiero J, Robb FT, Hill RT. 2000. Cloning and sequence analysis of the mercury resistance operon of Streptomyces sp. strain CHR28 reveals a novel putative second regulatory gene. *Journal of Bacteriology* **182(8)**:2345–2349 DOI 10.1128/JB.182.8.2345-2349.2000.

Ravel J, Schrempf H, Hill RT. 1998. Mercury resistance is encoded by transferable giant linear plasmids in two Chesapeake Bay *Streptomyces* strains. *Applied and Environmental Microbiology* **64**:3383–3388.

Rensing C, Pribyl T, Nies DH. 1997. New functions for the three subunits of the CzcCBA cation-proton antiporter. *Journal of Bacteriology* **179**:6871–6879.

Revenga JE, Campbell LM, Arribére MA, Ribeiro Guevara S. 2012. Arsenic, cobalt and chromium food web biodilution in a Patagonia mountain lake. *Ecotoxicology and Environmental Safety* **81**:1–10 DOI 10.1016/j.ecoenv.2012.03.014.

Ricci S, Janulczyk R, Björck L, Bjo L. 2002. The regulator PerR is involved in oxidative stress response and iron homeostasis and is necessary for full virulence of streptococcus pyogenes the regulator PerR is involved in oxidative stress response and iron homeostasis and is necessary for full virul. *Infection and Immunity* **70**:4968–4976 DOI 10.1128/IAI.70.9.4968.

Rodriguez-Rojas F, Diaz-Vasquez W, Undabarrena A, Munoz-Diaz P, Arenas F, Vasquez C. 2015. Mercury-mediated cross-resistance to tellurite in *Pseudomonas* spp. isolated from the Chilean Antarctic territory. *Metallomics* **8(1)**:108–117 DOI 10.1039/C5MT00256G.

Romero D, Traxler MF, López D, Kolter R. 2012. Antibiotics as signal molecules. *Chemical Reviews* **111**:5492–5505 DOI 10.1021/cr2000509.Antibiotics.

Rother D, Mattes R, Altenbuchner J. 1999. Purification and characterization of MerR, the regulator of the broad-spectrum mercury resistance genes in *Streptomyces* lividans 1326. *Molecular and General Genetics* **262**:154–162 DOI 10.1007/s004380051070.

Ruiz B, Chávez A, Forero A, García-Huante Y, Romero A, Sánchez M, Rocha D, Sánchez B, Rodríguez-Sanoja R, Sánchez S, Langley E. 2010. Production of microbial secondary metabolites: regulation by the carbon source. *Critical Reviews in Microbiology* **36**:146–67 DOI 10.3109/10408410903489576.

Sadler R, Connell D. 2012. Global distillation in an era of climate change. In: Puzyn T, ed. *Organic pollutants ten years after the stockholm convention—environmental and analytical update.* InpTech, 191–216.

Sánchez S, Chávez A, Forero A, García-Huante Y, Romero A, Sánchez M, Rocha D, Sánchez B, Avalos M, Guzmán-Trampe S, Rodríguez-Sanoja R, Langley E, Ruiz B. 2010. Carbon source regulation of antibiotic production. *Journal of Antibiotics* **63**:442–459 DOI 10.1038/ja.2010.78.

Sander R, Keene WC, Pszenny AAP, Arimoto R, Ayers GP, Baboukas E, Cainey JM, Crutzen PJ, Duce RA, Hönninger G, Huebert BJ, Maenhaut W, Mihalopoulos N, Turekian VC, Van Dingenen R. 2003. Inorganic bromine in the marine boundary layer: a critical review. *Atmospheric Chemistry and Physics Discussions* **3**:1301–1336 DOI 10.5194/acpd-3-2963-2003.

Schmidt A, Haferburg G, Schmidt A, Lischke U, Merten D, Ghergel F, Büchel G, Kothe E. 2009. Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. *Chemie der Erde—Geochemistry* **69**:35–44 DOI 10.1016/j.chemer.2007.11.002.

Schmidt A, Haferburg G, Sineriz M, Merten D, Büchel G, Kothe E. 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. *Chemie der Erde—Geochemistry* **65**:131–144 DOI 10.1016/j.chemer.2005.06.006.

Seaver LC, Imlay JA. 2001. Alkyl Hydroperoxide Reductase Is the Primary Scavenger of Endogenous Hydrogen Peroxide in *Escherichia coli*. *Journal of Bacteriology* **183**:7173–7181 DOI 10.1128/JB.183.24.7173.

Sedlmeier R, Altenbuchner J. 1992. Cloning and DNA-sequence analysis of the mercury resistance genes of *Streptomyces*-Lividans. *Molecular & General Genetics* **236**:76–85.

Seiler C, Berendonk TU. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology* **3**:1–10 DOI 10.3389/fmicb.2012.00399.

Siñeriz ML, Kothe E, Abate CM. 2009. Cadmium biosorption by *Streptomyces* sp. F4 isolated from former uranium mine. *Journal of Basic Microbiology* **49**:55–62 DOI 10.1002/jobm.200700376.

Silver S, Phung LT. 2005. A bacterial view of the periodic table: genes and proteins for toxic inorganic ions. *Journal of Industrial Microbiology and Biotechnology* **32**:587–605 DOI 10.1007/s10295-005-0019-6.

Sivaperumal P, Kamala K, Rajaram R. 2015. Bioactive DOPA melanin isolated and characterised from a marine actinobacterium *Streptomyces* sp. MVCS6 from Versova coast. *Natural Product Research* **29**:2117–2121 DOI 10.1080/14786419.2014.988712.

Sobarzo M. 2009. La región de los fiordos de la zona sur de Chile. In: *Aspectos Oceanográficos*. p. 6.

Storz G, Imlay JA. 1999. Oxidative stress. *Current Opinion in Microbiology* **2**:188–194 DOI 10.1016/S1369-5274(99)80033-2.

Studholme DJ. 2016. Genome update. Let the consumer beware: *Streptomyces* genome sequence quality. *Microbial Biotechnology* **9**:3–7 DOI 10.1111/1751-7915.12344.

Subramani R, Aalbersberg W. 2012. Marine actinomycetes: an ongoing source of novel bioactive metabolites. *Microbiological Research* **167**:571–580 DOI 10.1016/j.micres.2012.06.005.

Sun W, Zhang F, He L, Karthik L, Li Z. 2015. Actinomycetes from the South China Sea sponges: isolation, diversity, and potential for aromatic polyketides discovery. *Frontiers in Microbiology* **6**:1–15 DOI 10.3389/fmicb.2015.01048.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**:2725–2729 DOI 10.1093/molbev/mst197.

Tang J, Liu X, Peng J, Tang Y, Zhang Y. 2015a. Genome sequence and genome mining of a marine-derived antifungal bacterium *Streptomyces* sp. M10. *Applied Microbiology and Biotechnology* **99**:2763–2772 DOI 10.1007/s00253-015-6453-0.

Tang X, Li J, Millán-Aguiñaga N, Zhang JJ, O'Neill EC, Ugalde JA, Jensen PR, Mantovani SM, Moore BS. 2015b. Identification of thiotetronic acid antibiotic biosynthetic pathways by target-directed genome mining. *ACS Chemical Biology* **10**:2841–2849 DOI 10.1021/acschembio.5b00658.

Tatusova T, DiCuccio M, Badretdin A, Chetvernin V, Nawrocki EP, Zaslavsky L, Lomsadze A, Pruitt KD, Borodovsky M, Ostell J. 2016. NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research* gkw569 DOI 10.1093/nar/gkw569.

Taylor DE. 1999. Bacterial tellurite resistance. *Trends in Microbiology* **7**:111–115 DOI 10.1016/S0966-842X(99)01454-7.

Tian X, Zhang Z, Yang T, Chen M, Li J, Chen F, Yang J, Li W, Zhang B, Zhang Z, Wu J, Changsheng Z, Long L, Jingfa X. 2016. Comparative genomics analysis of *Streptomyces* species reveals their adaptation to the marine environment and their diversity at the genomic level. *Frontiers in Microbiology* **7**:1–16 DOI 10.3389/fmicb.2016.00998.

Tremaroli V, Fedi S, Zannoni D. 2007. Evidence for a tellurite-dependent generation of reactive oxygen species and absence of a tellurite-mediated adaptive response to oxidative stress in cells of Pseudomonas pseudoalcaligenes KF707. *Archives of Microbiology* **187**:127–135 DOI 10.1007/s00203-006-0179-4.

Turner RJ, Aharonowitz Y, Weiner JH, Taylor DE. 2001. Glutathione is a target in tellurite toxicity and is protected by tellurite resistance determinants in *Escherichia coli*. *Canadian Journal of Microbiology* **47**:33–40 DOI 10.1139/cjm-47-1-33.

Undabarrena A, Beltrametti F, Claverías FP, González M. 2016a. Exploring the diversity and antimicrobial potential of marine actinobacteria from the comau fjord in Northern Patagonia , Chile. *Frontiers in Microbiology* **7**:1–16 DOI 10.3389/fmicb.2016.01135.

Undabarrena A, Ugalde JA, Castro-Nallar E, Seeger M, Cámara BP. 2016b. Genome sequence of *Streptomyces* sp. strain H-KF8, a marine actinomycete isolated from a northern Chilean Patagonian fjord. *Genome Announcements* In Press.

Valliappan K, Sun W, Li Z. 2014. Marine actinobacteria associated with marine organisms and their potentials in producing pharmaceutical natural products. *Applied Microbiology and Biotechnology* **98**:7365–7377 DOI 10.1007/s00253-014-5954-6.

Van Lanen SG, Shen B. 2006. Microbial genomics for the improvement of natural product discovery. *Current Opinion in Microbiology* **9**:252–260 DOI 10.1016/j.mib.2006.04.002.

Van Nostrand JD, Khijniak TV, Gentry TJ, Novak MT, Sowder AG, Zhou JZ, Bertsch PM, Morris PJ. 2007. Isolation and characterization of four Gram-positive nickel-tolerant microorganisms from contaminated sediments. *Microbial Ecology* **53**:670–682 DOI 10.1007/s00248-006-9160-7.

Vicente J, Stewart A, Song B, Hill RT, Wright JL. 2013. Biodiversity of Actinomycetes associated with Caribbean sponges and their potential for natural product discovery. *Marine Biotechnology* **15**:413–424 DOI 10.1007/s10126-013-9493-4.

Vilos C, Morales FA, Solar PA, Herrera NS, Gonzalez-Nilo FD, Aguayo DA, Mendoza H, Comer J, Bravo ML, González PA, Kato S, Cuello MA, Alonso C, Bravo EJ, Bustamante EI, Owen GI, Velasquez LA. 2013. Paclitaxel-PHBV nanoparticles and their toxicity to endometrial and primary ovarian cancer cells. *Biomaterials* **34**:4098–4108.

Wacaster S. 2015. The mineral industry of Chile. *US Geological Survey*.

Wallhausser KH, Nesemann G, Prave P, Steigler A. 1965. Moenomycin, a new antibiotic. I. Fermentation and isolation. *Antimicrobial Agents Chemotherapy* 734–736.

Wang L, Chen S, Xiao X, Huang X, You D, Zhou X, Deng Z. 2006. arsRBOCT arsenic resistance system encoded by linear plasmid pHZ227 in *Streptomyces* sp. strain FR-008. *Applied and Environmental Microbiology* **72**:3738–3742 DOI 10.1128/AEM.72.5.3738-3742.2006.

Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Research* **43**:W237–W243 DOI 10.1093/nar/gkv437.

Weber T, Laiple KJ, Pross EK, Textor A, Grond S, Welzel K, Pelzer S, Vente A, Wohlleben W. 2008. Molecular Analysis of the Kirromycin Biosynthetic Gene Cluster Revealed β-Alanine as Precursor of the Pyridone Moiety. *Chemistry and Biology* **15**:175–188 DOI 10.1016/j.chembiol.2007.12.009.

Weber T, Welzel K, Pelzer S, Vente A, Wohlleben W. 2003. Exploiting the genetic potential of polyketide producing streptomycetes. *Journal of Biotechnology* **106**:221–232 DOI 10.1016/j.jbiotec.2003.08.004.

Westerdahl A, Olsson CJ, Kjelleberg S, Conway PL. 1991. Isolation and characterization of turbot (Scophtalmus maximus)—associated bacteria with inhibitory effects against vibrio anguillarum. *Applied and Environmental Microbiology* **57**:2223–2228.

Yagüe P, López-García MT, Rioseras B, Sánchez J, Manteca Á. 2013. Pre-sporulation stages of *Streptomyces* differentiation: state-of-the-art and future perspectives. *FEMS Microbiology Letters* **342**:79–88 DOI 10.1111/1574-6968.12128.

Yuan M, Yu Y, Li H-R, Dong N, Zhang X-H. 2014. Phylogenetic diversity and biological activity of actinobacteria isolated from the Chukchi Shelf marine sediments in the Arctic Ocean. *Marine Drugs* **12**:1281–1297 DOI 10.3390/md12031281.

Zazopoulos E, Huang K, Staffa A, Liu W, Bachmann BO, Nonaka K, Ahlert J, Thorson JS, Shen B, Farnet CM. 2003. A genomics-guided approach for discovering and expressing cryptic metabolic pathways. *Nature Biotechnology* **21**:187–190 DOI 10.1038/nbt784.

Zhang H, Lee YK, Zhang W, Lee HK. 2006. Culturable actinobacteria from the marine sponge Hymeniacidon perleve: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Antonie van Leeuwenhoek* **90**:159–169 DOI 10.1007/s10482-006-9070-1.

Zhang H, Wang H, Wang Y, Cui H, Xie Z, Pu Y, Pei S, Li F, Qin S. 2012. Genomic sequence-based discovery of novel angucyclinone antibiotics from marine *Streptomyces* sp. W007. *FEMS Microbiology Letters* **332**:105–112 DOI 10.1111/j.1574-6968.2012.02582.x.

Zhao B, Lin X, Lei L, Lamb DC, Kelly SL, Waterman MR, Cane DE. 2008. Biosynthesis of the sesquiterpene antibiotic albaflavenone in *Streptomyces* coelicolor A3(2). *The Journal of Biological Chemistry* **283**:8183–8189 DOI 10.1074/jbc.M710421200.

Zhu XM, Hackl S, Thaker MN, Kalan L, Weber C, Urgast DS, Krupp EM, Brewer A, Vanner S, Szawiola A, Yim G, Feldmann J, Bechthold A, Wright GD, Zechel DL. 2015. Biosynthesis of the fluorinated natural product nucleocidin in *Streptomyces* calvus is dependent on the bldA-specified Leu-tRNAUUA molecule. *ChemBioChem* **16**:2498–2506 DOI 10.1002/cbic.201500402.

Zotchev SB. 2014. Genomics-based insights into the evolution of secondary metabolite biosynthesis in actinomycete bacteria. In: Pontarotti P, ed. *Evolutionary biology: genome evolution, speciation, coevolution and origin of life.* Cham: Springer International Publishing, 35–46.

5.2.2 Addendum Chapter III

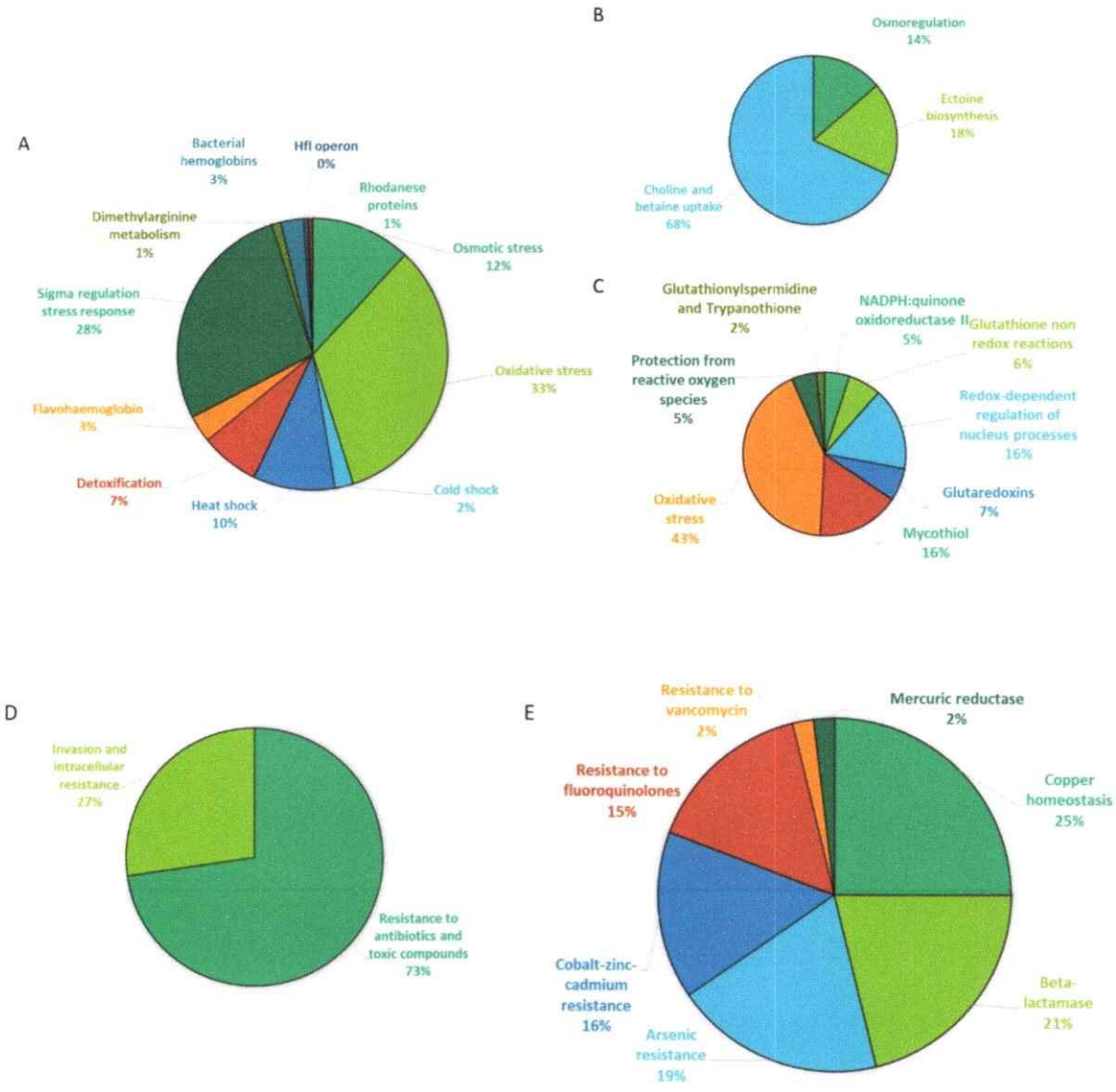


Figure 18: Coding sequences of *Streptomyces* sp. H-KF8 associated to biological traits. Percentage of genes grouped by functional categories, related to environmental adaptation responses. **A)** Stress response. **B)** Osmotic response. **C)** Oxidative stress response. **D)** Virulence and defense. **E)** Resistance to antibiotics and toxic compounds. These genetic determinants were evaluated by their functionality (**Chapter III**), as follows: metal tolerance response, **Figure 6**; oxidative stress response, **Figure 7**; antibiotic resistance response, **Figure 8**.

Table 8. Biosynthetic gene clusters (BGCs) present in the *Streptomyces* sp. H-KF8 genome. The detection was conducted by submitting the whole genome assembly to the antiSMASH platform.

CLUSTER (#)	antiSMASH TYPE DESCRIPTOR*	LENGTH (bp)	CONTIG
1	Terpene	20992	tig_00
2	Nucleoside-Phosphoglycolipid	35469	tig_00
3	Other	43290	tig_00
4	Terpene	21253	tig_02
5	NRPS	81285	tig_02
6	NRPS	79174	tig_02
7	Terpene	26858	tig_02
8	NRPS-PKS I	56103	tig_02
9	NRPS-PKS III	54318	tig_02
10	Terpene-Siderophore	50603	tig_02
11	Terpene	22162	tig_02
12	Bacteriocin	11412	tig_02
13	Lantipeptide	21819	tig_02
14	Lassopeptide	22692	tig_10
15	Butyrolactone	11073	tig_14
16	Oligosaccharide-PKS I	42574	tig_16
17	PKS I	33925	tig_138
18	Lantipeptide-PKS I	61004	tig_138
19	Terpene	21220	tig_138
20	NRPS-PKS I	50808	tig_138
21	NRPS-PKS I	52764	tig_139
22	PKS II	42512	tig_139
23	Ectoine	10398	tig_139
24	Melanin	10509	tig_139
25	Siderophore	11808	tig_139
26	Lantipeptide	24585	tig_139

*for comparison of BGCs with MIBiG database and prediction of each cluster product, see **Table 3, Chapter III.**

Chapter IV: Chemical detection of antimicrobial compounds in *Streptomyces* sp. H-KF8: unveiling their connection between its NRPS biosynthetic gene clusters

5.3 Chapter IV

5.3.1 Introduction

Metabolomics is defined as the comprehensive study of small molecules within a biological system and provides a direct measure of detectable secondary metabolite production within an organism of interest (Covington, McLean & Bachmann, 2016). Particularly, when the secondary metabolites are associated to a bioactivity, such as the antimicrobial one, the conventional approach consists in following the bacterial inhibition potential of the crude culture extract (Sharma, Kalita & Thakur, 2016; Balouiri, Sadiki & Ibnsouda, 2016). Another useful approach is based on genome mining, which addresses the genetic biosynthetic potential to produce compound(s) of interest in a specific strain (Gomez-Escribano, Alt & Bibb, 2016). However, none of these approaches allows drawing conclusions about the link between antibiotic potential and activity, nor does it clearly prove the involvement of a specific compound (Debois *et al.*, 2013). In this line, the Imaging mass spectrometry (Imaging MS) has recently been crucial for complementing the traditional mass techniques by enabling the preservation of molecular localization, yielding insights into the underlying biology. By providing a spatial snapshot for each observed mass signal, it is possible to directly visualize the metabolic exchange within and among microbial species on solid media (Yang et al., 2012). These developments have introduced a new concept of real-time mass spectrometry and data visualization tools, since Imaging MS is able to provide two-dimensional (2D) and even three-dimensional (3D) visualization of surface metabolites directly from microbial colonies (Fang & Dorrestein, 2014).

In order to understand it's genetic and metabolic relationship, for the antimicrobial-producer marine actinomycete *Streptomyces* sp. H-KF8, , a multiple procedure involving: i) the identification of its BGCs within the genome, with special focus in the NRPS clusters where a bioinformatics-based prediction of the monomers that compose the metabolites is proposed; ii) the conventional approach that involves the extraction of the antimicrobial compound(s) from cell culture was addressed; and iii) the analysis of crude extract using the mass spectrometry techniques of Electrospray Ionization – Fourier Transform – Ion Cyclotron Resonance (ESI-FT ICR MS), Matrix assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF MS) and Imaging-MS techniques (MALDI-FT ICR MS) was accomplished. Overall, the complement of these genomic and metabolic analyses will provide the basis for establishing the connection between the whole metabolome interaction and those specific molecules that are involved in an antagonistic inhibition interaction.

5.3.2 Materials and Methods

5.3.1.1 Bioinformatic prediction of the chemical structure of metabolites synthesized by NRPS biosynthetic gene clusters

Biosynthetic gene clusters were identified through the antiSMASH online platform (Weber *et al.*, 2015) using the whole genome sequence of the previously selected actinomycetes (*Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Kocuria* sp. H-KB5, *Rhodococcus* sp. H-CA8F and *Streptomyces* sp. H-KF8). *Streptomyces* sp. H-KF8 was considered for subsequent analyses, due to the presence of two complete NRPS BGCs. For this bacterium, each cluster was analyzed separately, in terms of it similarity with other known BGCs which are available in MIBiG Database (Medema *et al.*, 2015). For both NRPS BGCs, each gene function was manually analyzed through BLASTp, with subsequent analysis of their conserved domains. With the complementation of these bioinformatic tools it was possible to generate a predicted structure of the core molecule for each NRPS BGC, which was verified through PRISM software (Skinnider *et al.*, 2015). Genes involved in tailouring reactions were not used for the core prediction, although their functionality was suggested. Predicted molecules were drawn by ChemDraw software (PerkinElmer).

5.3.1.2 Extractions of antimicrobial compounds from actinomycetes in liquid cultures using organic solvents

Antimicrobial activity of actinomycetes were previously demonstrated, and assessed in several solid media (**Chapter I** and **Chapter III**). ISP2 and V media were selected to perform liquid cultures with *Streptomyces* sp. H-KF8. In ISP2 medium (Shirling & Gottlieb, 1966), *Streptomyces* sp. H-KF8 showed high antimicrobial activity mainly against *Staphylococcus aureus* NBRC

100910^T and *Listeria monocytogenes* 07PF0776 (see **Chapter I**). In medium V (Marcone *et al.*, 2010), *Streptomyces* sp. H-KF8 showed favourable and rapid growth. After 30 and 15 days of growth in ISP2 and V media, with constant shaking at 30 °C, solvent extractions were performed. For non-mycelial strains (*Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Kocuria* sp. H-KB5 and *Rhodococcus* sp. H-CA8F), a 50 mL culture incubated for 10 days in ISP2 medium with constant shaking at 30 °C was used. Due to their different polarities, several organic solvents were employed in a 1:1 ratio (v/v) for two times extraction of bioactive compounds. Hexane, ethyl acetate, methanol, ethanol, isopropanol, acetone, methanol-water (1:1) and acetone-water (8:2) were used to obtain extracts from the *Streptomyces* sp. H-KF8 cells supernatant and pellet. For non-mycelial strains, hexane, ethyl acetate and methanol were used to produce extracts from the cell supernatant and pellet. Solvent evaporation was carried out using a speed vacuum. Crude extracts was subsequently dissolved in methanol-water (1:1) for *Streptomyces* sp. H-KF8 and 10% dimethyl sulphoxide (DMSO) for non-mycelial extracts, until a final concentration of 5 mg/mL.

5.3.1.3 Biological activities assays of crude extracts

Activities of crude extracts were evaluated with several biological model targets. For antibacterial activity, bacterial reference strains *Staphylococcus aureus* NBRC 100910^T, *Listeria monocytogenes* 07PF0776, *Escherichia coli* FAP1, *Salmonella enterica* LT2^T and *Pseudomonas aeruginosa* DSM50071^T were grown overnight with constant shaking at 37 °C. LB-plates were spread with a final concentration of turbidity$_{600}$=0.3 for each bacterial reference strain, and once dried, 10 µL of each crude extract at a final concentration of 5 mg/mL was added on the plate. Inhibitions zones were evaluated 24 and 48 h after incubation at 37 °C. The respective medium

along with 10 % DMSO and methanol-water, were used as negative control (Balouiri, Sadiki & Ibnsouda, 2016).

Streptomyces sp. H-KF8 crude extracts were evaluated for antifungal activity. The methodology mentioned above was slightly modified, using *Saccharomyces cerevisiae* as model unicellular fungi. Inhibition zones were evaluated in Sabouraud agar after 24 h and 48 h of incubation at 25 °C (Balouiri, Sadiki & Ibnsouda, 2016).

To test anti-proliferative activity, four human cell lines were evaluated: MCF-7 cells (breast cancer), CACO-2 cells (colon cancer), SH-SY5Y (neuroblastoma) and fibroblasts. Cell line proliferation was measured using the resazurin method (Sarker, Nahar & Kumarasamy, 2007) after incubation with crude extracts obtained from *Streptomyces* sp. H-KF8. Experiment was performed in triplicate.

5.3.1.4 Exploring the chemical nature of *Streptomyces* sp. H-KF8 extract

Susceptibility of *Streptomyces* sp. H-KF8 crude extract to several temperatures (-80, -20, 4, 25, 37, 60, 80 and 100 °C) was tested after a one-hour incubation period. Additionally, susceptibility of the crude extract to various enzymes (proteinase K, lysozyme and trypsin) was carried out separately, and in combinations. Enzyme incubation was performed at 37 °C sequentially for 30 min. Enzyme were inactivated by heat shock at 80 °C for 15 min. Antibacterial activity after each treatment was assessed as previously described, using the bacterial reference strains *S. aureus* NBRC 100910^{T} and *L. monocytogenes* 07PF0776. After 24 and 48 h incubating at 37 °C, inhibition zones were measured. Experiments were performed in triplicate using three

biological replicates. A statistical Student's t-test analysis was applied to all data (n=9), using as significate a p value <0.01.

An absorbance spectrum scanning through several wavelengths starting from 230 to 600 nm was carried out with *Streptomyces* sp. H-KF8 crude extract, with a wavelength step size of 2 nm. Additionally, fluorescence emission spectrum starting from 280 to 700 nm was determined by excitation of the sample at a wavelength of 260 nm, also with a wavelength step size of 2 nm. A 1:10 dilution of the extract was used for these experiments. Aromatic amino acids (tyrosine, tryptophan and phenylalanine) were used for comparison, and methanol-water as negative control. Absorbance and fluorescence measurements were monitored using a microplate multireader Tecan Infinite 200 PRO.

Crude extract obtained from growth of *Streptomyces* sp. H-KF8 on different media (ISP2 and V medium) were evaluated through thin layer chromatography (TLC) using pre-coated silica gel aluminum sheets. Several different mixtures of solvents were used for migration of compounds such as: acetonitrile:chloroform:methanol (2:4:4); acetone:methanol (7:3), methanol:hexane:chloroform (6:2:2), acetate:hexane (1:1), ethyl acetate:methanol (7:3). Different detection procedures with several visualization reagents were used, such a UV light exposure at 312 and 565 nm and heat. Staining with ninhydrin/ethanol/glacial acetic acid; orcinol/glacial acetic acid; and heat shock at 110 °C were used for spots detection (http://lcso.epfl.ch/files/content/sites/lcso/files/load/TLC_Stains.pdf).

5.3.1.5 Chemical characterization of the antimicrobial compounds through HPLC-bioguided fractionation

Once conditions to obtain active crude extracts were standardized, middle-fermentations of *Streptomyces* sp. H-KF8 were carried out using a total culture of 2.4 L, with the aim of obtaining an increased amount of crude extract. Crude extracts were obtained from two different media (ISP2 and V medium) that were used for comparison. After 30 days of incubation, supernatant was separated and cells were left in methanol overnight under constant shaking. Solvent was evaporated and crude extracts were weighted, to obtain a final concentration of 50 mg/mL (Sharma, Kalita & Thakur, 2016). Extract was diluted in water-trifluoroacetic acid (TFA) 0.1%, centrifuged and filtered for high pressure liquid chromatography (HPLC) in a ÄKTA purifier chromatography system (GE Healthcare). A mobile phase with a TFA-water/methanol gradient was used, ranging from 0 to 100% of methanol in 7 column volumes. A Jupiter C18 250/10 column and a flow rate of 5 mL/min were used. In addition, a gradient with water-TFA/acetonitrile under the same conditions was also tested. Absorbance was simultaneously monitored at wavelengths 260 and 280 nm. Peaks were analyzed through the UNICORN software. Fractions with a volume of 1 mL were recovered and dried with a speed-vacuum. Fractions were resuspended in 15 µl of water-TFA 0.1% and antibacterial activity of each fraction was evaluated on LB-plates, by adding 3 µL of each fraction above a previously autoclaved paper disk, or directly above the plate. Bacterial strains *Staphylococcus aureus* ATCC 29740^T, *Staphylococcus epidermidis* ATCC 35984^T, *Escherichia coli* ATCC 8739^T, *Listeria monocytogenes* ATCC 19114^T, *Pseudomonas aeruginosa* ATCC 27853^T, *Klebsiella pneumoniae* ATCC 13883^T, *Enterococcus faecalis* ATCC 19433^T, *Micrococcus luteus* ATCC 9341^T and *Bacillus subtilis* ATCC 1668^T were used. Each bacterial strain was grown as a thin lawn from an overnight-liquid culture. Plates were incubated at 37 °C and

inhibition around paper disks was observed. Active fractions were evaluated through absorbance scanning from 200 to 500 nm, to test the presence of another possible peaks at wavelengths different that 260 nm or 280 nm.

5.3.1.6 Analysis of metabolite expression by analytical mass-spectrometry

Active fractions were further characterized through analytical mass spectrometry, using ESI-FT ICR MS, MALDI-TOF MS and Imaging-MS techniques SolariX 9.4T (Bruker, Bremen, Germany). For ESI-FT ICR MS, samples were diluted (up to 50×, depending of the UV absorption intensity) and tested in positive and negative ion mode (Debois *et al.*, 2013). Spectra were recorded from 72 to 3000 m/z. Ion accumulation time was optimized for every sample in order to avoid overfilling of the analyzer. MS/MS experiments were performed on ions with the higher intensity (Debois *et al.*, 2013).

Fractions that were obtained under different culture conditions and presented different profiles of activity were compared, leading to the selection of m/z ions candidates from each fraction which could be associated to metabolites responsible for the antibacterial activity. The specific m/z of these selected ions was compared with StreptomeDB online database (http://www.pharmaceutical-bioinformatics.de/streptomedb/) and those with no potential identification were further selected. Imaging-MS was performed under several conditions: *Streptomyces* sp. H-KF8 after 10 and 15 days incubation, was confronted either with *S. aureus* ATCC 29740^T, *S. epidermidis* ATCC 35984^T and *P. aeruginosa* ATCC 27853^T, in order to evaluate the metabolites that are expressed in the inhibition zone due to the specific interaction between these strains. As a control, *Streptomyces* sp. H-KF8 was also incubated alone during 10 and 15 days. All incubations were performed in modified-ISP2 medium over MALDI ITO glass slides (Yang

et al., 2012; Vergeiner *et al.*, 2013). Matrix deposition, α-cyano-4-hydroxycinnamic acid (HCCA) 5 mg/mL in ACN/H_2O-TFA 0.33 % (70/30 v/v) was performed using a SunCollect (Sunchrom) equipment with a flow of 20 µL/min (Debois *et al.*, 2013). Laser ionization zones and magnitude were set at 50 µm for the pixel size and 200 µm for the raster width (Debois *et al.*, 2013). Ions with a high expression in confrontation conditions were further selected for MALDI-TOF MS analysis. Samples were mixed with two different matrices: HCCA and 2,5-dihydroxybenzoic acid (DHB) (Sigma) in a 1:1 ratio (Debois *et al.*, 2013). Samples were dried at room temperature to prior analysis in both positive and negative LIFT mode. Fragmentation patterns and interpretation were analyzed manually. All data analysis was performed with the DataAnalysis or FlexAnalysis, Flex-Imaging and SCiLs software (Bruker).

5.3.2 Results

5.3.2.1 Bioinformatic prediction of the chemical structure of metabolites synthesized by NRPS biosynthetic gene clusters

Prediction of core chemical structures of the skeleton of the secondary metabolites that are synthesized by the NRPS biosynthetic gene clusters can be achieved by the analysis of the adenylation domains. The adenylation domains are conserved domains responsible for the selection of the amino acid monomer that is incorporated into each module. This selection is governed by the *Stachelhaus code*, which indicates potential amino acids to be incorporated (Stachelhaus, Mootz & Marahiel, 1999). This analysis was performed with *Streptomyces* sp. H-KF8, where two complete NRPS BGCs were detected. By merging information obtained by both antiSMASH and PRISM platforms, a prediction of the amino acid substrates was obtained for cluster #5 and cluster #6.

Cluster #5 harbours a total of 81,285 bp that bears 60 genes including 5 NRPS genes involved in the incorporation of six amino acids (valine + valine + tryptophan + tryptophan + tryptophan-valine) and a hybrid PKS-NRPS module which incorporates a modified phenylalanine (+Phe*), which undergoes a decarboxylation by the 3R-hydroxyacyl-CoA-dehydrogenase PKS gene which harbours a ketoreductase domain (KR). Additionally, genes encoding for glutamate synthase, glutamate/methyl aspartate mutase and asparagine ligase are within the cluster, suggesting an additional amino acid incorporation (asparragine). In summary, the peptidic core which is synthesized by the enzymes encoded in cluster #5 is composed by 7 amino acids (phenylalanine*-valine-valine-tryptophan-tryptophan-tryptophan-valine-asparragine). Two

genes encoding for thioesterase and one encoding for a cyclase are also present within the cluster #5, which may be involved in the cyclization of the linear assembly.

On the other hand, cluster #6 presents a total of 79,174 bp that harbour 33 genes, with two main NRPS genes responsible for the incorporation of ten amino acids (tryptophan-alanine-valine-alanine-tryptophan + ornithine-threonine-ornithine-valine-tryptophan). Five epimerization domains are encoded within the two biosynthetic NRPS genes, which are involved in the incorporation of the D-amino acid form of two tryptophan, one valine, threonine and one ornithine. An additional gene coding for a thioesterase domain situated upstream from the main biosynthetic NRPS genes, may be responsible for the release and subsequent cyclization of the predicted linear molecule. In addition, tailouring genes may be playing a role in the post-assembly modification of the peptidic core, acting on the incorporation of sugar moieties. Interestingly, an unusual gene (*aurF*) encoding for a p-amino benzoate *N*-oxygenase is present within cluster #6, which may be involved in the conversion to p-nitro benzoic acid. A general scheme for each BGC with the corresponding modules, domain and prediction of monomers incorporated is presented in **Figure 19** for cluster #5 and **Figure 20** for cluster #6.

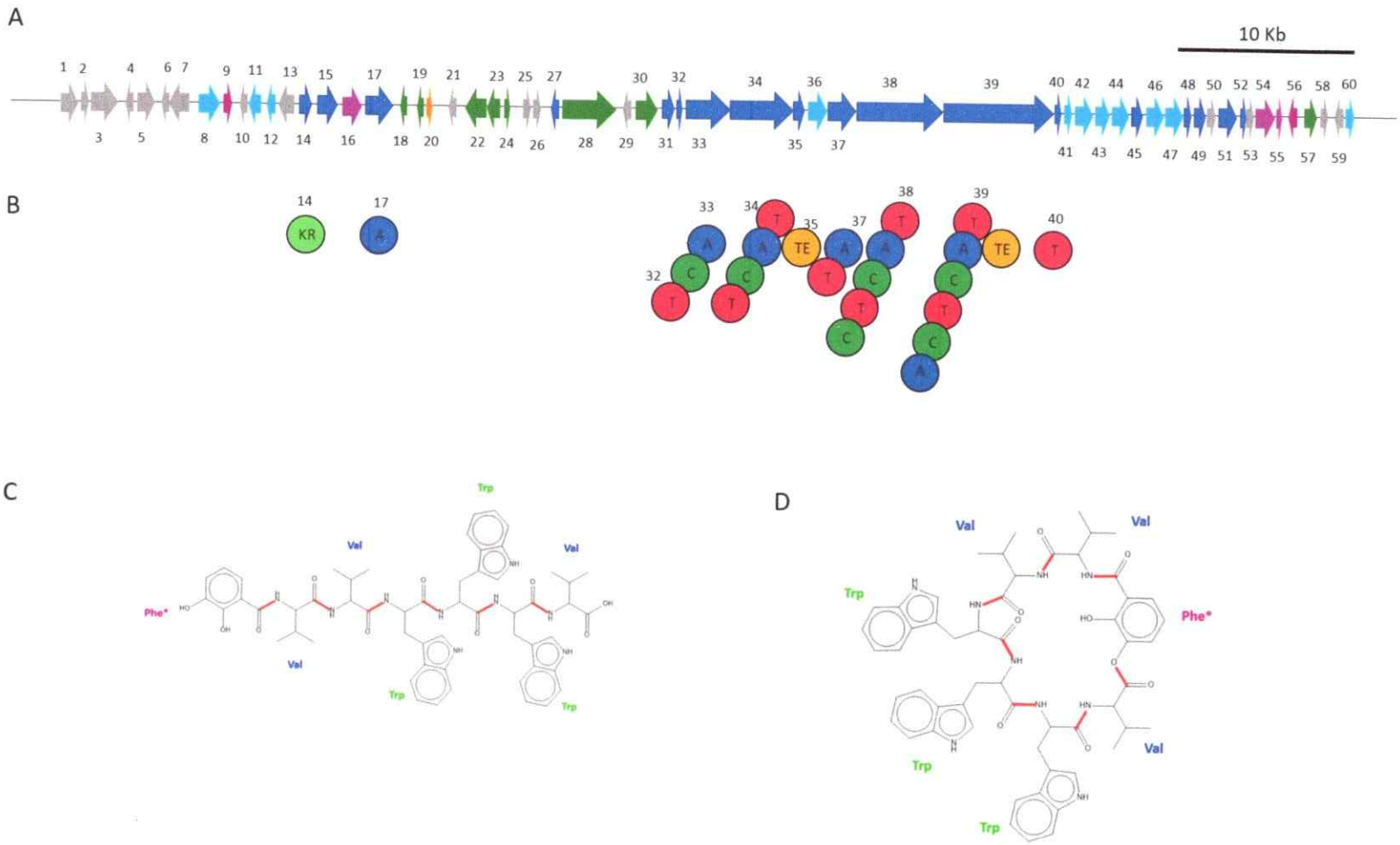


Figure 19: General scheme for cluster #5. A) 60 genes involving the 81,285 bp genetic cluster. Colours represent: blue, biosynthetic genes; light-blue, tailouring reactions; green, regulatory genes; purple, transport; orange, resistance; gray, unknown/non-directly related. **B)** Catalytic domains predicted for the biosynthetic genes. Letters represent: A, adenylation; C, condensation; T, thiolation; TE, thioesterase; KR, ketoreductase. **C)** Predicted amino acid linear assembly. **D)** Predicted amino acid cyclic assembly.

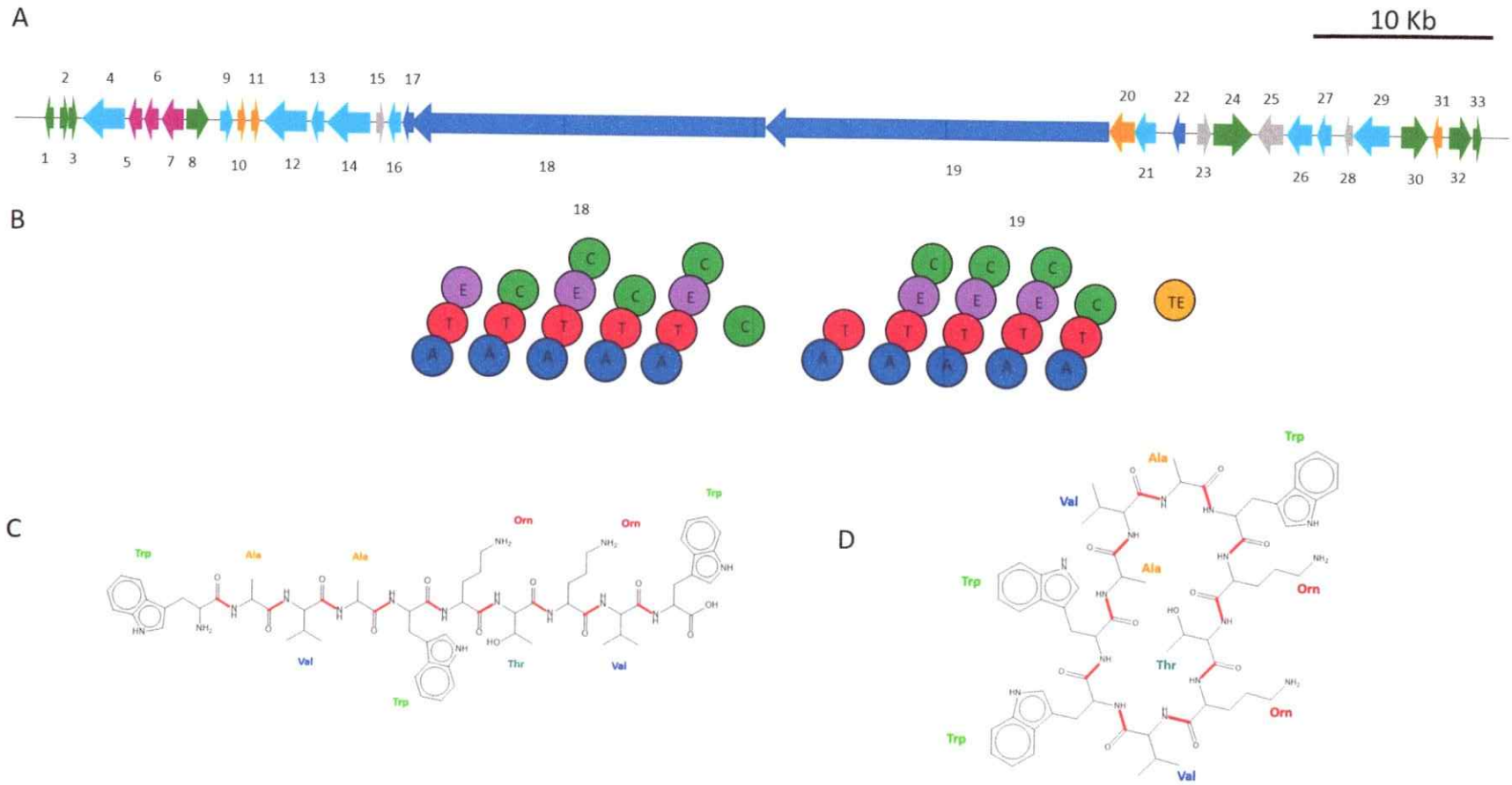


Figure 20: General scheme for cluster #6. A) 33 genes involving the 79,174 bp genetic cluster. Colours represent: blue, biosynthetic genes; light-blue, tailouring reactions; green, regulatory genes; purple, transport; orange, resistance; gray, unknown/non-directly related. **B)** Catalytic domains predicted for the biosynthetic genes. Letters represent: A, adenylation; C, condensation; T, thiolation; TE, thioesterase; E, epimerization. **C)** Predicted amino acid linear assembly. **D)** Predicted amino acid cyclic assembly.

5.3.2.2 Extractions of antimicrobial compounds from actinomycetes in liquid cultures using organic solvents

Extractions led to different results among the actinomycetes strains evaluated. Overall, the organic solvent hexane was not suitable to obtain metabolites with antibacterial activity against bacterial reference strains. On the contrary, ethyl acetate and methanol yielded better results. For non-mycelial strains, antibacterial activity against both Gram-positive and Gram-negative reference strains was observed with supernatant-extracts of *Rhodococcus* sp. H-CA8F obtained with ethyl acetate. For *Kocuria* sp. H-KB5, antibacterial activity of crude extracts was observed only against gram-negative bacterial reference strains, using ethyl acetate to perform supernatant-extracts. *Brevibacterium* sp. H-BE7 ethyl acetate extracts only displayed antibacterial activity against *S. enterica* LT2^{T}. Conversely, antibacterial activity in *Arthrobacter* sp. H-JH3 crude extracts was observed only when extractions were performed with methanol, from the cells, whereas no activity was observed from supernatant cultures. For complete antimicrobial activities of crude extracts, please see **Table 2** of **Chapter I.**

For mycelial strains, liquid cultures have to be adapted to the growth requirements of each strain. *Streptomyces* sp. H-KF8 is characterized for a slow growth and complex developmental cycle in liquid media. In ISP2 medium, morphology of clumps and fragmented hyphae was observed after 15 days of growth. When supernatants were tested directly, no antimicrobial activity was observed, although activity was observed when the cell pellet was used (**Figure 21A**). Regarding solvent extractions, neither hexane nor ethyl acetate were suitable to obtain a crude extract that displayed antibacterial activity. On the contrary, other polar organic

solvents were also evaluated, which indeed presented antibacterial activity (**Figure 21B**). In this line, activity against *S. aureus* NBRC 100910^T was observed when extraction was performed directly from mycelial cells using methanol, even when using only 1 µL of crude extract (**Figure 21C**). Overall, these results suggest that the chemical nature of the antibacterial compounds detected is clearly different in each actinomycete strain. Interestingly, antibacterial compound(s) from *Streptomyces* sp. H-KF8 have a polar nature that is reflected in the efficient extraction when using methanol, whereas antibacterial compounds from non-mycelial strains are observed when using a solvent of intermediate polarity. Moreover, in *Streptomyces* sp. H-KF8, antibacterial compound(s) remains attached to the cell surface when grown in liquid media, and are not observed when extractions are performed from the culture supernatant.

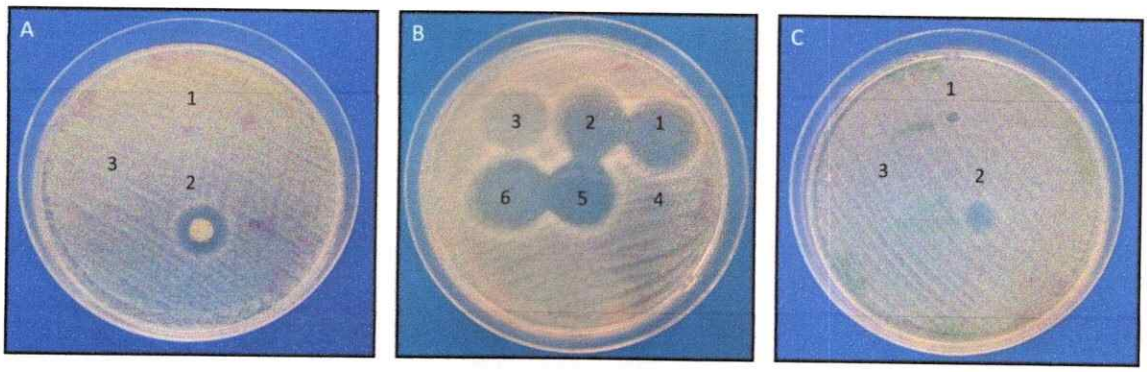


Figure 21: Solvent extractions of antimicrobial compounds for *Streptomyces* sp. H-KF8 against *S. aureus* NBRC 100910^T. A) Antibacterial activity directly from the culture after 30 days of incubation. 1, supernatant; 2, cell pellet; 3, fermentation media ISP2 as negative control. **B)** Antibacterial activity observed when extraction was performed with various polar solvents. 1, ethanol; 2, methanol; 3, isopropanol; 4, acetone; 5, methanol-water; 6, acetone-water. **C)** Antibacterial activity of the crude extract. 1, solvent methanol as technical control; 2, 1 µL of crude extract of *Streptomyces* sp. H-KF8; 3, methanol-water as negative control.

5.3.2.3 Exploring the different biological activities of *Streptomyces* sp. H-KF8 crude extract

To further explore other biological activities apart from the antibacterial ones against *S. aureus* NBRC 100910^T (**Figure 22A**) observed with *Streptomyces* sp. H-KF8 methanol-extract, different biological models were tested. In this line, activity against *L. monocytogenes* 07PF0776 (**Figure 22B**) and *E. coli* FAP1 (**Figure 22C**) was observed, although no antibacterial activity was observed against *P. aeruginosa* DSM 50071^T and *S. enterica* LT2^T when extraction with methanol was performed directly from mycelial cells. In addition, no antifungal activity was observed when tested against *S. cerevisiae* (data not shown).

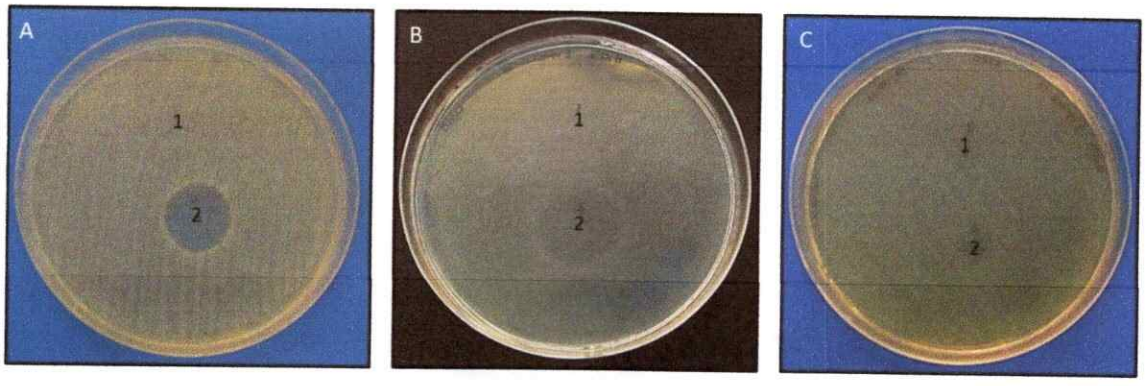


Figure 22: Antibacterial activity of *Streptomyces* sp. H-KF8 crude extract. 1, Methanol-water as negative control; 2, 5 µL of crude extract. **A)** *S. aureus* NBRC 100910^T. **B)** *L. monocytogenes* 07PF0776. **C)** *E. coli* FAP1.

In addition, crude extract activity against cancer cell lines was evaluated, and mainly an antiproliferative concentration-dependent effect was observed, at extract concentration up to 5 mg/mL (**Figure 23**). Notably, *Streptomyces* sp. H-KF8 crude extract showed a major decrease in the proliferation of SH-SY5Y neuroblastoma cells, leaving only up to 20 % of cell proliferation (**Figure 23A**); and up to a 40 % of cell proliferation in CACO-2 colon cancer cell lines (**Figure 23B**). On the other hand, no significant effect was observed for MCF-7 breast cancer cells (**Figure 23C**).

However, an important activity was also observed when the extract was tested against human fibroblasts as a model for non-cancer cell line (**Figure 23D**). Overall, our results show that the crude extract of *Streptomyces* sp. H-KF8 displays various bioactivities, which suggests that several compounds with different mode of action and different model targets are present within the extract obtained, or alternatively, it could be one family of compounds which present both antibacterial and antiproliferative activities.

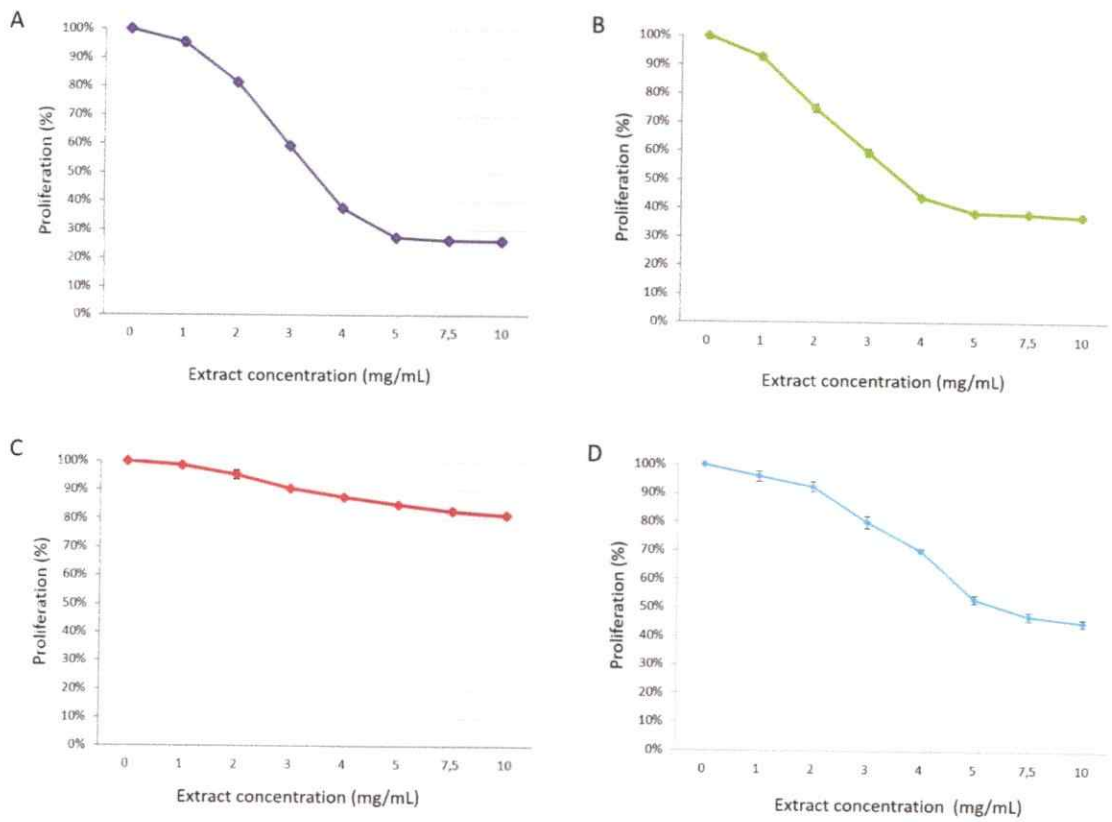


Figure 23: Antiproliferative activity of *Streptomyces* sp. H-KF8 crude extract. Antiproliferative activity was evaluated with the resazurin method. **A)** SH-SY5Y neuroblastoma cells. **B)** CACO-2 colon cancer cells. **C)** MCF-7 breast cancer cells. **D)** human fibroblasts cells.

5.3.2.4 Exploring the chemical nature of *Streptomyces* sp. H-KF8 crude extract

The *Streptomyces* sp. H-KF8 crude extract was evaluated in terms of it susceptibility to temperatures (**Figure 24**) and enzymes (**Figure 25**). Regarding the temperature assay, the inhibition areas against *S. aureus* NBRC 100910^T did not visibly change when the extract was incubated at different temperatures (**Figure 24A**). A decrease in the temperature of incubation did not affect the antibacterial activity observed (**Figure 24A**). Nevertheless, a statistically significant decrease of the inhibition zone was observed when the extract was incubated at 80 °C and this effect was more pronounced at 100 °C (**Figure 24B**), although the antimicrobial activity was rather stable. As protein denaturation usually occurs at high temperatures, our results suggests that the functional groups within the antibacterial compound are not constituted solely by amino acids. In this sense, an assay to test susceptibility to enzymes was proposed, using the following enzymes: lysozyme, which is a glycoside hydrolase; proteinase K, which targets between aliphatic amino acids and aromatic amino acids; and trypsin, which targets between positively charged amino acids such as lysine and arginine. Results showed that a decrease in the inhibition zone was observed in all the treatments were lysozyme was employed (**Figure 25A** and **Figure 25B**). The combination of enzymes did not present an accumulative effect, mainly because the activity of the extract was not affected by proteinase K and trypsin (**Figure 25B**). Due to the lysozyme susceptibility observed, our results suggest that sugar moieties may be necessary to preserve intact the bioactivity of the antibacterial compound and are somewhere added to the amino acidic core skeleton. The aglycone structure still maintains its bioactivity by itself, although with a lower effectivity.

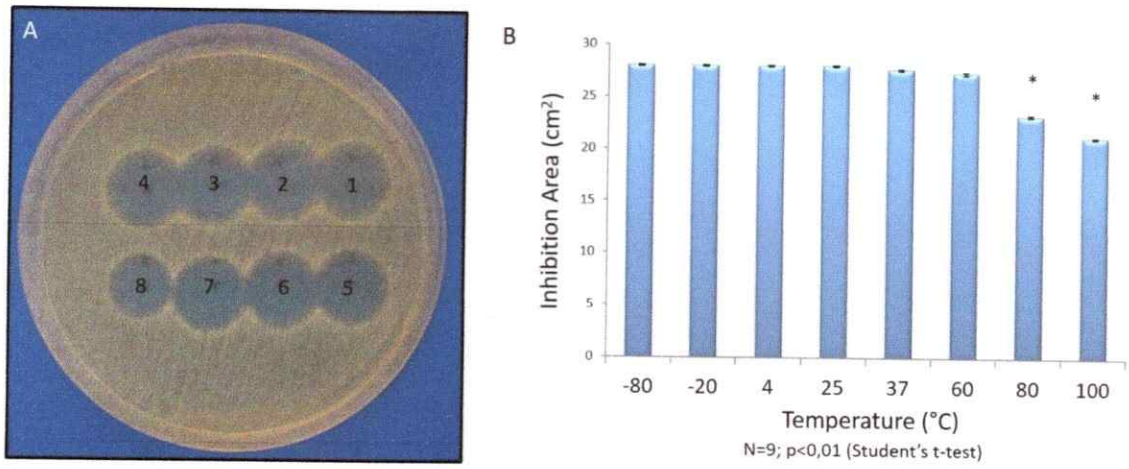


Figure 24: *Streptomyces* sp. H-KF8 crude extract thermal susceptibility assay. A) A representative plate showing inhibition areas against *S. aureus* NBRC 100910^T. 1, -80 °C; 2, -20 °C; 3, 4 °C ; 4, 25 °C, 5, 37 °C; 6, 60 °C; 7, 80 °C; 8, 100 °C. **B)** Effect of temperatures on the crude extract bioactivity. Biological and technical triplicates were performed (N=9; p<0,01 Student's *t*-test).

The *Streptomyces* sp. H-KF8 crude extract was evaluated regarding its absorbance and fluorescence spectra, to further gain insights into the functional groups that may be present in the antibacterial compound(s) (**Figure 26**). Absorbance spectrum demonstrated that the extract presented a maximum peak at a wavelength of 260 nm (**Figure 26A**). In parallel, absorbance of amino acids in solution was performed and phenylalanine showed a similar maximum peak at 260 nm (**Figure 26A**), suggesting the presence of this amino acid in the crude extract of *Streptomyces* sp. H-KF8. In addition, fluorescence was measured using a 260 nm excitation wavelength. Interestingly, the crude extract of *Streptomyces* sp. H-KF8 presented two peaks: one at 330 nm of wavelength, which resembles the tryptophan spectrum, and another at 460 nm of wavelength, which did not present similarities to the amino acids tested (**Figure 26B**). These results support the overall hypothesis, which suggests that the antibacterial compound(s) has a mixed and complex structure of, at least, amino acids and sugars, and by this technique, it was possible to determine the presence of phenylalanine and tryptophan.

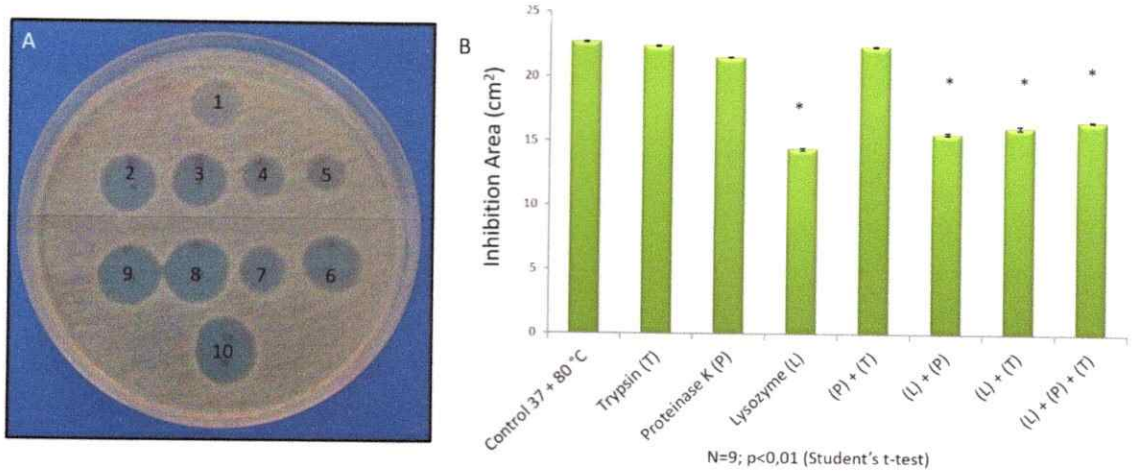


Figure 25: *Streptomyces* sp. H-KF8 crude extract susceptibility to hydrolytic enzymes. A) A representative plate showing inhibition areas against *S. aureus* NBRC 100910^{T}. 1, Lysozyme; 2, Proteinase K; 3, Trypsin; 4, Lysozyme + Proteinase K; 5, Lysozyme + Trypsin; 6, Proteinase K + Trypsin; 7, Lysozyme + Proteinase K + Trypsin; 8, Control without the effect of temperature (RT); 9, Control of effect of 37 °C; 10, Control of effect of 37 °C + 80 °C. **B)** Overall effect of several enzymes on the biological triplicates and technical triplicates over the bioactivity of the extract (N=9; p<0,01 Student's t-test).

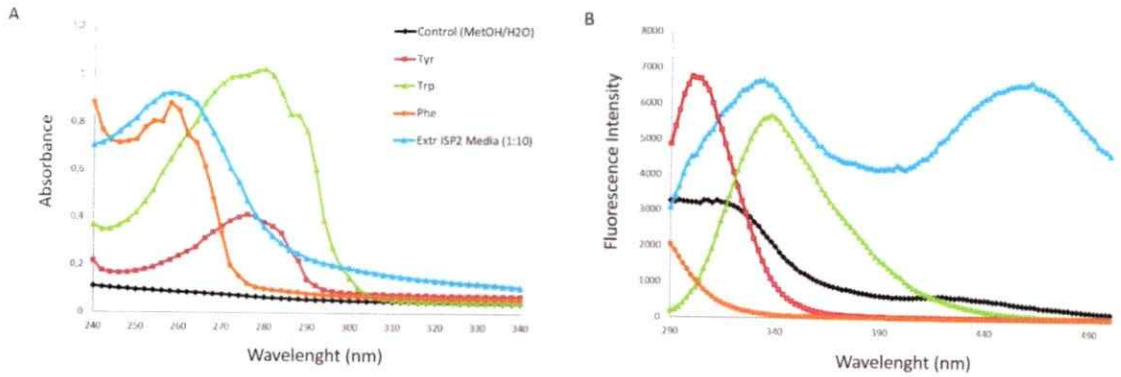


Figure 26: Absorbance and fluorescence spectra of crude extracts from *Streptomyces* sp. H-KF8. The 1:10 dilution of the extract is shown in light blue in both graphs. In colours, the following amino acids are shown: tyrosine, red; tryptophan, green; phenylalanine, orange. Negative control, methanol-water, the solvent in which the extract is resuspended, is shown in black. **A)** Absorbance spectrum showing a maximum crude extract peak at 260 nm of wavelength. **B)** Fluorescence spectrum showing the two crude extract peaks, one at 330 nm and the other at 460 nm of wavelength.

Thin layer chromatography (TLC) was performed in order to confirm the above hypotheses (**Figure 27**), and the extract of *Streptomyces* sp. H-KF8 was successfully stained with the ninhydrin reagent, which is commonly used to visualize amino acids, amino sugars and /or amines (**Figure 27A**). In addition, the orcinol reagent was also effective in revealing spots, indicating presence of glycosides and/or glycolipids (**Figure 27B**).

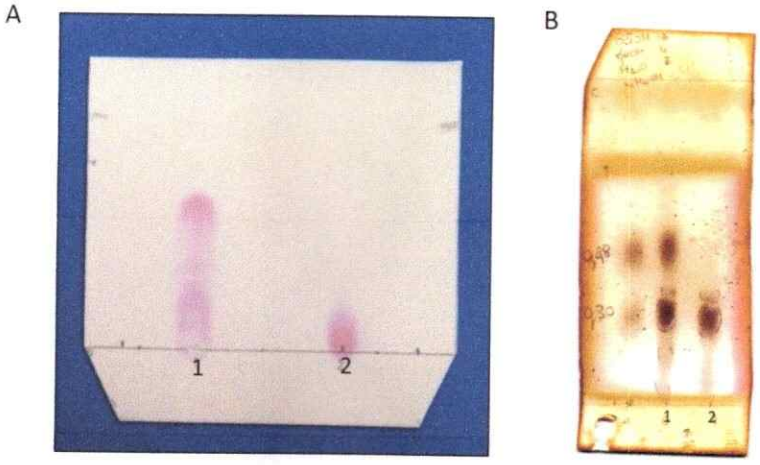


Figure 27: Thin layer chromatography of crude extract of *Streptomyces* sp. H-KF8. A) Ninhydrin reagent visualizing spots corresponding to amino acids, amino sugars and/or amines. 1, amino acid standard; 2, dilution 1:3 of the extract. **B)** Orcinol reagent visualizing spots corresponding to glycosides and/or glycolipids. 1, extract obtained from ISP2 medium; 2, extract obtained from medium V.

5.3.2.5 Chemical characterization of the antimicrobial compounds through HPLC-bioguided fractionation

Crude extract was resuspended in water-TFA 0.1%, centrifuged and filtered prior injection to HPLC, where a C18 column was used. Various mobile phases were tested, and as a result the water-TFA/methanol gradient was the best suited, from which several peaks were obtained (**Figure 28**). For comparison purposes, crude extracts obtained from two different

media were used: from ISP2 medium, where a total of 36 different fractions were retrieved (**Figure 28A**); and from V medium, where 12 fractions were obtained (**Figure 28B**). In both detections, an absorbance of 260 nm (blue) and 280 nm (pink) were selected for measurement. Each different fraction (of 1 mL volume) was collected and subsequently evaluated in terms of their antimicrobial activity.

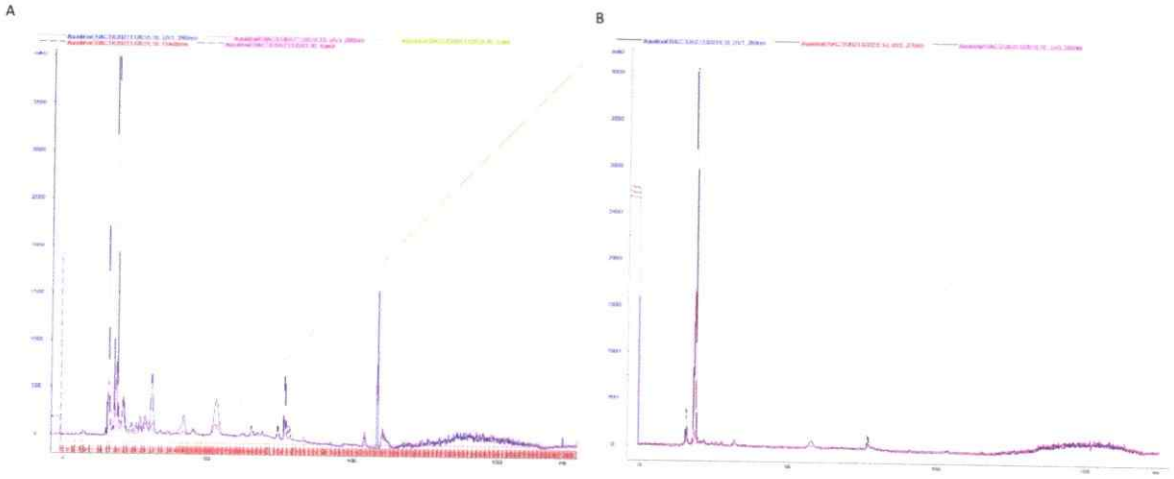


Figure 28: HPLC chromatograms of *Streptomyces* sp. H-KF8 crude extract. Diagonal line corresponds to the mobile phase gradient, water-TFA/methanol. A Jupiter C18 column was used. **A)** Extract obtained from fermentations in ISP2 medium. **B)** Extract obtained from fermentations in medium V.

Antibacterial activity of each fraction was evaluated against nine bacterial strains, and different inhibition patterns were observed. From the ISP2 extract, the most inhibited bacterial reference strains were *S. aureus* ATCC 29740^T (**Figure 29A**), *E. coli* ATCC 8739^T (**Figure 29B**) and *P. aeruginosa* ATCC 27853^T (**Figure 29C**) where 37 of 38 fractions (97.3 %) showed bioactivity. In addition, 86.1 % of the fractions showed growth inhibition against *S. epidermidis* ATCC 35984^T; 77.7 % against *L. monocytogenes* ATCC 19114^T and 61.1 % against *Bacillus subtilis* ATCC 1668^T. On the other hand, *Enterococcus faecalis* ATCC 19433^T, *Micrococcus luteus* ATCC 9341^T and *Klebsiella pneumoniae* ATCC 13883^T were less inhibited, with 36 to 39 % of fractions showing

activity. The three most active fractions and the detail of their inhibition results are depicted in **Table 9**. In contrast, when assessing the antimicrobial activity of the fractions retrieved from V medium, only one fraction from a total of 12 was active (fraction 114). Nevertheless, activity was observed as a broad-spectrum against all nine bacterial reference strains (**Table 9**).

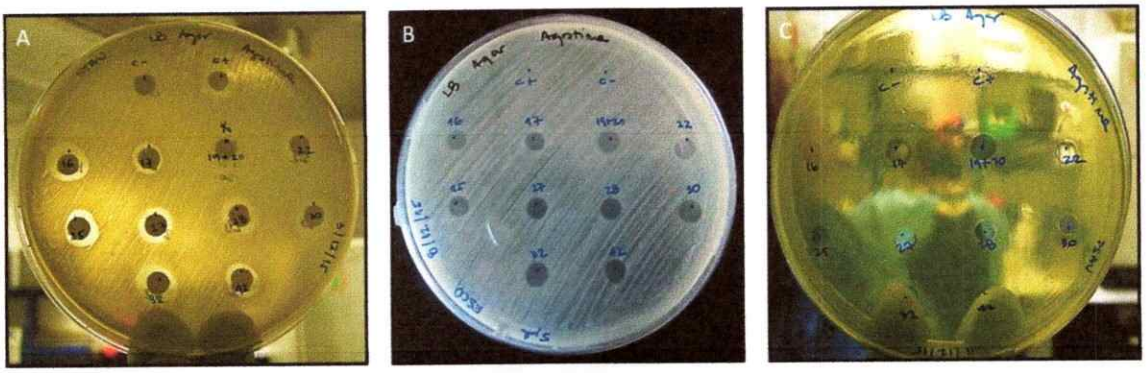


Figure 29: Antimicrobial activities from separated fractions obtained from *Streptomyces* sp. H-KF8 cultures grown in ISP2 medium. A) *S. aureus* ATCC 29740^T. **B)** *E. coli* ATCC 8739^T. **C)** *P. aeruginosa* ATCC 27853^T.

Table 9. Antibacterial activity of selected fractions against nine bacterial pathogens.

FRACTION	MEDIA	STAU	STEP	ESCO	LIMO	PSAU	KLPN	ENFA	MILU	BASU
19	ISP2	+	+	+	+	+	+	+	+	+
	V	-	-	-	-	-	-	-	-	-
168	ISP2	+	+	+	+	+	+	+	+	+
	V	-	-	-	-	-	-	-	-	-
114	V	+	+	+	+	+	+	+	+	+

+, with antibacterial activity; -, without antibacterial activity.
Bacterial strains: STAU, *Staphylococcus aureus* ATCC 29740^T; STEP, *Staphylococcus epidermidis* ATCC 35984^T; ESCO, *Escherichia coli* ATCC 8739^T; LIMO, *Listeria monocytogenes* ATCC 19114^T; PSAU, *Pseudomonas aeruginosa* ATCC 27853^T; KLPN, *Klebsiella pneumoniae* ATCC 13883^T; ENFA, *Enterococcus faecalis* ATCC 19433^T; MILU, *Micrococcus luteus* ATCC 9341^T; BASU, *Bacillus subtilis* ATCC 1668^T.

5.3.2.6 Analysis of overall metabolite expression by analytical mass-techniques

Active fractions were subsequently analyzed through mass-spectrometry techniques. Altogether, the 36 samples from ISP2 medium together with the 6 samples from V medium were analyzed with two different matrices, and in positive and negative mode each, reaching a total sample number of 176. Several criteria to perform data analysis of the 176 samples were applied. Firstly, the ESI-FT ICR MS was employed to evaluate the amount of different ions present in active fractions. In general, several different masses were observed in each active fraction (**Figure 30:** *e.g.*, fraction 168). Nevertheless, a primary composition of the functional groups in samples was determined (**Figure 30:** *e.g*, fraction 168) where some sugar moieties were observed. All samples were analyzed in order to look for masses corresponding to matrix adducts, to discard them. Concerning the antibacterial activity criteria, the fractions 19 and 168 from ISP2 medium were selected, and fraction 114 from V medium (**Table 9**). A comparison between the mass spectra of the fractions that were present in both media was performed to discard the masses which appear in both spectra, as only the fraction from one medium (ISP2) was the active one. An example of this analysis is shown in **Figure 31** for fraction 19, which presented activity in ISP2 medium, and not in V medium. As shown in **Figure 31,** some masses appears in both spectra of the same fraction obtained from the two different media. Therefore, these masses may not be responsible for the antibacterial activity previously demonstrated, and these masses were discarded from further analyses. This analysis was performed with all samples, leading to a candidate list of 66 different masses, corresponding to three distinct fractions, shown as follows: 8 masses in positive mode and 5 masses in negative mode for fraction 19; 26 masses in positive mode and 22 masses in negative mode for fraction 168 and 2 masses in positive mode and 3 masses in negative mode for fraction 114.

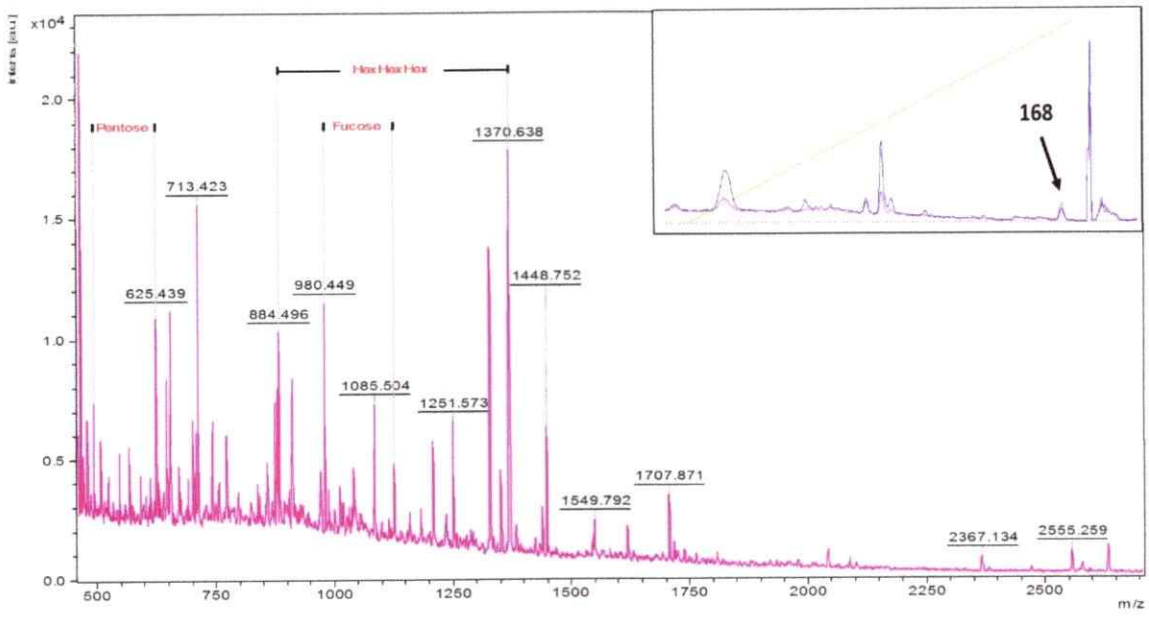


Figure 30: ESI-FT ICR MS mass spectrum for fraction 168 obtained from *Streptomyces* sp. H-KF8 grown in ISP2 medium. In pink, several masses can be appreciated. Some mass differences can be explained by the loss of sugar moieties, such as hexoses, a pentose and fucose. In the inset panel, the HPLC chromatogram is shown, depicting fraction 168 obtained from the bioguided fractionation of the crude extract that derives from ISP2 medium.

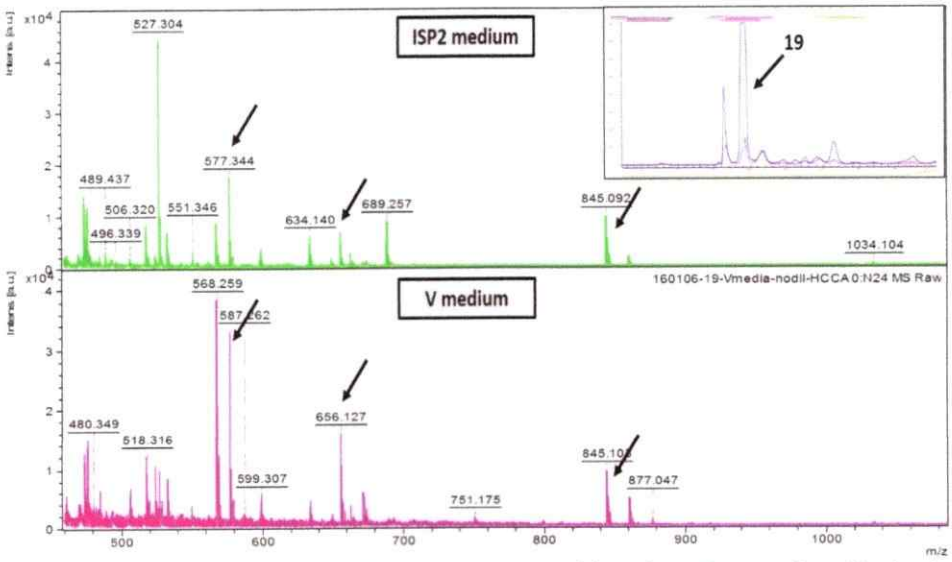


Figure 31: Comparison of ESI-FT ICR MS mass spectra of fraction 19. Fraction 19 obtained from ISP2 medium (with antimicrobial activity) is depicted in green, whereas fraction 19 obtained from V medium (without antimicrobial activity) is shown in pink. Black arrows point to masses that are common between both spectra, and therefore are discarded for further analysis. In the inset panel, the HPLC chromatogram is shown, depicting fraction 19 obtained from the bioguided fractionation of the crude extract that derives from ISP2 medium.

Imaging-MS was performed to select those masses from the candidate list that are specifically expressed in an antagonist condition of bacterial inhibition. To test this, several conditions were proposed (see materials and methods). Overall analysis consisted of recording, through images, those metabolites that the bacterium *Streptomyces* sp. H-KF8 is releasing to the agar in conditions where it is incubated on its own, or, when it is confronting another bacterium. These metabolites can be visualized through an average mass spectrum of all conditions, and also, in each condition separately, allowing to compare all treatments tested. Moreover, association of metabolite intensity can be noticed, where blue is no expression and light-blue to yellow means an increase in intensity of its production. Therefore, this technique is crucial for understanding when a specific metabolite is produced and determining the exact condition of its expression. Sample preparation after matrix deposition (HCCA) and selected areas (black rectangles) to be analyzed by the laser, are shown in **Figure 32**.

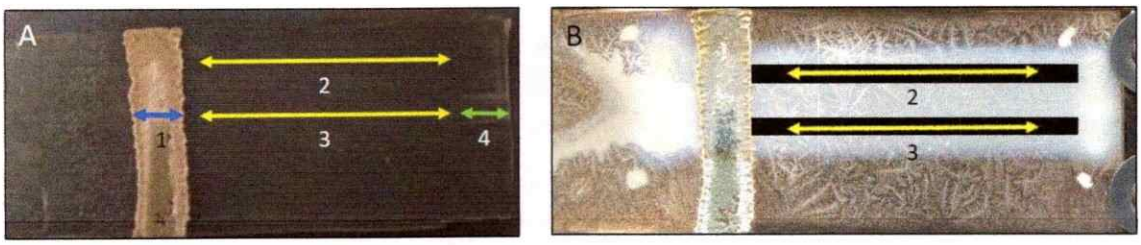


Figure 32: Imaging-MS slides showing analyzed surrounding areas for *Streptomyces* sp. H-KF8. **A)** A slide where *Streptomyces* sp. H-KF8 (1, blue arrow) was incubated confronting *S. aureus* ATCC 29740^T (4, green arrow). The inhibition area is shown as 3 (yellow arrow). As a control, the metabolites released to the agar but not confined solely to the inhibition area are shown as 2 (yellow arrow). **B)** A slide after matrix deposition (white area). Black rectangles shows areas 2 and 3, respectively, where the laser will be deposited.

Comparison with the masses predicted by genome mining (see **Chapter III**) was performed in order to determine if one of these masses was expressed under an antagonist condition. Imaging-MS results regarding incubation-day 15 are listed in **Table 10**. A 39% of the

predicted masses were observed by Imaging-MS. However, not all of them were expressed in the condition of antibacterial inhibition. For example, masses 219.5 m/z and 476.9 m/z corresponding to the albaflavenone and isorenieratene compounds, respectively, both predicted with a 100 % identity, were observed only when *Streptomyces* sp. H-KF8 is incubated alone, and the expression of these masses is lost under a confrontation condition (**Table 10**). By contrast, the observed mass 1450.1 m/z was expressed in all three antagonistic interactions and it is also present when *Streptomyces* sp. H-KF8 is incubated alone (**Table 10**). This mass presents a match with the mannopeptimycin compound, although it only has a 7 % of identity with it. These results suggest that mass 1450.1 m/z can be related to the mannopeptimycin family of compounds, although the overall chemical structure may be probably different, due to the very low similarity reflected between them.

Table 10. Imaging-MS of the molecules predicted by genome mining of *Streptomyces* sp. H-KF8, after 15 incubation days.

MOLECULE (IDENTITY* %)	PREDICTED m/z (Da)	OBSERVED m/z (Da)	H-KF8	H-KF8 +STAU	H-KF8 +STEP	H-KF8 +PSAU
Moenomycin (100)	1580.5	N/O				
Stenothricin (13)	1118.6	N/O				
Streptolydigin (13)	601.7	N/O				
Mannopeptimycin (7)	1449.2	1450.1	+ -	+ -	+ -	+ -
Hopene	410.7	411.0	-	-	-	-
Himastatin (12)	1484.7	N/O				
Furaquinocin A	402.4	402.0	-	-	-	-
Isorenieratene (100)	528.8	527.1	-	-	-	- +
Griseoviridin (11)	477.1	476.9	+ -	- +	-	- +
Stambomycin (52)	1376.9	N/O				
Kirromycin (6)	796.9	796.1	-	-	-	-
Albaflavenone (100)	218.3	219.5	+ -	-	-	-
SGR PTMS (100)	511.2	N/O				
Neomycin (5)	614.6	N/O				
Ectoine (100)	142.1	N/O				
Melanin (100)	318.2	N/O				
Desferrioxiamine B (83)	560.6	N/O				

*Identity corresponding to the similarity of *Streptomyces* sp. H-KF8 biosynthetic gene clusters with known-BGCs from MIBiG database.
Imaging-MS expression results determined as: N/O, not observed; -, no change in intensity between conditions; - +, change in the intensity close to the antagonistic bacterium; + -, change in intensity close to *Streptomyces* sp. H-KF8. Bacterial antagonistic strains: STAU, *Staphylococcus aureus* ATCC 29740^T; STEP, *Staphylococcus epidermidis* ATCC 35984^T; PSAU, *Pseudomonas aeruginosa* ATCC 27853^T.

An Imaging-MS analysis of the selected masses from the candidate list obtained by the ESI-FT ICR MS results was also performed. From the 66 masses selected, 28 were shown to appear under the various confrontational conditions tested, as observed by Imaging-MS. Nine of these masses corresponded to masses that were visualized under the positive mode, and are presented in **Table 11**. Overall, a total of 20 masses showed some degree of intensity at least in one condition tested. Masses shown to be expressed by the antagonistic bacterium, such as 489.1 m/z and 527.1 m/z and 619.1 m/z, were discarded for further analysis. On the contrary, masses where expression was observed from *Streptomyces* sp. H-KF8 in the condition of antibacterial inhibition (*i.e.*, masses 550.0 m/z and 689.2 m/z from fraction 19; and masses 820.1 m/z, 981.1 m/z and 1450.1 m/z from fraction 168), were compared according to its image patterns, and those with an interesting pattern were further selected for MALDI-TOF MS fragmentation. From fraction 114 (obtained from fermentation in V medium), only two masses were observed in the Imaging-MS experiment. However, these masses did not present intensity; hence, they were not expressed in the conditions tested. This is in accordance with the medium-dependent expression of metabolites, and the hypothesis that metabolite synthesis changes when growth media is different.

Table 11. ESI-FT ICR MS selected masses that presented an expression by Imaging-MS after 15 days of incubation.

FRACTION	OBSERVED m/z (Da)	H-KF8	H-KF8 +STAU	H-KF8 +STEP	H-KF8 +PSAU
19	489.1	-	-+	-	-+
	527.1	-	-	-	-+
	550.0	+-	+-	-	+-
	619.1	+-	-+	-	-+
	689.2	-	+-	+-	+-
168	209.2	-	+-	+-	+-
	820.1	-	-	-	+-
	981.1	+-	+-	+-	+-
	1450.1	+-	+-	+-	+-

Imaging-MS expression results: -, no change in intensity between conditions; -+, change in the intensity close to the antagonistic bacterium; +-, change in intensity close to *Streptomyces* sp. H-KF8. Bacterial antagonistic strains: STAU, *Staphylococcus aureus* ATCC 29740^T; STEP, *Staphylococcus epidermidis* ATCC 35984^T; PSAU, *Pseudomonas aeruginosa* ATCC 27853^T.

Interestingly, two of these masses (1450.1 m/z and 981.1 m/z) showed an intensity in their expression in all the antagonistic conditions tested. These masses derived from the active fraction 168, obtained from fermentation in ISP2 medium. As an example, the Imaging-MS result for mass 1450.1 m/z in all conditions (when incubated *Streptomyces* sp. H-KF8 alone or in confrontation, at 10 or 15 days of incubation) is shown in **Figure 33**. Although a mild expression without a specific location was observed at 10 days of incubation (**Figure 33A**), its expression became even more intense at 15 days of incubation (**Figure 33B**). Moreover, at day 15, intensity of this mass decreases when *Streptomyces* sp. H-KF8 is incubated alone, and the expression of this metabolite is enhanced when *Streptomyces* sp. H-KF8 was confronted with other bacteria, independent if it is a Gram-positive (*i.e., Staphylococcus aureus* and *Staphylococcus epidermidis*) or Gram-negative bacterium (*i.e., Pseudomonas aeruginosa*) (**Figure 33B**). Therefore, the mass 1450.1 m/z is expressed only in a context of antagonistic confrontation, suggesting a crucial activity as an antibacterial metabolite.

The masses selected by ESI-FT ICR MS whose expression was positive by Imaging-MS, were selected to further perform MALDI-TOF MS/MS. This technique will gain insights into the composition of the metabolites selected, as a consequence of the fragmentation of the molecule. Thus, it will provide valuable information about the functional groups present in such masses, therefore generating the link between the bioinformatic prediction and the experimental observation.

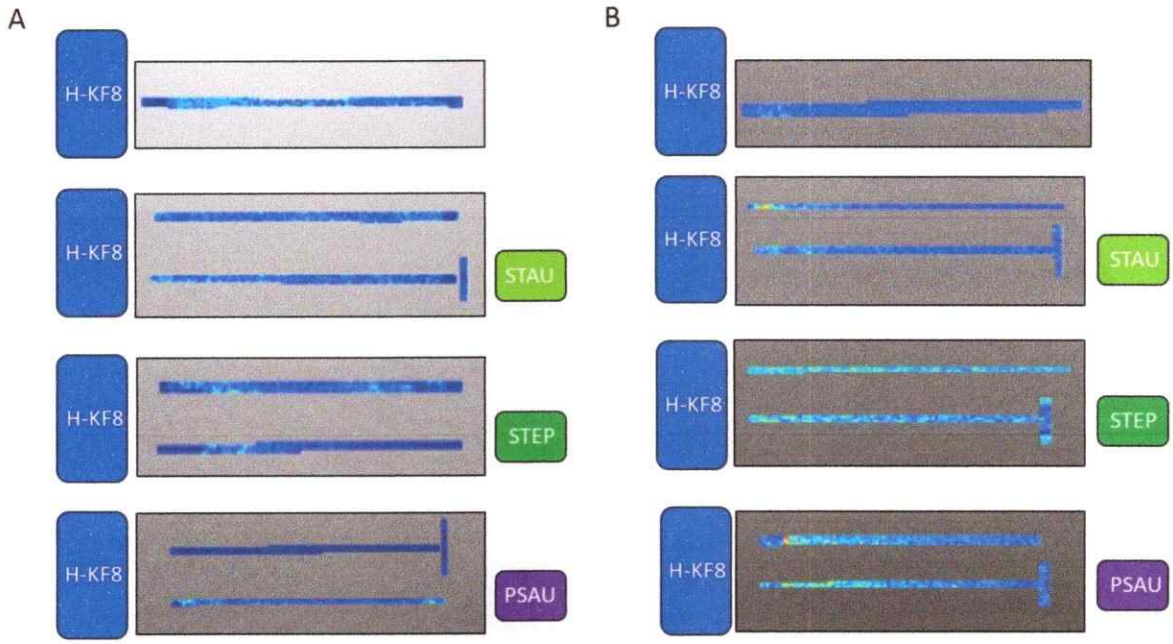


Figure 33: Imaging-MS for the selected ion 1450.1 m/z. First line corresponds to *Streptomyces* sp. H-KF8 (blue) incubated alone. Second and third line, corresponds to the confrontation against the Gram-positive bacteria *S. aureus* ATCC 29740^T (STAU, light-green) and *S. epidermidis* ATCC 35984^T (STEP, green), respectively. Fourth line corresponds to the confrontation against a Gram-negative bacterium, *P. aeruginosa* ATCC 27853^T (PSAU, purple). **A)** Experiment performed at 10 days of incubation. **B)** Experiment performed at 15 days of incubation.

The parental ion 1450.1 m/z previously depicted in **Figure 33** was isolated and its MALDI-TOF MS/MS fragmentation pattern is shown in **Figure 34** (observed mass 1448.7520 m/z by MALDI-TOF MS/MS). In effect, it was possible to visualize several amino acids, such as one

tryptophan, two valines, two alanines (one as dehydroalanine) and two of the non-conventional amino acids ornithines. In addition, sugar moieties were also observed, such as hexoses and a pentose, in agreement with the ESI-FT ICR MS spectrum previously shown. The amino acids observed in this sample resemble the amino acids predicted in cluster #6 (**Figure 20**), where 8 of the 10 amino acids presents a correlation. Interestingly, a nitro-tyrosine (mass 209.19 m/z) was observed during the fragmentation of this parental ion (1450.1 m/z), and also in the 820.114 m/z ion. Accordingly, it was possible to observe the expression of this mass (209.19 m/z) through Imaging-MS (**Table 11**). Therefore, we suggest that a nitro-tyrosine is present in the core metabolite structure instead of a tryptophan, thus resulting in two tryptophans instead of the three tryptophans that were originally predicted (**Figure 20**). As fragmentation patterns of these parental ions observed by MALDI-TOF MS are related, they might represent different fragmentation sections of the overall metabolite. This is also in agreement with the similar amino acid composition among the three parental ions, which confirms the presence of the predicted amino acids of cluster #6 (**Table 12**).

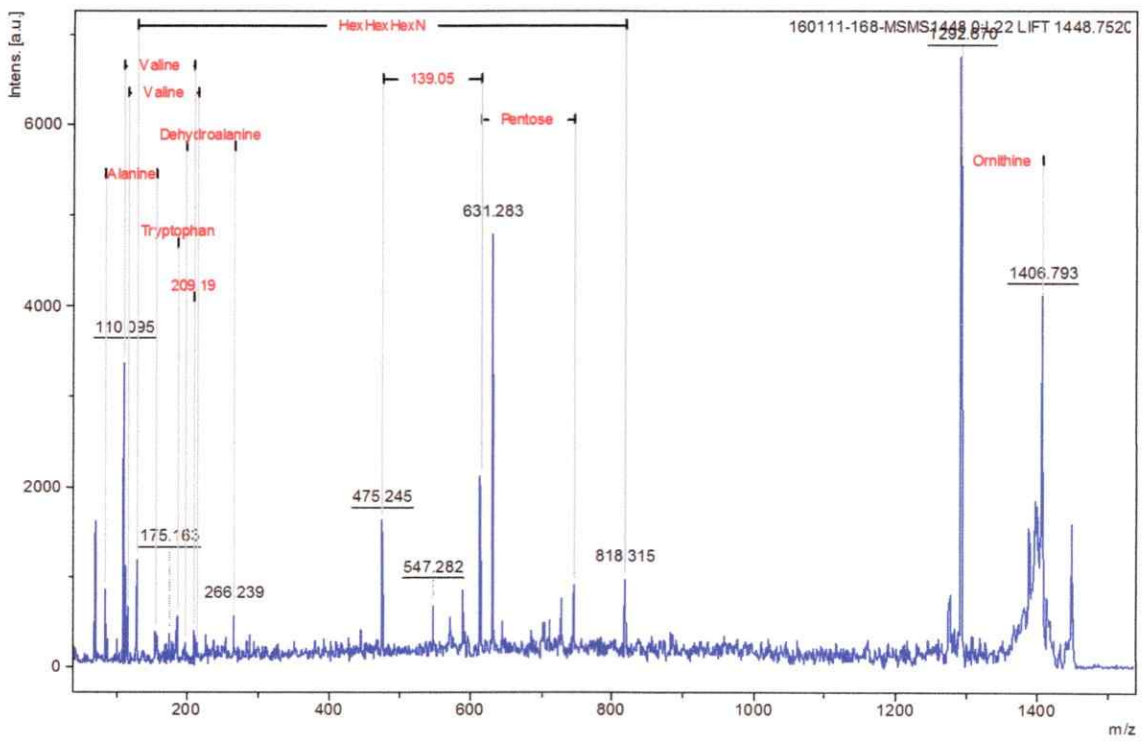


Figure 34: MALDI-TOF MS/MS of parental ion 1450.1 m/z. In red, the monomer composition of the fragmentation pattern of the parental ion show several amino acids, which are related to the prediction of the NRPS BGC of cluster #6.

In the same line, the analysis of the mass 981.1 m/z whose expression was observed in all the conditions tested (**Table 11**) was performed. The intensity of its expression is depicted in **Figure 35**. Interestingly, this ion shows a similar pattern to ion 1450.1 m/z (**Figure 33**), where an intense expression is observed when *Streptomyces* sp. H-KF8 is incubated alone (**Figure 35A;** first line) and a disperse location is observed when it is confronted to an antagonistic interaction (**Figure 35A**; second, third and fourth line) at 10 days of incubation. On the contrary, the expression of this metabolite decreases at 15 days of growth when it is incubated alone (**Figure 35B;** first line) and its intensity increases when it is in a confrontation condition (**Figure 35B**; second, third and fourth line). In addition, the mass 981.1 m/z can be observed in conditions involving both Gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis*,

and even in the Gram-negative bacterium *Pseudomonas aeruginosa* (**Figure 35B**). As the expression of the mass 981.1 m/z is enhanced in an antagonistic condition rather than when it is incubated by itself, the presence of this mass can be related to bacterial inhibition. Therefore, this mass was also selected for MALDI-TOF MS/MS.

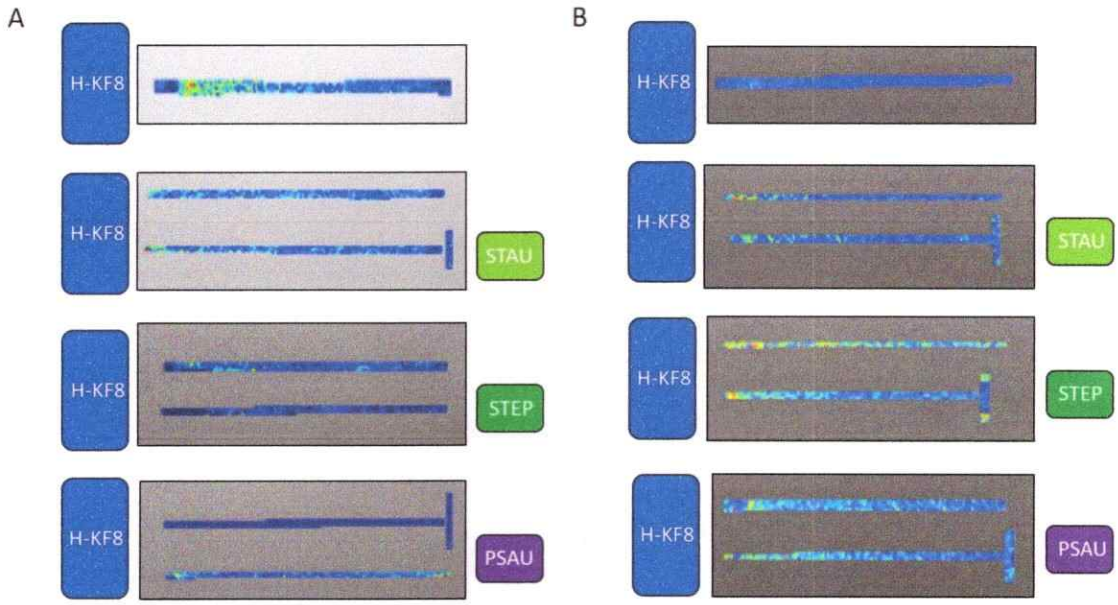


Figure 35: Imaging-MS for the selected ion 981.1 m/z. First line corresponds to *Streptomyces* sp. H-KF8 (blue) incubated alone. Second and third line, corresponds to the confrontation against the Gram-positive reference strains *S. aureus* ATCC 29740^T (STAU, light-green) and *S. epidermidis* ATCC 35984^T (STEP, green), respectively. Fourth line corresponds to the confrontation against a Gram-negative reference strain, *P. aeruginosa* ATCC 27853^T (PSAU, purple). **A)** Experiment performed at 10 days of incubation. **B)** Experiment performed at 15 days of incubation.

The fragmentation pattern of parental ion 981.1 m/z (observed mass 980.4 m/z by MALDI-TOF) is shown in **Figure 36**. In red, the functional groups product of the fragmentation can be observed. Specifically, the amino acids threonine, two valines, two alanines were depicted, in addition to the sugar pentose and the amino sugar *N*-acetyl-hexosamin (**Figure 36**). These monomers show a relationship to the MALDI-TOF MS/MS fragmentation pattern observed for the parental ion 1450.1 m/z (**Figure 34**). Moreover, their Imaging-MS expression pattern of

both ions (1450.1 m/z and 981.1 m/z) are also related (**Figure 33** and **Figure 35**, respectively). Therefore, these results suggests that these masses may be related, and, in addition to mass 820.1 m/z that presents a similar pattern (data not shown), these three masses are proposed to explain the functional groups present into the overall metabolite, responsible for the antibacterial activity observed (**Table 12**). In addition, the observed amino acids from the fragmentation pattern of the three masses indicate that this metabolite is likely to be synthesized by the NRPS enzymes which were predicted from the BGCs, specially to cluster #6 (**Table 12**). Overall, these results strongly suggests that it is possible to correlate the predicted bioinformatic genomic data to the metabolic profiles obtained experimentally, which in turn, may explain the core structure and functional nature of the antimicrobial metabolite(s) observed in the marine actinomycete *Streptomyces* sp. H-KF8.

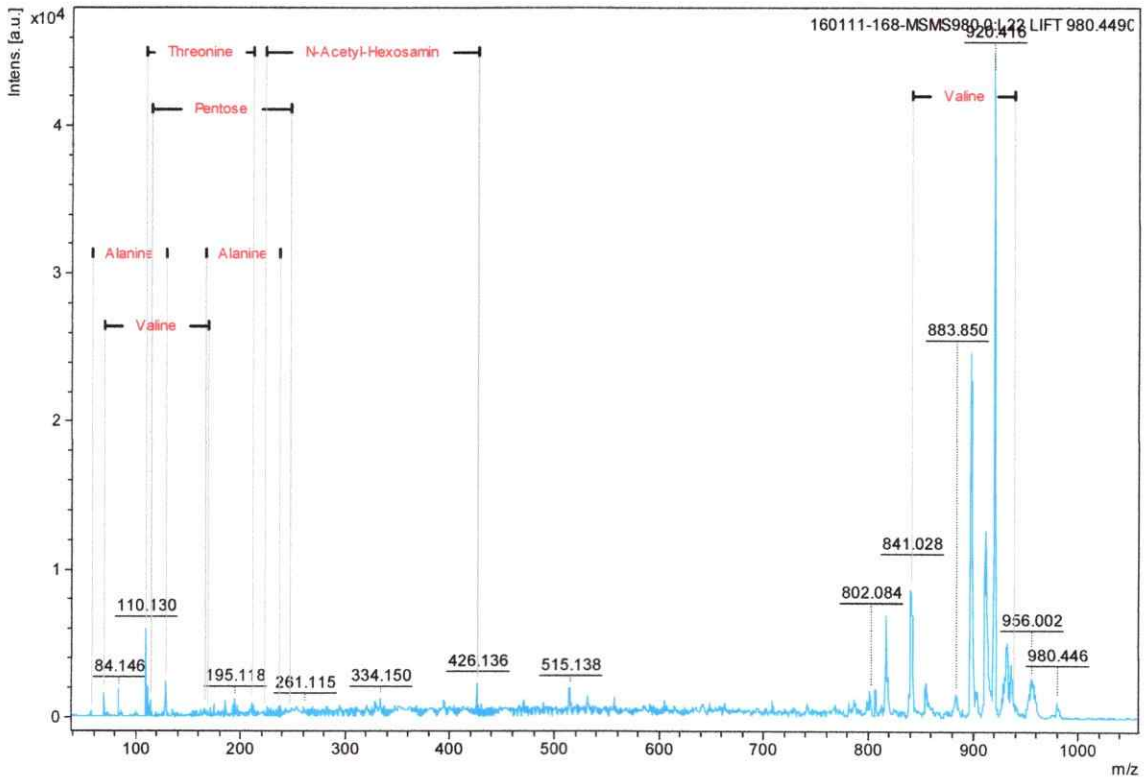


Figure 36: MALDI-TOF MS/MS of parental ion 981.1 m/z. In red, the monomer composition of the fragmentation pattern of the parental ion show several amino acids, which are related to the prediction of the NRPS BGC cluster #6. In addition, sugar moieties (pentose + *N*-acetyl-hexosamine) are present.

Table 12. MALDI-TOF MS/MS parental ions with their respective monomer composition, and their relationship with the amino acid prediction of NRPS BGC cluster #6.

OBSERVED m/z (Da)	Trp	Ala	Val	Ala	Trp	Orn	Thr	Orn	Val	Tyr-NO_2
820.1		×	×	×		×		×		×
981.1		×	×	×			×		×	
1450.1		×	×	×	×	×	×	×	×	×

Amino acids are shown as: Trp, tryptophan; Ala, Alanine; Val, Valine; Orn, Ornithine; Thr, Threonine; Tyr-NO_2, Nitro-tyrosine.

5.3.1 Addendum Chapter IV

Table 13. Genes present in the NRPS BGC corresponding to cluster #5 of *Streptomyces* sp. H-KF8. Genes are categorized according to their respective functions depicted with the following colours: blue, biosynthetic genes; light-blue, tailouring reactions; green, regulatory genes; purple, transport; orange, resistance; black, unknown/non-directly related.

GENE ID	START (bp)	END (bp)	PREDICTED FUNCTION
A4U61_01965	144765	146073	Precorrin 3B synthase (CobG)
A4U61_01970	146108	146741	Precorrin 8X methylmutase (CobH)
A4U61_01975	146737	148234	Precorrin 3B-C17 methyltransferase (CobF)
A4U61_01980	148247	149018	Cobalt precorrin 6X reductase (CobK)
A4U61_01985	149077	150196	Cobalt precorrin 6A synthase (CobE)
A4U61_01990	150299	151049	Precorrin 4-C11 methyltransferase (CobM)
A4U61_01995	151045	152365	Precorrin 6Y methyltransferase (CobL)
A4U61_02000	152769	154140	Sphingolipid synthesis
A4U61_02005	154478	155000	ABC transporter type I (peptide exporter)
A4U61_02010	155148	155691	Hypothetical protein
A4U61_02015	155674	156646	Dioxygenase
A4U61_02020	156742	157333	Hemerythrin binding protein
A4U61_02025	157341	158541	Hypothetical protein
A4U61_02030	158821	159730	3R-hydroxyacyl-CoA-dehydrogenase (PKS KR)
A4U61_02035	159726	160926	Acetyl CoA-Acetyltransferase (PKS TE)
A4U61_02040	161127	162468	Major facilitator superfamily (MFS)
A4U61_02045	162506	164135	Long Chain Fatty Acid CoA-Ligase (PKSA KS)
A4U61_02050	164187	164820	Transcriptional regulator multidrug efflux pump
A4U61_02055	165177	165804	Transcriptional regulator multidrug efflux pump
A4U61_02060	165904	166558	Acyltransferase resistance protein
A4U61_02065	166933	167182	Hypothetical Protein
A4U61_02070	167465	167837	Hypothetical Protein
A4U61_02075	168451	169552	Two-component system histidine kinase sensor
A4U61_02080	169548	170214	DNA-binding response regulator
A4U61_02085	170534	171086	Histidine phosphatase (PhoE)
A4U61_02090	172056	172704	Hypothetical protein
A4U61_02095	172771	173380	Sortase
A4U61_02100	173636	174056	Snoal-like domain (PKS cyclase)
A4U61_02105	174390	177366	Transcriptional regulator SARP
A4U61_02110	177928	178174	Hypothetical protein
A4U61_02115	178422	179736	Transcriptional regulator
A4U61_02120	179933	180968	Ketoacyl-Acyl carrier protein synthase (PKS ACP)
A4U61_02125	181188	181449	Phosphopantetheine attachment site (PKS AT)
A4U61_02130	181486	184231	Non-ribosomal peptide synthetase (NRPS C+A)
A4U61_02135	184227	187866	Non-ribosomal peptide synthetase (NRPS C+A)

A4U61_02140	187965	188766	Thioesterase (NRPS TE)
A4U61_02145	188919	189951	Dioxygenase
A4U61_02150	191755	196252	Non-ribosomal peptide synthetase (NRPS A+T)
A4U61_02155	196490	203792	Non-ribosomal peptide synthetase (NRPS C+A)
A4U61_02160	196490	203792	Non-ribosomal peptide synthetase (NRPS 2C+2A)
A4U61_02165	203788	203992	MbtH protein (NRPS T)
A4U61_02170	204200	204674	Glutamate/Methyl-aspartate mutase
A4U61_02175	204808	206074	Glutamate/Methyl-aspartate mutase
A4U61_02180	206088	207090	Asparragine ligase
A4U61_02185	207113	208427	Glutamate synthase
A4U61_02190	208476	209406	3-Oxoacyl-ACP synthase (PKS KS)
A4U61_02195	209474	210890	Carboxylate amine ligase
A4U61_02200	210882	212160	Diaminoepimelate decarboxylase
A4U61_02205	212278	212560	Phosphopanteteine binding protein (PKS AT)
A4U61_02210	212564	213479	Condensing β-ketoacyl ACP synthase (PKS KS)
A4U61_02215	213475	214192	Hypothetical Protein
A4U61_02220	214188	215358	Acyl-CoA-Dehydrogenase (PKS DH)
A4U61_02225	215241	216202	Phosphopantetheine transferase (PKS AT)
A4U61_02230	216264	216510	Hypothetical protein
A4U61_02235	216557	217904	MFS transporter
A4U61_02240	218414	218852	ABC transporter
A4U61_02245	219002	219584	Transporter
A4U61_02250	220209	220962	Transcriptional regulator
A4U61_02255	221146	221704	Hypothetical protein
A4U61_02260	222070	222514	Universal stress protein
A4U61_02265	222833	223295	Chaperone
A4U61_02270	223433	224303	Transcriptional regulator

In PKS: KR, ketoreductase domain; KS, ketosynthase domain; AT, acyl-transferase domain; ACP, acyl carrier protein; DH, dehydratase domain. In NRPS: A, adenylation domain; T, thiolation domain, C, condensation domain; TE, thioesterase.

Table 14. Genes present in the NRPS BGC corresponding to cluster #6 of *Streptomyces* sp. H-KF8. Genes are categorized according to their respective functions depicted with the following colours: blue, biosynthetic genes; light-blue, tailouring reactions; green, regulatory genes; purple, transport; orange, resistance; black, unknown/non-directly related.

GENE ID	START (bp)	END (bp)	PREDICTED FUNCTION
A4U61_02855	364271	364742	Anti-sigma regulatory factor
A4U61_02860	364905	365778	DNA-binding protein
A4U61_02865	365774	365975	Antibiotics Transcriptional regulator
A4U61_02870	366101	368477	β-glucosidase (glycosyl hydrolase)
A4U61_02875	368485	369304	ABC transporter permease (TM)
A4U61_02880	369300	370218	ABC transporter permease (TM)
A4U61_02885	370228	371533	Sugar-binding protein (PER)
A4U61_02890	371701	372694	LacI transcriptional regulator
A4U61_02895	373838	374861	Acetyltransferase (Nudix hydrolase)
A4U61_02900	374875	375508	Dipeptidase D-Ala-D-Ala
A4U61_02905	375685	376420	Antibiotic resistance protein
A4U61_02910	376601	378368	β-galactosidase (glycosyl hydrolase)
A4U61_02915	378894	379518	Galactosidase *O*-Acetyltransferase
A4U61_02920	380202	381486	Neuraminidase (glycosyl hydrolase)
A4U61_02925	382139	382778	Hypothetical protein
A4U61_02930	383058	383571	Aminoglycoside *N*-Acetyltransferase
A4U61_02935	383680	383884	MbtH-like protein
A4U61_02940	383978	403979	Non-ribosomal peptide synthetase
A4U61_02945	403992	422721	Non-ribosomal peptide synthetase
A4U61_02950	422845	424252	β-lactamase D-Ala-D-Ala carboxypeptidase
A4U61_02955	424287	425496	Cytochrome P450
A4U61_02960	426378	427131	Thioesterase
A4U61_02965	427394	428000	Hypothetical protein
A4U61_02970	428346	430416	PAS sensor protein (light & O_2)
A4U61_02975	430491	431634	Hypothetical protein
A4U61_02980	431873	433421	Ferredoxin-NADP(+) reductase
A4U61_02985	433444	434359	p-aminobenzoate N-oxigenase (AurF)
A4U61_02990	435431	435911	Hypothetical protein
A4U61_02995	435994	438502	Chaperone
A4U61_03000	439096	440821	PAS sensor DNA-binding protein
A4U61_03005	440908	441508	VanZ resistance protein
A4U61_03010	441724	442858	Histidine kinase
A4U61_03015	442854	443502	LuxR regulator

TM, transmembrane; PER, periplasmic

6 Discussion

In this Ph.D. thesis, the isolation of actinomycetes from an underexplored environment, the study of its cultivable biodiversity along with their physiological and phylogenetic features as well as the evaluation of their antimicrobial activity were assessed (**Chapter I**). In addition, whole genome sequencing of selected isolates was performed to unveil the BGCs and perform comparative genomics (**Chapter II**), with a special focus on PKS and NRPS anabolic routes of secondary metabolites (**Chapter III**). Genome sequencing led to the identification of genetic determinants related to marine adaptations and abiotic stress (such as metal and antibiotic resistances, along with oxidative and osmotic stress defense systems) (**Chapter II** and **Chapter III**). Finally, middle scale fermentations were pursued to analyze the chemical nature of crude extracts, using HPLC bio-guided fractionation and mass-spectrometry techniques to obtain the subsequent characterization of the antimicrobial compound(s) produced by *Streptomyces* sp. H-KF8 (**Chapter IV**).

Actinomycetes from marine sediments have demonstrated to be a prolific source for natural bioactive products (Zotchev, 2012; Subramani & Aalbersberg, 2012; Manivasagan *et al.*, 2013). The bioprospection strategy involves the search for novel bioactive compounds from environmental sources. Members of the *Actinomycetales* remain the richest source of NP. This is the first report concerning bioprospection of bioactive cultivable actinomycetes and its ecophysiological characterization from marine sediments of a Northern Patagonian fjord from Chile. The Comau fjord, located in the X Region of Los Lagos, is a unique environment suitable for microbial exploration (**Figure 5**, Addendum **Chapter I**). Comau fjord is especially attractive due to their unique geographic features, such as its nearby geothermic activity, high precipitation

rates (~5,600 mm annual) and its depth (350-500 m) with surrounding mountains of 2,000 m height, and fluctuable water salinities and temperatures (Lagger *et al.*, 2009; Pantoja, Luis Iriarte & Daneri, 2011). This fjord demonstrated to be a rich source of antimicrobial-producing actinomycetes (**Chapter I**). Twenty-five actinomycetes were isolated from marine sediments and characterized (**Figure 6** and **Table 1**, Addendum **Chapter I**). The largest amount of these bacteria were obtained from deeper samples (>20 m) from Lilihuapi Island (**Figure 7**, Addendum **Chapter I**). Despite that a relatively low number of actinomycetes were retrieved from Comau fjord, a rather high cultivable biodiversity was observed (**Figure 8**, Addendum **Chapter I**). This biodiversity was composed of 10 different genera using five different media (**Figure 2**, **Chapter I**). Interestingly, major abundance of isolates were obtained from media with complex carbon sources (*e.g.*, starch in M1 medium) or low nutrient composition (*e.g.*, NaST21Cx medium) (**Figure 7**, Addendum **Chapter I**). Media composed of relatively simple nutrients has been reported to yield more actinomycetes from diverse environments (Zhang *et al.*, 2006; Gontang, Fenical & Jensen, 2007; Qin *et al.*, 2011); which is consistent with the negligible amount of nutrients that are available in the natural marine environment (Kurtböke, Grkovic & Quinn, 2007). A trend where an improved growth rate was evidenced when artificial sea water (ASW) was added in the media preparation, denoting a special nutrient requirement for salts (**Table 4**, Addendum **Chapter I**). This requirement has been used as a decisive criterion when demonstrating marine environmental adaptation (Penn & Jensen, 2012). NaCl tolerance was also evaluated, where the 91 % of the actinomycetes were able to grow even with ≥ 3.5 % of NaCl, which is the average salinity of sea water (**Table 3**, Addendum **Chapter I**). Also, 72% of the actinomycetes were able to grow at 4 °C, indicating a psychrotolerant behaviour (**Table 2**, Addendum **Chapter I**). Overall, these features demonstrate that actinomycetes from Comau

fjord exhibit a wide range of tolerance to abiotic stressful parameters, which is crucial for the adaptation to the dynamics of the fjord-marine environment.

Phylogenetic trees for actinomycetes are depicted in **Figure 13** (Addendum **Chapter I**). In general, actinomycetes showed more 16S rRNA gene identity with isolates derived from polar marine environments (**Figure 4, Chapter I**). Four putative new taxa are proposed based on numerical thresholds for species definition using 16S rRNA gene (Rosselló-Móra & Amann, 2015): *Arthrobacter* sp. H-JH3, *Brevibacterium* sp. H-BE7, *Corynebacterium* sp. EH3 and *Kocuria* sp. H-KB5 (**Table 3, Chapter I**). *Corynebacterium* sp. EH3 was not further characterized due to low cell viability. Instead, *Rhodococcus* sp. H-CA8F was used as it also presented low 16S rRNA gene identity (**Table 3, Chapter I**). For *Streptomyces* sp. H-KF8, a further evolutionary analysis based on comparative genomics was performed, which suggested that it may represent a novel specie due to its low Average Nucleotide Identity (ANI) value, pan-genome and Multi-Locus Sequence Analysis (MLSA) (Goris *et al.*, 2007) (data not shown).

In order to assess antimicrobial activities, an initial screening using the cross streak method (Haber & Ilan, 2014) in two different media was proposed. Notably, all isolates were able to display inhibition towards to at least one of the reference bacteria tested (**Figure 3, Chapter I**). Various inhibition patterns were observed depending on the media tested. In general, antimicrobial activities were enhanced in TSA-ASW medium than in ISP2-ASW medium (**Table 1, Chapter I**). For example in TSA-ASW medium, 84 % of the actinomycetes were able to inhibit *E. coli* growth regarding the Gram-negative bacteria tested, and 64 % of the actinomycetes were able to inhibit *S. aureus* NBRC 100910^T and *L. monocytogenes* 07PF0776 growth, respectively, regarding the Gram-positive bacteria tested (**Table 1, Chapter I**). A second approach to test

inhibition activities using extractions with organic solvents was pursued, where 67% of the antimicrobial activities observed with the cross-streak method were retrieved (**Table 2, Chapter I**). These results suggest that our actinomycetes collection obtained from Comau fjord present a wide biotechnological potential to produce biologically active compounds with antimicrobial activities. Actinomycetes generally showed resistance to almost all antibiotics tested (**Table 4, Chapter I**). As each biosynthetic cluster normally harbours a gene for the resistance of it bioactive product (Zotchev, 2014), the widespread resistance phenomena could be due to the presence of multiple biosynthetic clusters within the bacterial genome, involving different classes of antibiotic compounds, that could remain cryptic under the above conditions tested (Zazopoulos *et al.*, 2003). In nature, resource competition is thought to be a major drive of evolutionary diversification (Svanbäck *et al.*, 2007). Mainly, there are two competitive strategies by which organisms compete for resources: i) the exploitation competition, which is characterized by rapid nutrient utilization; and ii) interference competition, which occurs when one organism directly harms another (Hibbing *et al.*, 2010). By our first screening approach to evaluate antimicrobial activity, the cross streak method, it is possible to address responses by both types of competition. However, when using liquid fermentations and crude extracts it is possible to obtain activities by interference competition exclusively, which is mainly due to antibiotic compounds (Patin *et al.*, 2015). Chemical mediated interference competition has been linked to improve fitness and stimulate biodiversity (Hibbing *et al.*, 2010). In marine environments, interference competition is more common among particle-associated bacteria that those that are free living (Long & Azam, 2001). As bacteria in marine environments are often submitted to harsh conditions in terms of pressure, temperature, salinity and depletion of micronutrients, the ability of microbes to survive and proliferate is dependent on the ability to produce small molecules resulting in antagonistic

interactions (de Carvalho & Fernandes, 2010; Wietz *et al.*, 2013). Symbiotic relationships with sessile organisms, such as sponges and corals, where actinomycetes account for 13% of their biomass, support the chemical welfare hypothesis (Abdelmohsen *et al.*, 2010; Vicente *et al.*, 2013). Therefore, in natural environments these metabolites play important roles as signal molecules, facilitating intra- or inter-species interactions within microbial communities, related to virulence, colonization, motility, stress response and biofilm formation (Romero *et al.*, 2011). NP have been selected by nature for specific biological interactions, evolving to bind biomolecules and thus presenting drug-like properties (Nisbet & Moore, 1997). Nevertheless, in a laboratory scenario, several considerations must be taken into account when searching for NP. For example, the optimal conditions for biosynthesis of secondary metabolites are not necessarily identical to those for growth. Regarding carbon sources, glucose is a good source for growth, but, in most cases, represses the production of some metabolites. Secondary metabolic pathways are often negatively affected by nitrogen sources favourable for rapid growth, although complex natural sources of nitrogen such as soybeans and casamino acids are indeed favourable. The optimal phosphate concentration for secondary metabolism is generally lower than concentrations required for growth (Knight *et al.*, 2003). The antimicrobial activity of *Streptomyces* sp. H-KF8 was tested in several different agar media, where the carbon, nitrogen and phosphorous sources differ. Accordingly, different patterns of antimicrobial inhibition were observed (**Table 1, Chapter III**). Therefore, as production of microbial secondary metabolites is finely regulated by the carbon sources, this could explain the different patterns exhibited by the same strain in various media with different carbon sources (Ruiz *et al.*, 2010; Sánchez *et al.*, 2010). *Streptomyces* possess a complex developmental life cycle, that affects the secondary metabolism as well, and changes considerably between solid and liquid media. In solid media,

Streptomyces substrate mycelium is made of multiple hyphae that grows by tip extension, and branches in search for nutrients (Chater *et al.*, 2010). In contrast, in liquid media *Streptomyces* undergoes programmed cell death of the mycelium to form four morphological classes: pellets, clumps, branched hyphae and non-branched hyphae (Yagüe *et al.*, 2013). For *Streptomyces* sp. H-KF8, these differences in morphology were noticed (**Figure 1** and **Figure 2, Chapter III**) and, in addition, differences in antimicrobial activity were also observed (**Figure 14,** Addendum **Chapter I** & **Table 1, Chapter III**). An important activity against *S. aureus* NBRC 100910^T was seen after 9 days of incubation in ISP2-agar plates (**Figure 3, Chapter III**). In liquid medium, 30 incubation days were necessary to obtain an active crude extract (**Chapter IV**). These results suggest that secondary metabolites are indeed produced by microorganisms to respond to environmental stimuli, which is supported by the selective activation of BGCs with various stimulatory methods (Covington, McLean & Bachmann, 2016). In addition, the intrinsically complex nature attributed to the regulation of secondary metabolite gene clusters also plays an important role (Bibb & Hesketh, 2009), where normally there is specific regulation for each product within each cluster, as well as pleiotropic mechanisms of regulation that can simultaneously control several pathways (Martin & Liras, 2012). New approaches including the elucidation of the signals that trigger antibiotic biosynthetic pathways, to improve culture media and co-cultures to mimic competition-collaboration scenarios in nature are proposed for future studies with the aim to enhance antibiotic production in *Streptomyces* (Antoraz *et al.*, 2015).

Marine actinomycetes isolated from Comau fjord presented a widespread distribution of the presence of PKS (type I and II) and NRPS genes (**Chapter I**). Most isolates bear at least one biosynthetic gene, where NRPS genes resulted to be the predominant one (91 % of the actinomycetes) (**Table 3, Chapter I** and **Table 5,** Addendum **Chapter I**). However, primers used

to detect the presence of these genes are degenerated, and are specialized to target only one conserved domain of all the possible modules that one gene may have (Ayuso-Sacido & Genilloud, 2005; Ayuso *et al.*, 2005; Gontang *et al.*, 2010). Therefore, this approach may introduce some bias in the amplification of false positives, although it may serve well as a primary screening method (Hodges, Slattery & Olson, 2012). On the other hand, next generation sequencing has been fundamental to the discovery of NP, including in the identification of their biosynthetic pathways through genome mining (Gomez-Escribano, Alt & Bibb, 2016). In this line, whole genome sequencing of five selected actinomycetes was performed (**Chapter II** and **Table 6** Addendum **Chapter II**) to confirm the presence of these genes and to study the broad spectrum of all types of BGCs (**Table 3, Chapter III**). These isolates, along with the criteria for their selection, were as follows: 1) *Kocuria* sp. H-KB5, presents the lowest 16S rRNA gene identity (96.97 %) of all isolates and possibly may represent a new taxon; it is PCR-positive for PKS II and NRPS genes; it tolerates up to 10% of NaCl, and was isolated from a shallow sample under low tide conditions (only 0.25 m of depth) (**Table 3, Chapter I**). 2) *Arthrobacter* sp. H-JH3, is below the 16S rRNA threshold for species definition (with 98.26 % identity); is PCR-positive for NRPS gene; it has a wide temperature and NaCl tolerance (4-37 °C and 0-10 %, respectively) (**Table 3, Chapter I**) and it showed antimicrobial activity against *P. aeruginosa*, *E. coli* and *L. monocytogenes* (**Table 1, Chapter I**). 3) *Brevibacterium* sp. H-BE7, has a low 16S rRNA gene identity (97.94 %); is PCR-positive for all three biosynthetic genes tested (PKS I, PKS II and NRPS); was isolated from the deepest sample (25.1 m of depth); is able to widely tolerate temperature and salinity (4-37 °C and 0-10 %, respectively) (**Table 3, Chapter I**); showed resistance to all commercial antibiotic families tested with exception of sulfonamides (**Table 4, Chapter I**) and presents inhibitory activity against *S. aureus* NBRC 100910^T, and all three Gram-negative reference bacteria tested

(**Table 1, Chapter I**). 4) *Rhodococcus* sp. H-CA8F, is PCR-positive for PKS II and NRPS genes, it cannot tolerate any addition of NaCl although it grows well with ASW (**Table 3, Chapter I**); it showed resistance for all commercial antibiotics tested with exception of erythromycin (**Table 4, Chapter I**) and it showed a strong broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria (**Table 1, Chapter I**). In addition, a mycelial strain 5) *Streptomyces* sp. H-KF8 was also selected due to the presence of PKS II and NRPS biosynthetic genes and tolerance to temperature and NaCl (4-37 °C and 0-7 %, respectively) (**Table 3, Chapter I**); along with its activity against Gram-positives reference bacteria and *E. coli* (**Table 1, Chapter I**). Moreover, antimicrobial activities of crude extracts using various organic solvents with these strains was corroborated, demonstrating the potential of these selected strains to produce antibiotic compounds (**Table 2, Chapter I**).

Currently, the access to microbial genome sequencing due to its low cost and rapid results, has revolutionized the NP research field (Doroghazi & Metcalf, 2013; Antoraz *et al.*, 2015; Katz & Baltz, 2016). Genome sequencing provides a highly informed approach by which strains can be prioritized based on a bioinformatic assessment of their biosynthetic potential. This potential can be used to: i) make generalized predictions about the type of compounds that can be expected; ii) make specific structural predictions of the produced molecules; and/or iii) infer the production of known compounds trough the de-replication process (Jensen *et al.*, 2013). In this study, whole genome sequencing was used to achieve the i) and ii) previously mentioned aims. In this line, a total of 26 BGCs were detected through genome mining in the marine actinomycete *Streptomyces* sp. H-KF8 (**Figure 5, Chapter III**). Also, BGCs were detected in the non-mycelial actinomycetes as follows: 9 BGCs for *Kocuria* sp. H-KB5, 1 BGC for *Arthrobacter* sp. H-JH3, 6 BGCs for *Brevibacterium* sp. H-BE7 and 21 BGCs for *Rhodococcus* sp. H-CA8F (**Table 7,**

Addendum **Chapter II**). These results are in agreement with the fact that mycelial strains are known to harbour more BGCs than non-mycelial strains (Donadio, Monciardini & Sosio, 2007). Technical disadvantages, such as large number of short reads difficult to assemble in large contigs, product of Illumina sequencing (**Table 6**, Addendum **Chapter II**), can be a major challenge when looking for BGCs. As BGCs can usually exceed 100 kb of length, it is mostly uncommon to capture large pathways on a single contig. Therefore, in this study, a second whole genome sequencing strategy was pursued to obtain non-fragmented BGCs, involving PacBIO SMRT technology for *Streptomyces* sp. H-KF8, due to the possibility to obtain improved assembled contigs with complete BGCs (**Chapter II**). PacBIO provides sequence reads of several kb in length, meaning that an entire BGC could be represented on a single sequence read, avoiding the difficulties of assembling repetitive sequence from short fragments (Harrison & Studholme, 2014). Merging the information of both platforms have been demonstrated to give more confident results than when using these platforms separately, as seen for *Streptomyces* sp. Mg1 (Hoefler, Konganti & Straight, 2013). Nevertheless, each genome sequence assembly is a hypothesis, although there are several bioinformatic methods for assessing length, completeness and accuracy (Studholme, 2016). In this Ph.D. thesis, we are reporting the *Streptomyces* sp. H-KF8 whole genome sequence (**Figure 16**, Addendum **Chapter II**). *Streptomyces* sp. H-KF8 genome consists of a linear chromosome of 7,684,888 bp, which is represented in 11 scaffolds obtained from Illumina and PacBIO sequencing, with a 500-fold coverage (**Chapter II**). Annotation grouped in RAST subsystems is presented in **Figure 17**, (Addendum **Chapter II**). Detailed COGs distribution of coding-sequence genes is shown in **Table 2** (**Chapter III**) and comparative genomics of COGs with a terrestrial and a marine-derived *Streptomyces* strains are depicted in **Figure 4** (**Chapter III**). In general, *Streptomyces* genus

possess unstable linear chromosomes that undergo rearrangements, including large deletion and amplifications, presenting huge terminal inverted repeats at both ends ensuring homologous sequences for recombination (Hopwood, 1999; Kinashi, 2008). Even more, NRPS and PKS are long, modular proteins made up of many repeated domain units; thus, genes encoding these key enzymes can be particularly difficult to assemble accurately from short sequence reads. These particular features, along with their commonly high GC content and large genome sizes, have positioned *Streptomyces* genome assemblies as a real challenge, leading to obtain only 19 complete available *Streptomyces* genomes among the 125 draft assemblies over the 30,000 strains that have been identified so far (Harrison & Studholme, 2014).

The term 'genome mining' has been used to describe the exploitation of genomic information for the discovery of new processes, targets and products (Challis, 2008). Natural product biosynthetic potential can be estimated from genomic sequence data through automated bioinformatic platforms, such as antiSMASH and PRISM (Skinnider *et al.*, 2015; Weber *et al.*, 2015). These platforms are capable of comparing sequenced BGCs from previously sequenced microorganisms available in the MIBiG database (Medema *et al.*, 2015), thus inferring putative structures of NP. These comparison relations are particularly useful in the understanding of BGCs that seem novel due to their low identity value with other known-BGCs, and therefore, in the comprehension of the putative molecule produced. The 81 % of the BGCs detected in *Streptomyces* sp. H-KF8 presented low similitudes with known BGCs. From a total of 26 BGCs, two of them corresponded to NRPSs, two PKSs and with four NRPS-PKS hybrids (**Table 8**, Addendum **Chapter II**). Interestingly, the two NRPS clusters presented very low identity with known BGCs from the MIBiG database, where NRPS cluster #5 showed a 13 % of similarity with the streptolydigin BGC (Olano *et al.*, 2009), and NRPS cluster #6 showed a 7 % of similarity with

the mannopeptimycin BGC (Magarvey *et al.*, 2006) (**Table 3, Chapter III**). In addition, the distribution of the BGCs was assessed and the two previously mentioned clusters were found in the same contig (**Figure 5, Chapter III**). As *Streptomyces* sp. H-KF8 presented nine BGCs with a rather low similarity (between 5 and 52%), in addition to eight BGCs with no matches to known BGCs, these results suggest that most secondary metabolites produced by this marine actinomycete have a novel nature (**Table 3, Chapter III**). In this Ph.D. thesis, a focus on the two NRPS clusters with the subsequent analysis of their genes is presented (**Figure 19, Chapter IV** and **Table 13**, Addendum **Chapter IV** for cluster #5 & **Figure 20, Chapter IV** and **Table 14**, Addendum **Chapter IV** for cluster #6). We predicted the peptidic core structure, and infer a few tailouring reactions occurring post-assembly due to the organization of their modules and domains (**Figure 19** for cluster #5 and **Figure 20** for cluster #6, **Chapter IV**). This bioinformatic approach was fundamental to guide further experiments to gain knowledge into the nature of the compound(s) produced by *Streptomyces* sp. H-KF8. The importance of genome mining has been reported for other *Streptomyces* strains, where this approach has led to the identification of novel BGCs and their metabolites (Gomez-Escribano *et al.*, 2015; Iftime *et al.*, 2015; Tang *et al.*, 2015).

In addition to genome mining for BGCs, the genome sequence was analyzed to gain information about ecological abiotic tolerance features (**Chapter III**). The sea is a unique and extreme environment characterized by high pressure, low temperature, lack of light and variable salinity and oxygen concentration (Manivasagan *et al.*, 2013). Thus, microorganisms have to adapt through functional biological traits in order to survive in this extreme environment. This process of adaptability may, in turn, modulate the synthesis of their bioactive metabolites. *Streptomyces* sp. H-KF8 showed a high amount of genes (69 genes) related to oxidative stress,

with nine genes for thioerdoxins and alkyl hydroperoxide reductases each. An efficient response against several concentrations of H_2O_2 was observed, being able to tolerate the toxicity even better than the model strain *S. coelicolor* A3(2) **(Figure 7, Chapter III)**. The antibiotic-resistance response was evaluated. *Streptomyces* sp. H-KF8 showed resistance to 14 of the 16 commercial antibiotics tested, and presented more than 90 genes that could be involved in antibiotic resistance **(Figure 8, Chapter III)**. Due to the naturally high concentrations of certain heavy metals in Chilean Northern Patagonia (Revenga *et al.*, 2012; Hermanns & Biester, 2013), the ability of *Streptomyces* sp. H-KF8 to tolerate exposure to metal(loid)s was evaluated. Interestingly, resistance to copper, cobalt, mercury, tellurite, chromate and nickel was revealed **(Figure 6, Chapter III)**. Accordingly, several genetic determinants for metal(loid)s resistance were found within the *Streptomyces* sp. H-KF8 genome. The most abundant genes were related to tellurite resistance. To our knowledge, this is the first tellurite-resistant *Streptomyces* strain described so far. Metal exposure, especially trace metal ions and rare earth elements have been demonstrated to modulate secondary metabolite production (Locatelli, Goo & Ulanova, 2016). In fact, contrary to the widely held hypothesis that metals are a hindrance in secondary metabolism, they can induce or enhance the synthesis of potent and medically relevant metabolites in metal tolerant *Streptomyces* strains (Haferburg *et al.*, 2008). Even more, it has been shown that cryptic gene clusters can be induced under conditions of heavy metal stress (Haferburg *et al.*, 2008; Locatelli, Goo & Ulanova, 2016), opening new possibilities for NP discovery. Overall, these results suggest a molecular crosstalk between the biological responses to these abiotic stressors, where *Streptomyces* sp. H-KF8 proved to be a genetically well-prepared bacterium with the ability to confront the dynamics of the marine environment.

Conventional approaches to address the metabolites produced by a bioactive bacterium involves the fermentation in liquid media and subsequent extraction of the compound(s) using organic solvents (Sarmiento-Vizcaíno *et al.*, 2016). In this study, extraction was performed with several organic solvents with varying polarities, where polar solvents yielded better results (**Figure 21, Chapter IV**). Several biological models were tested to evaluate the different bioactivities. *Streptomyces* sp. H-KF8 crude extract showed inhibition to *S. aureus* NBRC 100910^T, *L. monocytogenes* 07PF0776 and, to a lesser extent inhibition of *E. coli* FAP1 (**Figure 22, Chapter IV**). Cytotoxic activity with cancer cells was also observed (**Figure 23, Chapter IV**). No inhibition against a model yeast was noticed. These results support the hypothesis that several compounds with different modes of action and different model targets are present within the crude extract, or alternatively, it may be one family of compounds which present both antibacterial and antiproliferative activities. Bioguided HPLC fractionation led to the identification of 36 fractions with different bioactivity patterns when *Streptomyces* sp. H-KF8 was incubated in ISP2 medium, and 12 fractions when it was incubated in V medium (**Figure 28, Chapter IV**). These two media were used for comparison purposes, as antimicrobial activity of fractions considerably differed from each other (**Figure 29** and **Table 9, Chapter IV**). Active fractions were analyzed by three different mass-techniques: ESI-FT ICR MS, MALDI-TOF MS and MALDI-FT ICR IMS, to profile the chemical output from the marine actinomycete *Streptomyces* sp. H-KF8, and, more importantly, analyze the metabolites in an antagonistic interaction condition of inhibition. These metabolites that are produced when a *Streptomyces* strain is co-cultivated with another strain, but do not necessarily appear under mono-cultivating conditions, are suggested to be interaction-specific (Hopwood, 2013; Traxler *et al.*, 2013). Co-culturing metabolomic assays proved that *Streptomyces* displays an idiosyncratic response (Traxler *et al.*, 2013). Thus, performing MALDI-

FT ICR IMS allows a high-resolution mapping of several ions within a sample (Watrous & Dorrestein, 2011). ESI-FT ICR MS and MALDI-TOF allows to obtain the mass-to-charge ratio (m/z) of the detected metabolites with high accuracy and fragmentation of parental ions (**Figure 30** and **Figure 34**, respectively, **Chapter IV**). TOF MS is able to determine mass accuracy with an error under 1 ppm, and FT ICR MS may perform at sub-ppm mass errors (Covington, McLean & Bachmann, 2016). Therefore, the utilization of both techniques is useful to analyze the metabolites of an antibiotic producer-microorganism, and was applied to understand the *Streptomyces* sp. H-KF8 metabolome. Moreover, IMS techniques complement conventional mass analyzers by providing a two-dimensional visualization of surface metabolites directly secreted from microbial colonies, enabling the preservation of molecular localization within the agar plate and spatially mapping microbial molecules (Yang *et al.*, 2012). MALDI IMS can be summarized in five steps: i) microbe cultivation on thin agar, ii) transfer of the excised culture-containing agar slice onto the MALDI target plate, iii) solid matrix application, iv) dehydration of the sample and v) data acquisition and interpretation (Vergeiner *et al.*, 2013). In an IMS experiment, the desorption and ionization of the molecules from the sample occurs when a desorption probe hits the surface of a sample at defined positions controlled by the X-Y stage (**Figure 32, Chapter IV**), dislodging ions that are then weighed in a mass spectrometer (Fang & Dorrestein, 2014). As a result, it provides a mass spectrum for each position. The collection of several mass spectra at each position will be shown as a single image, with each specific mass displayed as a false-colour gradient, showing the specific molecular distribution and relative abundance of the metabolites on the sample (**Figure 33** and **Figure 35, Chapter IV**) (Fang & Dorrestein, 2014). The number of detectable compounds from a metabolomics extract will vary depending on the analytical methods used during acquisition, where neutral or poorly ionizing

species will be transparent to MS detection (Covington, McLean & Bachmann, 2016). In MALDI-IMS, sample is first covered with a layer of matrix (**Figure 32B, Chapter IV**), which is a UV-absorbing organic acid such as DHB (used for glycans, lipids, peptides and polymers) or HCCA (used for glycans, peptides and proteins) (Yang *et al.*, 2012; Fang & Dorrestein, 2014). Matrix facilitates desorption and ionization of compounds from the sample surface, where molecules with m/z up to 110 kDa and higher can be detected (Remoortere, Ze & Oever, 2010). Application of solid matrix is the crucial step of sample preparation procedures, since the homogeneity of the coating directly influences sensitivity and achievable MS resolution (Vergeiner *et al.*, 2013). Several sample preparation techniques can be used, and results obtained differ considerably (Vergeiner *et al.*, 2013; Hoffmann & Dorrestein, 2015). In this study, a sprayed HCCA matrix solution onto dried agar-based sample plates was used (**Figure 32B, Chapter IV**), to allow a homogeneous matrix layer where improved ionization efficiency has been demonstrated (Hoffmann & Dorrestein, 2015). This technique was fundamental to reveal specialized metabolites that are produced when a *Streptomyces* sp. H-KF8 was co-cultivated with another bacterium in an antagonistic interaction, resembling chemical interference competition (**Table 10** and **Table 11, Chapter IV**). The selected ions were then analyzed through MALDI TOF MS, and amino acid composition of parental ions suggest that they were related with the bioinformatic prediction of cluster #6 (**Table 12, Chapter IV**), therefore suggesting that this cluster could be involved in the synthesis of a specialized compound (or family of compounds), which may explain the antimicrobial activity observed.

The NRPS cluster #6, is located in the contig *tig_02* and bears a length of 79,174 bp covering at least 33 genes, involved in the biosynthesis, tailouring reaction, regulation, transport and resistance functions (**Table 14,** Addendum **Chapter IV**). An antiSMASH result showed that

7% of the genes within this cluster showed similarity with the known BGC of mannopeptimycins (**Table 3, Chapter III**). Mannopeptimycins are produced by *Streptomyces hygroscopicus* NRRL 30439, and represent a novel lipoglycopeptide antibiotic with activity against methicillin-resistant *S. aureus* and vancomycin-resistant enterococci (Magarvey *et al.*, 2006). Interestingly, exploration of the different biological activities of *Streptomyces* sp. H-KF8 crude extracts revealed inhibition mainly on Gram-positives (**Figure 22A** and **Figure 22B, Chapter IV**), but also on *E. coli* (**Figure 22C, Chapter IV**). These results suggest the presence of different compounds in the crude extract, each one with a selective mode of action and/or, the presence of a family of related molecules that have a broad spectrum of inhibition. The exploration of the chemical nature of the crude extract by TLC (**Figure 27**) using ninhydrin (**Figure 27A**) and orcinol (**Figure 27B**), suggested the presence of compound(s) with amino acids, amino sugars and/or amines, and a glycoside and/or a glycolipid. Evidence of the complex and mixed structure was also observed in the crude extract resistant to temperature and enzymes assays (**Figure 24** and **25**, respectively, **Chapter IV**), and the two peaks obtained in the fluorescence spectrum of *Streptomyces* sp. H-KF8 crude extract (**Figure 26, Chapter IV**). These results suggest that *Streptomyces* sp. H-KF8 crude extract harbours a glycopeptide type antimicrobial compound, which presents a rather low similarity with the lipoglycopeptide mannopeptimycins. These molecules have a cyclic hexapeptide core containing a unique combination of two proteinogenic (serine and glycine) and four non-proteinogenic (two β-hydroxy-enduracididines, a β-methyl-phenylalanine and a β-hydroxy-tyrosine) amino acids. Additionally, the hexapeptide is tailoured with one *N*-linked mannose and an *O*-linked di-mannose. Finally, the terminal O-linked mannose is modified with an isovaleryl group at one of three different positions, resulting in the γ-, δ- and ε-mannopeptimycins (Magarvey *et al.*, 2006). Similarly to the mannopeptimycin BGC, the

Streptomyces sp. H-KF8 cluster #6 bears two main biosynthetic NRPS genes, A4U61_02940 and A4U61_02945 **(Figure 20, Chapter IV)**. They are involved in the incorporation of ten amino acids (tryptophan-alanine-valine-alanine-tryptophan + ornithine-threonine-ornithine-valine-tryptophan). In contrast the mannopeptimycin NRPS genes only incorporate six amino acids. Specifically, the gene A4U61_02940 from *Streptomyces* sp. H-KF8 is similar to the *mmpB* gene from the mannopeptimycin cluster, which is responsible for incorporating three non-proteinogenic amino acids (two β-hydroxy-enduracididines and a β-hydroxy-tyrosine). However, these amino acids are not predicted to be incorporated by the NRPS of cluster #6, although a modified tyrosine (nitro-tyrosine) is proposed instead of one tryptophan **(Table 12, Chapter IV)**. Another similarity between these BGCs, is that both present the carboxypeptidase resistance genes resembling to *vanY* and *vanZ* (A4U61_02950 and A4U61_03005 genes, respectively, in *Streptomyces* sp. H-KF8) from the vancomycin BGC (Recktenwald *et al.*, 2002). VanY is a D-Ala-D-Ala carboxypeptidase/carboxyesterase that cleaves off the terminal D-Ala residue of the forming-peptidoglycan pentapeptide and acts in series with VanX, which is a D-Ala-D-Ala dipeptidase, to prevent the accumulation of the substrate for the vancomycin-type glycopeptides (Wright *et al.*, 1992). This glycopeptide antibiotic family targets Gram-positive bacteria, by binding to the *N*-acyl-D-Ala-D-Ala termini of the peptidoglycan and its precursor Lipid II. This binding sequesters the substrates of two key enzymes critical to cell wall synthesis: the transglycosylases and D,D-transpeptidases. The target microorganism rigidifies the cell wall and has the inability to grow, thus blocking cell division and weakening the wall, ultimately resulting in cell death (Yim *et al.*, 2014). Since these resistance genes are present within the cluster #6 of *Streptomyces* sp. H-KF8, in addition to another gene for glycopeptide resistance (A4U61_02900), a similar mode of action of the metabolite concerning inhibition of the cell wall biosynthesis is proposed. Also, these

genes may be responsible for structural changes within the cell wall of the antibiotic-producer bacterium, that may render it immune to its own antibiotic. Nevertheless, an additional bleomycin resistance gene (A4U61_02905) is also located in cluster #6. Since bleomycin mode of action is primarily involved in the induction of DNA cleavage, the presence of this resistance gene may suggest probably a dual mechanism of inhibition. Another structural similarity with mannopeptimycins is the sugar moieties that are added to the peptidic core structure. In this molecule, three genes encoding mannosyltransferases are in charge of transferring three mannoses to the aglycone. Although no glycosyltransferase(s) were found within cluster #6 from *Streptomyces* sp. H-KF8, it was present in the genetic neighbourhood approximately ~100 genes upstream of cluster #6. Within cluster #6, several genes involved in sugar modifications post-assembly are present. These involve three glycosyl hydrolases (A4U61_02870, A4U61_02910 and A4U61_02910 genes) and three sugar acetyltransferases (A4U61_02895, A4U61_02915 and A4U61_02930 genes) along with sugar transporters (A4U61_02875, A4U61_02880 and A4U61_02885 genes) (**Table 14,** Addendum **Chapter IV**). Altogether, these results suggests that sugar moieties are crucial functional groups that are being added, and subsequently incorporated to the peptidic core, conferring additional glycosylation diversity in the structure. Sugar moieties are often found in NP, for example in the antibiotics erythromycin (Staunton & Wilkinson, 1997), vancomycin (Recktenwald *et al.*, 2002) or teicoplanin (Li *et al.*, 2004; Hadatsch *et al.*, 2007). Usually, the glycosyl groups play an important role in NP, where the sugar unit(s) directly mediate the bioactivity of the compound; *e.g.*, by promoting binding to a target biomolecule (La Ferla *et al.*, 2011). This is in agreement with the results obtained with the crude extract resistance to enzymes assay, where reduced antimicrobial activity resulted when incubated with lysozyme (**Figure 25, Chapter IV**). From a structural point of view, glycopeptides

have a complex architecture due to their highly crosslinked state, where three or four biaryl and biaryl-ether cross-links between the side chains of aromatic amino acids are present in the heptapeptide backbone (Butler *et al.*, 2014; Yim *et al.*, 2014). The cytochrome P450s monooxygenase enzymes install these crosslinks with a strict order of cyclization steps, which confers the characteristic cup-shape of the aglycone (Peschke, Brieke & Cryle, 2016). In *Streptomyces* sp. H-KF8 cluster #6, the A4U61_02955 gene encoding for a cytochrome P450 is contiguous to one of the NRPS genes, A4U61_029550 (**Table 14,** Addendum **Chapter IV**). It has been demonstrated that P450 monooxygenases acts directly on the NRPS-bounded substrates (Bischoff *et al.*, 2005). Interestingly, glycopeptides require a β-hydroxytyrosine as a precursor for NRPS-catalyzed peptide assembly, where this oxidation is P450-catalyzed (Recktenwald *et al.*, 2002). As aromatic amino acids are being predicted in the two main NRPS genes that conform cluster #6, a role for the gene encoding the P450 monooxygenase in the crosslink of these aromatic side chains and subsequent formation of an ether bond is proposed. These enzymes play many roles in the biosynthesis of NP, specially in glycopeptides, being able to perform hydroxylation, epoxidation and aromatic crosslinking with high degrees of selectivity (Cryle, Stok & De Voss, 2003). In addition, it also has activity as a glycosyl transferase activator, suggesting a relation with the sugar moieties predicted to be added to the peptidic core structure, as discussed previously.

Overall, based on the similarities with genes related to known glycopeptide BGCs, we hypothesize that cluster #6 may be generating a family of glycopeptides whose peptidic core structure consists of a cyclic decapeptide, that has additional *O*-linked sugar moieties possibly in at least one of the aromatic amino acids that are part of the core molecule. To our knowledge, this NRPS BGC has not been previously described, thus, the proposed structure for its metabolite

should be unique, suggesting a novel glycopeptide compound. Moreover, Additional analysis such as NMR will be crucial to fully elucidate the molecular composition of the cluster #6 product(s).

7 Conclusions

- This study showed that the Comau fjord is a rich source for bioprospecting actinomycetes that produce bioactive compounds, especially those with antimicrobial activity.

- The presence of NRPS and/or PKS metabolic routes within the actinomycetes sequenced genomes, indicated the genetic potential of the selected isolates to produce bioactive compounds.

- *Streptomyces* sp. H-KF8 harbours novel secondary metabolites biosynthetic gene clusters. Two NRPS clusters were studied, and the peptidic core of the respective metabolites was predicted. These peptidic core molecules were composed of modified amino acids and non-proteinogenic ones, with additional decorating functional groups such as sugar moieties.

- A sustained correlation among the predicted bioinformatic data and one of the NRPS clusters (cluster #6) of *Streptomyces* sp. H-KF8 is proposed, together with the molecular composition from experimental data obtained with mass techniques (ESI FT ICR MS, Imaging MS and MALDI TOF MS/MS) was accomplished, suggesting the synthesis of a novel antimicrobial compound of glycopeptide nature.

8 Research needs

- NMR studies will be crucial to fully elucidate the chemical structure of the previously purified compound(s) which were predicted by bioinformatic tools in this study, and therefore, demonstrating the role of the genes that conforms cluster #6.

- A whole comparative genomic study involving marine actinomycetes from other geographic locations will be useful to understand the distribution and the uniqueness of the biosynthetic gene clusters described in this study.

- A metagenomics approach will gain insights into the biodiversity of the bacterial community in the remote Comau fjord, which will be useful to understand the role of actinomycetes in this marine ecosystem.

9 References

Abdel-Mageed WM., Milne BF., Wagner M., Schumacher M., Sandor P., Pathom-aree W., Goodfellow M., Bull AT., Horikoshi K., Ebel R., Diederich M., Fiedler H-P., Jaspars M. 2010. Dermacozines, a new phenazine family from deep-sea dermacocci isolated from a Mariana Trench sediment. *Organic & biomolecular chemistry* 8:2352–2362.

Abdelmohsen UR., Pimentel-Elardo SM., Hanora A., Radwan M., Abou-El-Ela SH., Ahmed S., Hentschel U. 2010. Isolation, phylogenetic analysis and anti-infective activity screening of marine sponge-associated actinomycetes. *Marine Drugs* 8:399–412.

Antoraz S., Santamaria RI., Diaz M., Sanz D., Rodriguez H. 2015. Toward a new focus in antibiotic and drug discovery from the *Streptomyces* arsenal. *Frontiers in Microbiology* 6:1–8.

Ayuso A., Clark D., González I., Salazar O., Anderson A., Genilloud O. 2005. A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. *Applied microbiology and biotechnology* 67:795–806.

Ayuso-Sacido A., Genilloud O. 2005. New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups. *Microbial ecology* 49:10–24.

Balouiri M., Sadiki M., Ibnsouda S. 2016. Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, 6(2): 71–79.

Baltz RH. 2008. Renaissance in antibacterial discovery from actinomycetes. *Current Opinion in Pharmacology* 8:557–563.

Barka EA., Vatsa P., Sanchez L., Gaveau-Vaillant N., Jacquard C., Klenk HP., Clément C., Ouhdouch Y., van Wezel GP. 2016. Taxonomy, Physiology, and Natural Products of *Actinobacteria*. *Microbiology and Molecular Biology Reviews* 80:1-43.

Baskaran R., Vijayakumar R., Mohan P. 2011. Enrichment method for the isolation of bioactive actinomycetes from mangrove sediments of Andaman Islands India. *Malaysian Journal of Microbiology* 7:26–32.

Bentley S., Chater K., Cerdeño-Tárraga A-M., Challis GL., Thomson NR., James KD., Harris DE., Quail M a., Kieser H., Harper D., Bateman A., Brown S., Chandra G., Chen CW., Collins M., Cronin A., Fraser A., Goble A., Hidalgo J., Hornsby T., Howarth S., Huang C-H., Kieser T., Larke L., Murphy L., Oliver K., O'Neil S., Rabbinowitsch E., Rajandream M., Rutherford K., Rutter S., Seeger K., Saunders D., Sharp S., Squares R., Squares S., Taylor K., Warren T., Wietzorrek A., Woodward J., Barrell BG., Parkhill J., Hopwood D a. 2002. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature* 417:141–147.

Bérdy J. 2005. Bioactive Microbial Metabolites. *Journal of Antibiotics* 58:1–26.

Bérdy J. 2012. Thoughts and facts about antibiotics: Where we are now and where we are heading. *The Journal of Antibiotics* 65:441–441.

Bernan VS., Greenstein M., Maiese WM. 1997. Marine microorganisms as a source of new natural products. *Advances in applied microbiology* 43:57–90.

Bibb M., Hesketh A. 2009. *Analyzing the Regulation of Antibiotic Production in Streptomycetes*. Elsevier Inc.

Bischoff D., Bister B., Bertazzo M., Pfeifer V., Stegmann E., Nicholson GJ., Keller S., Pelzer S., Wohlleben W., Süssmuth RD. 2005. The biosynthesis of vancomycin-type glycopeptide antibiotics - A model for oxidative side-chain cross-linking by oxygenases coupled to the action of peptide synthetases. *ChemBioChem* 6:267–272.

Blunt JW., Copp BR., Hu WP., Munro MH., Northcote PT., Prinsep MR. 2009. Marine natural products. *Nat Prod Rep* 26:170–244.

Bredholdt H., Galatenko O., Engelhardt K., Fjaervik E., Terekhova LP., Zotchev SB. 2007. Rare actinomycete bacteria from the shallow water sediments of the Trondheim fjord, Norway: isolation, diversity and biological activity. *Environmental microbiology* 9:2756–64.

Brown KD., Kulis J., Thomson B., Chapman TH., Mawhinney DB. 2006. Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Science of the Total Environment* 366:772–783.

Bull A., Stach J. 2007. Marine actinobacteria: new opportunities for natural product search and discovery. *Trends in microbiology* 15:491–9.

Bustamante MS. 2009. The southern Chilean fjord region: oceanographic aspects. In: *Marine benthic fauna of chilean patagonia*. 53–60.

Butler MS., Hansford KA., Blaskovich MAT., Halai R., Cooper MA. 2014. Glycopeptide antibiotics: Back to the future.

The Journal of Antibiotics 67:631–644.
Cane DE., Walsh CT., Khosla C. 1998. Harnessing the biosynthetic code: combinations, permutations, and mutations. *Science* 282:63–68.
de Carvalho CCCR., Fernandes P. 2010. Production of metabolites as bacterial responses to the marine environment. *Marine drugs* 8:705–27.
Challis GL. 2008. Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology* 154:1555–1569.
Chater KF., Biro S., Lee KJ., Palmer T., Schrempf H. 2010. The complex extracellular biology of *Streptomyces*. *FEMS Microbiology Reviews* 34:171–198.
Chen Y., Wang Y., Zhang Y., Tang S., Liu Z., Xiao H., Xu L., Cui X., Li W., Chen Y. 2016. *Nocardiopsis litoralis* sp. nov., a halophilic marine actinomycete isolated from a sea anemone. *International journal of systematic and evolutionary microbiology*:2708–2713.
Chopra I., Roberts M. 2001. Tetracycline Antibiotics : Mode of Action , Applications , Molecular Biology , and Epidemiology of Bacterial Resistance Tetracycline Antibiotics : Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews* 65:232–260.
Claverías FP., Undabarrena A., González M., Seeger M., Cámara B. 2015. Culturable diversity and antimicrobial activity of *Actinobacteria* from marine sediments in Valparaíso bay, Chile. *Frontiers in Microbiology* 6:1–11.
Coates A., Hu Y., Bax R., Page C. 2002. The future challenges facing the development of new antimicrobial drugs. *Nature reviews. Drug discovery* 1:895–910.
Colquhoun JA., Heald SC., Li L., Tamaoka J., Kato C., Horikoshi K., Bull AT. 1998. Taxonomy and biotransformation activities of some deep-sea actinomycetes. *Extremophiles* 2:269–277.
Covington BC., McLean JA., Bachmann BO. 2016. Comparative mass spectrometry-based metabolomics strategies for the investigation of microbial secondary metabolites. *Nat. Prod. Rep.* 0:1–19.
Cryle MJ., Stok JE., De Voss JJ. 2003. Reactions catalyzed by bacterial cytochromes P 450. *Aust. J. Chem. FIELD Full Journal Title:Australian Journal of Chemistry* 56:749–762.
Dai HQ., Wang J., Xin YH., Pei G., Tang SK., Ren B., Ward A., Ruan JS., Li WJ., Zhang LX. 2010. *Verrucosispora sediminis* sp. nov., a cyclodipeptide-producing actinomycete from deep-sea sediment. *International Journal of Systematic and Evolutionary Microbiology* 60:1807–1812.
Das S., Lyla PS., Khan SA. 2006. Marine microbial diversity and ecology: importance and future perspectives. *Current Science* 90:1325–1335.
Debois D., Ongena M., Cawoy H., De Pauw E. 2013. MALDI-FTICR MS Imaging as a powerful tool to identify *Paenibacillus* antibiotics involved in the inhibition of plant pathogens. *Journal of American Society for Mass Spectrometry*, 24: 1202–1213.
Demain A., Fang A. 2000. The natural functions of secondary metabolites. In: *Advances in Biochemical Engineering/Biotechnology*. 222.
Donadio S., Monciardini P., Sosio M. 2007. Polyketide synthases and nonribosomal peptide synthetases: the emerging view from bacterial genomics. *Natural product reports* 24:1073–109.
Donia M., Hamann MT. 2003. Marine natural products and their potential applications as anti-infective agents. *Lancet Infectious Diseases* 3:338–348.
Doroghazi JR., Metcalf WW. 2013a. Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *BMC genomics* 14:611.
Duncan K., Haltli B., Gill K a., Kerr RG. 2014. Bioprospecting from marine sediments of New Brunswick, Canada: exploring the relationship between total bacterial diversity and actinobacteria diversity. *Marine drugs* 12:899–925.
Engelhardt K., Degnes KF., Kemmler M., Bredholt H., Fjaervik E., Klinkenberg G., Sletta H., Ellingsen TE., Zotchev SB. 2010. Production of a new thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species. *Applied and Environmental Microbiology* 76:4969–4976.
Eustáquio AS., McGlinchey RP., Liu Y., Hazzard C., Beer LL., Florova G., Alhamadsheh MM., Lechner A., Kale AJ., Kobayashi Y., Reynolds K a., Moore BS. 2009. Biosynthesis of the salinosporamide A polyketide synthase substrate chloroethylmalonyl-coenzyme A from S-adenosyl-L-methionine. *Proceedings of the National Academy of Sciences of the United States of America* 106:12295–12300.
Euzéby J. 2011. Notification that new names and new combinations have appeared in volume 59, part 8, of the IJSEM. *International journal of systematic and evolutionary microbiology* 61:477–478.
Fang J., Dorrestein PC. 2014. Emerging mass spectrometry techniques for the direct analysis of microbial colonies. *Current Opinion in Microbiology* 19:120–129.
Feling RH., Buchanan GO., Mincer TJ., Kauffman CA., Jensen PR., Fenical W. 2003. Salinosporamide A: A highly cytotoxic

proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*. *Angewandte Chemie - International Edition* 42:355–357.

Fenical W. 1993. Chemical Studies of Marine Bacteria: Developing a New Resource. *Chemical Reviews* 93:1673–1683.

Fenical W., Jensen PR., Palladino MA., Lam KS., Lloyd GK., Potts BC. 2009. Discovery and development of the anticancer agent salinosporamide A (NPI-0052). *Bioorganic and Medicinal Chemistry* 17:2175–2180.

La Ferla B., Airoldi C., Zona C., Orsato A., Cardona F., Merlo S., Sironi E., D'Orazio G., Nicotra F. 2011. Natural glycoconjugates with antitumor activity. *Natural product reports* 28:630–648.

Fiedler H-P., Bruntner C., Bull AT., Ward AC., Goodfellow M., Potterat O., Puder C., Mihm G. 2005. Marine actinomycetes as a source of novel secondary metabolites. *Antonie van Leeuwenhoek* 87:37–42.

Fischbach M a., Walsh CT. 2006. Assembly-Line Enzymology for Polyketide and Nonribosomal Peptide Antibiotics: Logic, Machinery, and Mechanisms. *ChemInform* 37:3468–3496.

Flärdh K., Buttner MJ. 2009. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nature Reviews Microbiology* 7:36–49.

Gao B., Gupta RS. 2005. Conserved indels in protein sequences that are characteristic of the *phylum Actinobacteria*. *International journal of systematic and evolutionary microbiology* 55:2401–12.

Gao B., Gupta RS. 2012. Phylogenetic framework and molecular signatures for the main clades of the *phylum Actinobacteria*. *Microbiology and molecular biology reviews : MMBR* 76:66–112.

Genilloud O. 2014. The re-emerging role of microbial natural products in antibiotic discovery. *Antonie van Leeuwenhoek* 106:173–88.

Gomez-Escribano JP., Castro JF., Razmilic V., Chandra G., Andrews B., Asenjo J., Bibb MJ. 2015. The *Streptomyces leeuwenhoekii* genome: de novo sequencing and assembly in single contigs of the chromosome, circular plasmid pSLE1 and linear plasmid pSLE2. *BMC genomics* 16:485.

Gomez-Escribano JP., Alt S., Bibb MJ. 2016. Next generation sequencing of actinobacteria for the discovery of novel natural products. *Marine Drugs* 14:6–8.

Gontang E., Gaudêncio SP., Fenical W., Jensen PR. 2010. Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria. *Applied and environmental microbiology* 76:2487–99.

Gontang E., Fenical W., Jensen P. 2007. Phylogenetic diversity of gram-positive bacteria cultured from marine sediments. *Applied and environmental microbiology* 73:3272–82.

Goodfellow M., Fiedler H-P. 2010. A guide to successful bioprospecting: informed by actinobacterial systematics. *Antonie van Leeuwenhoek* 98:119–42.

Goodfellow M., Williams ST. 1983. Ecology of actinomycetes. *Annual review of microbiology* 37:189–216.

Goris J., Konstantinidis KT., Klappenbach JA., Coenye T., Vandamme P., Tiedje JM. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *International Journal of Systematic and Evolutionary Microbiology* 57:81–91.

Gulder TAM., Moore BS. 2010. Chasing the treasures of the sea – bacterial marine natural products. *Current opinion in microbiology* 12:252–260.

Haber M., Ilan M. 2014. Diversity and antibacterial activity of bacteria cultured from Mediterranean *Axinella* spp. sponges. *Journal of applied microbiology* 116:519–32.

Hadatsch B., Butz D., Schmiederer T., Steudle J., Wohlleben W., Süssmuth R., Stegmann E. 2007. The Biosynthesis of Teicoplanin-Type Glycopeptide Antibiotics: Assignment of P450 Mono-Oxygenases to Side Chain Cyclizations of Glycopeptide A47934. *Chemistry and Biology* 14:1078–1089.

Haefner B. 2003. Drugs from the deep: marine natural products as drug candidates. *Research Focus* 8:536–544.

Haferburg G., Kloess G., Schmitz W., Kothe E. 2008. "Ni-struvite" - A new biomineral formed by a nickel resistant *Streptomyces acidiscabies*. *Chemosphere* 72:517–523.

Hamada M., Iino T., Iwami T., Tamura T., Shigeaki H., Ken-ichiro S. 2009. *Arsenicicoccus piscis* sp. nov., a Mesophilic Actinobacterium Isolated from the Intestinal Tract of a Fish. *Actinomycetologica* 23:40–45.

Han SK., Nedashkovskaya OI., Mikhailov V V., Kim SB., Bae KS. 2003. *Salinibacium amurskyense* gen. nov., sp. nov., a novel genus of the family *Microbacteriaceae* from the marine environment. *International journal of systematic and evolutionary microbiology* 53:2061–2066.

Harrison J., Studholme DJ. 2014. Recently published *Streptomyces* genome sequences. *Microbial Biotechnology* 7:373–380.

Helmke E., Weyland H. 1984. *Rhodococcus marinonascens* sp. nov., an actinomycete from the sea. *International Journal of Systematic Bacteriology* 49:127–138.

Hermanns YM., Biester H. 2013. Anthropogenic mercury signals in lake sediments from southernmost Patagonia, Chile. *Science of the Total Environment* 445–446:126–135.

Hibbing ME., Fuqua C., Parsek MR., Peterson SB. 2010. Bacterial competition: surviving and thriving in the microbial

jungle. *Nature reviews. Microbiology* 8:15–25.
Hodges TW., Slattery M., Olson JB. 2012. Unique actinomycetes from marine caves and coral reef sediments provide novel PKS and NRPS biosynthetic gene clusters. *Marine biotechnology* 14:270–80.
Hoefler BC., Konganti K., Straight PD. 2013. De Novo Assembly of the *Streptomyces* sp. strain Mg1 Genome Using PacBio Single-Molecule Sequencing. *Genome Announcements* 1:1–2.
Hoffmann T., Dorrestein PC. 2015. Homogeneous Matrix Deposition on Dried Agar for MALDI Imaging Mass Spectrometry of Microbial Cultures. *Journal of the American Society for Mass Spectrometry* 26:1959–1962.
Hogan D., Kolter R. 2002. Why are bacteria refractory to antimicrobials? *Current Opinion in Microbiology* 5:472–477.
Hong K., Gao A-H., Xie Q-Y., Gao HG., Zhuang L., Lin H-P., Yu H-P., Li J., Yao X-S., Goodfellow M., Ruan J-S. 2009. Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. *Marine Drugs* 7:24–44.
Hopwood DA. 1999. Forty years of genetics with *Streptomyces*: from in vivo through in vitro to in silico. *Microbiology* 145:2183–2202.
Hopwood DA. 2007. How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? *Molecular Microbiology* 63:937–940.
Hopwood DA. 2013. Imaging Mass Spectrometry reveals highly specific interactions between actinomycetes to activate specialized metabolic gene clusters. *mBio* 4:612–613.
Iftime D., Kulik A., Härtner T., Rohrer S., Niedermeyer THJ., Stegmann E., Weber T., Wohlleben W. 2015. Identification and activation of novel biosynthetic gene clusters by genome mining in the kirromycin producer *Streptomyces collinus* Tü 365. *Journal of Industrial Microbiology and Biotechnology* 43:277–291.
Ikeda H., Ishikawa J., Hanamoto A., Shinose M., Kikuchi H., Shiba T., Sakaki Y., Hattori M., Omura S. 2003. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nature biotechnology* 21:526–531.
Jang KH., Nam S-J., Locke JB., Kauffman C a., Beatty DS., Paul L a., Fenical W. 2013. Anthracimycin, a potent anthrax antibiotic from a marine-derived actinomycete. *Angewandte Chemie (International ed. in English)*:1–4.
Jensen PR., Mincer TJ., Williams PG., Fenical W. 2005a. Marine actinomycete diversity and natural product discovery. *Antonie van Leeuwenhoek* 87:43–48.
Jensen PR., Gontang E., Mafnas C., Mincer TJ., Fenical W. 2005b. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. *Environmental microbiology* 7:1039–48.
Jensen PR., Chavarria KL., Fenical W., Moore BS., Ziemert N. 2013. Challenges and triumphs to genomics-based natural product discovery. *Journal of Industrial Microbiology and Biotechnology* 41:203–209.
Jiang S., Sun W., Chen M., Dai S., Zhang L., Liu Y., Lee KJ., Li X. 2007. Diversity of culturable actinobacteria isolated from marine sponge *Haliclona* sp. *Antonie van Leeuwenhoek* 92:405–16.
Jiao W., Zhang F., Zhao X., Hu J., Suh JW. 2013. A novel alkaloid from marine-derived actinomycete *Streptomyces xinghaiensis* with broad-spectrum antibacterial and cytotoxic Activities. *PLoS ONE* 8:e75994.
Joint I., Mühling M., Querellou J. 2010. Culturing marine bacteria - an essential prerequisite for biodiscovery. *Microbial biotechnology* 3:564–75.
Jose PA., Jha B. 2016. New dimensions of research on actinomycetes: quest for next generation zntibiotics. *Frontiers in Microbiology* 7:1–5.
Katz L., Baltz RH. 2016. Natural product discovery: past, present, and future. *Journal of Industrial Microbiology and Biotechnology* 43:155–176
Khan ST., Tamura T., Takagi M., Shin-ya K. 2010. *Streptomyces tateyamensis* sp. nov., *Streptomyces marinus* sp. nov. and *Streptomyces haliclonae* sp. nov., isolated from the marine sponge *Haliclona* sp. *International Journal of Systematic and Evolutionary Microbiology* 60:2775–2779.
Kim TK., Garson MJ., Fuerst JA. 2005. Marine actinomycetes related to the "*Salinospora*" group from the Great Barrier Reef sponge *Pseudoceratina clavata*. *Environmental microbiology* 7:509–518.
Kinashi H. 2008. Antibiotic production, linear plasmids and linear chromosomes in *Streptomyces*. *Actinomycetologica* 22:20–29.
Knight V., Sanglier JJ., DiTullio D., Braccili S., Bonner P., Waters J., Hughes D., Zhang L. 2003. Diversifying microbial natural products for drug discovery. *Applied microbiology and biotechnology* 62:446–58.
Krassilnikov NA. 1941. Key to the Actinomycetales. In: *Cassification of ray fungi: Actinomycetales*. Moscow, Leningrad, Acd. Sci. USSR, 148.
Kuang W., Li J., Zhang S., Long L. 2015. Diversity and distribution of *Actinobacteria* associated with reef coral *Porites lutea*. *Frontiers in Microbiology* 6:1–13.
Kunst F., Ogasawara N., Moszer I., Albertini a M., Alloni G., Azevedo V., Bertero MG., Bessières P., Bolotin A., Borchert S., Borriss R., Boursier L., Brans A., Braun M., Brignell SC., Bron S., Brouillet S., Bruschi C V., Caldwell B., Capuano

V., Carter NM., Choi S-K., Codani J-J., Connerton IF., Cummings NJ., Daniel RA., Denizot F., Devine KM., Düsterhöft A., Ehrlich SD., Emmerson PT., Entian KD., Errington J., Fabret C., Ferrari E., Foulger D., Fritz C., Fujita M., Fujita Y., Fuma S., Galizzi A., Galleron N., Ghim S-Y., Glaser P., Goffeau A., Golightly EJ., Grandi G., Guiseppi G., Guy BJ., Haga K., Haiech J., Harwood CR., Hénaut A., Hilbert H., Holsappel S., Hosono S., Hullo M-F., Itaya M., Jones L., Joris B., Karamata D., Kasahara Y., Klaerr-Blanchard M., Klein C., Kobayashi Y., Koetter P., Koningstein G., Krogh S., Kumano M., Kurita K., Lapidus A., Lardinois S., Lauber J., Lazarevic V., Lee S-M., Levine A., Liu H., Masuda S., Mauël C., Médigue C., Medina N., Mellado RP., Mizuno M., Moestl D., Nakai S., Noback M., Noone D., O'Reilly M., Ogawa K., Ogiwara A., Oudega B., Park S-H., Parro V., Pohl TM., Portetelle D., Porwollik S., Prescott AM., Presecan E., Pujic P., Purnelle B., Rapoport G., Rey M., Reynolds S., Rieger M., Rivolta C., Rocha E., Roche B., Rose M., Sadaie Y., Sato T., Scanlan E., Schleich S., Schroeter R., Scoffone F., Sekiguchi J., Sekowska A., Seror SJ., Serror P., Shin B-S., Soldo B., Sorokin A., Tacconi E., Takagi T., Takahashi H., Takemaru K., Takeuchi M., Tamakoshi A., Tanaka T., Terpstra P., Tognoni A., Tosato V., Uchiyama S., Vandenbol M., Vannier F., Vassarotti A., Viari A., Wambutt R., Wedler E., Wedler H., Weitzenegger T., Winters P., Wipat A., Yamamoto H., Yamane K., Yasumoto K., Yata K., Yoshida K., Yoshikawa H-F., Zumstein E., Yoshikawa H., Danchin A. 1997. The complete genome sequence of the Gram-positive bacterium Bacillus subtillis. *Nature* 390:249–256.

Kurahashi M., Fukunaga Y., Sakiyama Y., Harayama S., Yokota A. 2010. *Euzebya tangerina* gen. nov., sp. nov., a deeply branching marine actinobacterium isolated from the sea cucumber *Holothuria edulis*, and proposal of *Euzebyaceae* fam. nov., *Euzebyales* ord. nov. and *Nitriliruptoridae* subclassis nov. *International journal of systematic and evolutionary microbiology* 60:2314–9.

Kurtböke Dİ., Grkovic T., Quinn RJ. 2007. Marine Actinomycetes in biodiscovery. In: *Marine Microbiology and Biotechnology*. 663–676.

Lacret R., Oves-Costales D., Gómez C., Díaz C., De La Cruz M., Pérez-Victoria I., Vicente F., Genilloud O., Reyes F. 2015. New ikarugamycin derivatives with antifungal and antibacterial properties from *Streptomyces zhaozhouensis*. *Marine Drugs* 13:128–140. DOI: 10.3390/md13010128.

Lagger C., Häussermann V., Försterra G., Tatián M. 2009. Ascidians from the southern Chilean Comau Fjord. *Spixiana* 32:173–186.

Lam KS. 2006. Discovery of novel metabolites from marine actinomycetes. *Current opinion in microbiology* 9:245–51.

Lam KS. 2007. New aspects of natural products in drug discovery. *Trends in Microbiology* 15:279–289.

Lee SD. 2006. *Phycicoccus jejuensis* gen. nov., sp., an actinomycete isolated from seaweed. *International Journal of Systematic and Evolutionary Microbiology* 56:2369–2373.

Lee DW., Lee JM., Seo JP., Schumann P., Kim SJ., Lee SD. 2008. *Phycicola gilvus* gen. nov., sp. nov., an actinobacterium isolated from living seaweed. *International journal of systematic and evolutionary microbiology* 58:1318–23.

Lee SH., Moon K., Kim H., Shin J., Oh DC., Oh KB. 2014. Bahamaolide A from the marine-derived *Streptomyces* sp. CNQ343 inhibits isocitrate lyase in *Candida albicans*. *Bioorganic and Medicinal Chemistry Letters* 24:4291–4293.

León J., Liza L., Soto I., Cuadra DL., Patiño L. 2007. Actinomycetes bioactivos de sedimento marino de la costa central del Perú. *Revista Peruana de Microbiologia* 14:259–270.

Li T-L., Huang F., Haydock SF., Mironenko T., Leadlay PF., Spencer JB. 2004. Biosynthetic gene cluster of the glycopeptide antibiotic teicoplanin: characterization of two glucosyltransferases and key acyltransferase. *Chemistry & Biology* 11:107–119.

Li MH., Ung PM., Zajkowski J., Garneau-Tsodikova S., Sherman DH. 2009. Automated genome mining for natural products. *BMC bioinformatics* 10:1.

Li H., Yu Y., Luo W., Zeng Y. 2010. *Marisediminicola antartica* gen. nov., sp. nov., an actinobacterium isolated from the Antarctic. *International Journal of Systematic Bacteriology* 60:2535–2539.

Li F., Jiang P., Zheng H., Wang S., Zhao G., Qin S., Liu Z. 2011. Draft genome sequence of the marine bacterium *Streptomyces griseoaurantiacus* M045, which produces novel manumycin-type antibiotics with a pABA core component. *Journal of Bacteriology* 193:3417–3418.

Liao ZL., Tang SK., Guo L., Zhang YQ., Tian XP., Jiang CL., Li WJ. 2009. *Verrucosispora lutea* sp. nov., isolated from a mangrove sediment sample. *International Journal of Systematic and Evolutionary Microbiology* 59:2269–2273.

Liu Z., Li Y., Zheng L., Huang Y., Li W. 2010. *Saccharomonospora marina* sp. nov., isolated from an ocean sediment of the East China Sea. *International journal of systematic and evolutionary microbiology* 60:1854–1857.

Livermore DM. 2009. Has the era of untreatable infections arrived? *The Journal of antimicrobial chemotherapy* 64:i29-36.

Locatelli FM., Goo K-S., Ulanova D. 2016. Effects of trace metal ions on secondary metabolism and the morphological development of streptomycetes. *Metallomics*.

Long RA., Azam F. 2001. Antagonistic Interactions among Marine Pelagic Bacteria. *Applied and Environmental Microbiology* 67:4975–4983.

Magarvey N., Keller J., Bernan V., Dworkin M., Sherman DH. 2004. Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. *Applied and environmental microbiology* 70:7520–9.

Magarvey NA., Haltli B., He M., Greenstein M., Hucul JA. 2006. Biosynthetic pathway for mannopeptimycins, lipoglycopeptide antibiotics active against drug-resistant gram-positive pathogens. *Antimicrobial Agents and Chemotherapy* 50:2167–2177.

Mahmoud HM., Kalendar AA. 2016. Coral-associated *Actinobacteria*: Diversity, abundance, and biotechnological potentials. *Frontiers in Microbiology* 7:1–13.

Maldonado L a., Fenical W., Jensen PR., Kauffman C a., Mincer TJ., Ward AC., Bull AT., Goodfellow M. 2005. *Salinispora arenicola* gen. nov., sp. nov. and *Salinispora tropica* sp. nov., obligate marine actinomycetes belonging to the family *Micromonosporaceae*. *International journal of systematic and evolutionary microbiology* 55:1759–66.

Maldonado L., Stach J., Ward A., Bull A., Goodfellow M. 2008. Characterisation of *Micromonosporae* from aquatic environments using molecular taxonomic methods. *Antonie van Leeuwenhoek* 94:289–98.

Manivasagan P., Venkatesan J., Sivakumar K., Kim S-K. 2013. Marine actinobacterial metabolites: Current status and future perspectives. *Microbiological Research* 168:311–332.

Marcone GL., Carrano L., Marinelli F., Beltrametti F. 2010. Protoplast preparation and reversion to the normal filamentous growth in antibiotic-producing uncommon actinomycetes. *The Journal of Antibiotics* 63:83–88.

Martin JF., Liras P. 2012. Cascades and networks of regulatory genes that control antibiotic biosynthesis. In: *Reprogrammin Microbial Metabolic Pathways*. 159–179.

Medema MH., Kottmann R., Yilmaz P., Cummings M., Biggins JB., Blin K., de Bruijn I., Chooi YH., Claesen J., Coates RC., Cruz-Morales P., Duddela S., Düsterhus S., Edwards DJ., Fewer DP., Garg N., Geiger C., Gomez-Escribano JP., Greule A., Hadjithomas M., Haines AS., Helfrich EJN., Hillwig ML., Ishida K., Jones AC., Jones CS., Jungmann K., Kegler C., Kim HU., Kötter P., Krug D., Masschelein J., Melnik A V., Mantovani SM., Monroe EA., Moore M., Moss N., Nützmann H-W., Pan G., Pati A., Petras D., Reen FJ., Rosconi F., Rui Z., Tian Z., Tobias NJ., Tsunematsu Y., Wiemann P., Wyckoff E., Yan X., Yim G., Yu F., Xie Y., Aigle B., Apel AK., Balibar CJ., Balskus EP., Barona-Gómez F., Bechthold A., Bode HB., Borriss R., Brady SF., Brakhage AA., Caffrey P., Cheng Y-Q., Clardy J., Cox RJ., De Mot R., Donadio S., Donia MS., van der Donk WA., Dorrestein PC., Doyle S., Driessen AJM., Ehling-Schulz M., Entian K-D., Fischbach MA., Gerwick L., Gerwick WH., Gross H., Gust B., Hertweck C., Höfte M., Jensen SE., Ju J., Katz L., Kaysser L., Klassen JL., Keller NP., Kormanec J., Kuipers OP., Kuzuyama T., Kyrpides NC., Kwon H-J., Lautru S., Lavigne R., Lee CY., Linquan B., Liu X., Liu W., Luzhetskyy A., Mahmud T., Mast Y., Méndez C., Metsä-Ketelä M., Micklefield J., Mitchell DA., Moore BS., Moreira LM., Müller R., Neilan BA., Nett M., Nielsen J., O'Gara F., Oikawa H., Osbourn A., Osburne MS., Ostash B., Payne SM., Pernodet J-L., Petricek M., Piel J., Ploux O., Raaijmakers JM., Salas JA., Schmitt EK., Scott B., Seipke RF., Shen B., Sherman DH., Sivonen K., Smanski MJ., Sosio M., Stegmann E., Süssmuth RD., Tahlan K., Thomas CM., Tang Y., Truman AW., Viaud M., Walton JD., Walsh CT., Weber T., van Wezel GP., Wilkinson B., Willey JM., Wohlleben W., Wright GD., Ziemert N., Zhang C., Zotchev SB., Breitling R., Takano E., Glöckner FO. 2015. The Minimum Information about a Biosynthetic Gene cluster (MIBIG) specification. *Nature chemical biology* 11:625–631.

Mincer TJ., Jensen PR., Kauffman C a., Fenical W. 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Applied and Environmental Microbiology* 68:5005–5011.

Montalvo NF., Mohamed NM., Enticknap JJ., Hill RT. 2005. Novel actinobacteria from marine sponges. *Antonie van Leeuwenhoek* 87:29–36.

Morlon H., O'Connor TK., Bryant JA., Charkoudian LK., Docherty KM., Jones E., Kembel SW., Green JL., Bohannan BJM. 2015. The biogeography of putative microbial antibiotic production. *PLoS ONE* 10:1–15.

Newman DJ., Cragg GM. 2016. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products* 75:311–335.

Nisbet LJ., Moore M. 1997. Will natural products remain an important source of drug research for the future? *Current opinion in biotechnology* 8:708–712.

Oh D., Gontang EA., Kauffman CA., Jensen PR., Fenical W. 2008. Salinipyrones and Pacificanones, mixed-precursor polyketides from the marine actinomycete *Salinispora pacifica*. *Jounal of Natural Products* 71:1–21.

Ohnishi Y., Ishikawa J., Hara H., Suzuki H., Ikenoya M., Ikeda H., Yamashita A., Hattori M., Horinouchi S. 2008. Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *Journal of Bacteriology* 190:4050–4060.

Olano C., Gómez C., Pérez M., Palomino M., Pineda-Lucena A., Carbajo RJ., Braña AF., Méndez C., Salas JA. 2009. Deciphering Biosynthesis of the RNA Polymerase Inhibitor Streptolydigin and Generation of Glycosylated Derivatives. *Chemistry and Biology* 16:1031–1044.

Opal SM., Mayer KH., Madeiros AA. 2000. Mechanisms of bacterial resistance. In: Mandell GL, Bennett JE, Dolin R eds. *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*. Churchill Livingstone,

Philadelphia, Pa, 236–258.
Palomo S., González I., de la Cruz M., Martín J., Tormo JR., Anderson M., Hill RT., Vicente F., Reyes F., Genilloud O. 2013. Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Marine drugs* 11:1071–86.
Pantoja S., Luis Iriarte J., Daneri G. 2011. Oceanography of the Chilean Patagonia. *Continental Shelf Research* 31:149–153.
Pathom-aree W., Nogi Y., Sutcliffe IC., Ward AC., Horikoshi K., Bull AT., Goodfellow M. 2006a. *Williamsia marianensis* sp. nov., a novel actinomycete isolated from the Mariana Trench. *International journal of systematic and evolutionary microbiology* 56:1123–1126.
Pathom-aree W., Nogi Y., Sutcliffe IC., Ward AC., Horikoshi K., Bull AT., Goodfellow M. 2006b. *Dermacoccus abyssi* sp. nov., a piezotolerant actinomycete isolated from the Mariana Trench. *International journal of systematic and evolutionary microbiology* 56:1233–7.
Pathom-Aree W., Stach JEM., Ward AC., Horikoshi K., Bull AT., Goodfellow M. 2006. Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. *Extremophiles : life under extreme conditions* 10:181–9.
Patin N V., Duncan KR., Dorrestein PC., Jensen PR. 2015. Competitive strategies differentiate closely related species of marine actinobacteria. *The ISME journal*:1–13.
Payne DJ., Gwynn MN., Holmes DJ., Pompliano DL. 2007. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nature Reviews. Drug Discovery* 6:29–40.
Peláez F. 2006. The historical delivery of antibiotics from microbial natural products - Can history repeat? *Biochemical Pharmacology* 71:981–990.
Penn K., Jenkins C., Nett M., Udwary DW., Gontang EA., McGlinchey RP., Foster B., Lapidus A., Podell S., Allen EE., Moore BS., Jensen PR. 2009. Genomic islands link secondary metabolism to functional adaptation in marine Actinobacteria. *The ISME journal* 3:1193–1203.
Penn K., Jensen PR. 2012. Comparative genomics reveals evidence of marine adaptation in *Salinispora* species. *BMC genomics* 13:86.
Peschke M., Brieke C., Cryle MJ. 2016. F-O-G Ring formation in glycopeptide antibiotic biosynthesis is catalysed by OxyE. *Scientific Reports* 6:35584.
Pesic A., Baumann H., Kleinschmidt K., Ensle P., Wiese J., Süssmuth R., Imhoff J. 2013. Champacyclin, a New Cyclic Octapeptide from *Streptomyces* Strain C42 Isolated from the Baltic Sea. *Marine Drugs* 11:4834–4857.
Pham TM., Wiese J., Wenzel-Storjohann A., Imhoff JF. 2016. Diversity and antimicrobial potential of bacterial isolates associated with the soft coral *Alcyonium digitatum* from the Baltic Sea. *Antonie van Leeuwenhoek,* 109:105–119.
Piel J., Hertweck C., Shipley PR., Hunt DM., Newman MS., Moore BS. 2000. Cloning, sequencing and analysis of the enterocin biosynthesis gene cluster from the marine isolate "*Streptomyces maritimus*": Evidence for the derailment of an aromatic polyketide synthase. *Chemistry and Biology* 7:943–955.
Pimentel-elardo SM., Scheuermayer M. 2009. *Streptomyces axinellae* sp. nov., isolated from the Mediterranean sponge *Axinella polypoides* (*Porifera*). *International journal of systematic and evolutionary microbiology* 59:1433–1437.
Qin Q-L., Li Y., Zhang Y-J., Zhou Z-M., Zhang W-X., Chen X-L., Zhang X-Y., Zhou B-C., Wang L., Zhang Y-Z. 2011. Comparative genomics reveals a deep-sea sediment-adapted life style of *Pseudoalteromonas* sp. SM9913. *The ISME journal* 5:274–284.
Recktenwald J., Shawky R., Puk O., Pfenning F., Keller U., Wohlleben W., Pelzer S. 2002. Nonribosomal biosynthesis of vancomycin-type antibiotics: A heptapeptide backbone and eight peptide synthetase modules. *Microbiology* 148:1105–1118.
Remoortere A Van., Ze JM Van., Oever V Den. 2010. MALDI Imaging and Profiling MS of Higher Mass Proteins from Tissue. *American Society of Mass Spectromtry* 21:1922–1929.
Revenga JE., Campbell LM., Arribére MA., Ribeiro Guevara S. 2012. Arsenic, cobalt and chromium food web biodilution in a Patagonia mountain lake. *Ecotoxicology and Environmental Safety* 81:1–10.
Reynolds PE. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 8:943–950.
Romero D., Traxler MF., López D., Kolter R. 2011. Antibiotics as Signal Molecules. *Chemical Reviews* 111:5492–5505.
Rosselló-Móra R., Amann R. 2015. Past and future species definitions for *Bacteria* and *Archaea*. *Systematic and applied microbiology*:1–8.
Ruiz B., Chávez A., Forero A., García-Huante Y., Romero A., Sánchez M., Rocha D., Sánchez B., Rodríguez-Sanoja R.,

Sánchez S., Langley E. 2010. Production of microbial secondary metabolites: regulation by the carbon source. *Critical reviews in microbiology* 36:146–67.

Salanoubat M., Genin S., Artiguenave F., Gouzy J., Mangenot S., Arlat M., Billault A., Brottier P., Camus JC., Cattolico L., Chandler M., Choisne N., Claudel-Renard C., Cunnac S., Demange N., Gaspin C., Lavie M., Moisan A., Robert C., Saurin W., Schiex T., Siguier P., Thébault P., Whalen M., Wincker P., Levy M., Weissenbach J., Boucher C a. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* 415:497–502.

Salomon CE., Magarvey N a., Sherman DH. 2004. Merging the potential of microbial genetics with biological and chemical diversity: an even brighter future for marine natural product drug discovery. *Natural product reports* 21:105–21.

Sánchez S., Chávez A., Forero A., García-Huante Y., Romero A., Sánchez M., Rocha D., Sánchez B., Avalos M., Guzmán-Trampe S., Rodríguez-Sanoja R., Langley E., Ruiz B. 2010. Carbon source regulation of antibiotic production. *Journal of Antibiotics* 63:442–59.

Santhanam R., Okoro CK., Rong X., Huang Y., Bull AT., Andrews B., Asenjo J., Weon HY., Goodfellow M. 2012a. *Streptomyces deserti* sp. nov., isolated from hyper-arid Atacama Desert soil. *Antonie van Leeuwenhoek* 101:575–81.

Santhanam R., Okoro CK., Rong X., Huang Y., Bull AT., Weon HY., Andrews B., Asenjo J., Goodfellow M. 2012b. *Streptomyces atacamensis* sp. nov., isolated from an extreme hyper-arid soil of the Atacama Desert, Chile. *International journal of systematic and evolutionary microbiology* 62:2680–4.

Santhanam R., Rong X., Huang Y., Andrews B., Asenjo J., Goodfellow M. 2013. *Streptomyces bullii* sp. nov., isolated from a hyper-arid Atacama Desert soil. *Antonie van Leeuwenhoek* 103:367–73.

Sarker SD., Nahar L., Kumarasamy Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods* 42:321–324.

Sarmiento-Vizcaíno A., González V., Braña AF., Palacios JJ., Otero L., Fernández J., Molina A., Kulik A., Vázquez F., Acuña JL., García LA., Blanco G. 2016. Pharmacological potential of phylogenetically diverse *Actinobacteria* isolated from deep-sea coral ecosystems of the submarine Avilés Canyon in the Cantabrian Sea. *Microbial Ecology*.

Sen A., Daubin V., Abrouk D., Gifford I., Berry AM., Normand P. 2014. Phylogeny of the class *Actinobacteria* revisited in the light of complete genomes. The orders *"Frankiales"* and *Micrococcales* should be split into coherent entities: proposal of *Frankiales* ord. nov., *Geodermatophilales* ord. nov., *Acidothermales* ord. nov. an. *International journal of systematic and evolutionary microbiology* 64:3821–32.

Seo YB., Kim D., Kim G., Kim H., Nam S., Kim YT., Lee JH. 2009. *Kocuria gwangalliensis* sp. nov., an actinobacterium isolated from seawater. *International journal of systematic and evolutionary microbiology* 59:2769–2772.

Sharma P., Kalita M., Thakur D. 2016. Broad spectrum antimicrobial activity of forest-derived soil actinimycete, *Nocardia* sp. PB-52. *Frontiers in Microbiology*, 7:347 1–11.

Shirling E., Gottlieb D. 1966. Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology* 16:313–340.

Silva N. 2006. Oxígeno disuelto, pH y nutrientes en canales y fiordos australes. In: *Avances en el conocimiento oceanográfico de las aguas interiores chilenas*. 37–43.

Skinnider MA., Dejong CA., Rees PN., Johnston CW., Li H., Webster ALH., Wyatt MA., Magarvey NA. 2015. Genomes to natural products PRediction Informatics for Secondary Metabolomes (PRISM). *Nucleic Acids Research* 43:9645–9662.

Sobarzo M. 2009. La Región de los fiordos de la zona sur de Chile. In: *Aspectos oceanográficos*. 6.

Spiekermann P., Rehm B.H., Kalscheuer R., Baumeister D., & Steinbüchel A. 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Archives of Microbiology*, *171*(2), 73-80.

Stachelhaus T., Mootz HD., Marahiel MA. 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chemistry & Biology* 6:493–505. Staunton J., Wilkinson B. 1997. Biosynthesis of Erythromycin and Rapamycin. 2665.

Stover CK., Pham XQ., Erwin AL., Mizoguchi SD., Warrener P., Hickey MJ., Brinkman FS., Hufnagle WO., Kowalik DJ., Lagrou M., Garber RL., Goltry L., Tolentino E., Westbrock-Wadman S., Yuan Y., Brody LL., Coulter SN., Folger KR., Kas A., Larbig K., Lim R., Smith K., Spencer D., Wong GK., Wu Z., Paulsen IT., Reizer J., Saier MH., Hancock RE., Lory S., Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959–964.

Studholme DJ. 2016. Genome Update. Let the consumer beware: *Streptomyces* genome sequence quality. *Microbial Biotechnology* 9:3–7.

Subramani R., Aalbersberg W. 2012. Marine actinomycetes: an ongoing source of novel bioactive metabolites.

Microbiological research 167:571–80.

Sun W., Zhang F., He L., Karthik L., Li Z. 2015. Actinomycetes from the South China Sea sponges: Isolation, diversity, and potential for aromatic polyketides discovery. *Frontiers in Microbiology* 6:1–15.

Svanbäck R., Bolnick DI., Svanback R., Bolnick DI., Svanbäck R., Bolnick DI. 2007. Intraspecific competition drives increased resource use diversity within a natural population. *Proceedings of the Royal Society B: Biological Sciences* 274:839–844.

Tang J., Liu X., Peng J., Tang Y., Zhang Y. 2015. Genome sequence and genome mining of a marine-derived antifungal bacterium *Streptomyces* sp. M10. *Applied Microbiology and Biotechnology* 99:2763–2772.

Tian X., Tang S., Dong J., Zhang Y., Xu L., Zhang S., Li W. 2009a. *Marinactinospora thermotolerans* gen. nov., sp. nov., a marine actinomycete isolated from a sediment in the northern South China Sea. *International journal of systematic and evolutionary microbiology* 59:948–952.

Tian XP., Zhi XY., Qiu YQ., Zhang YQ., Tang SK., Xu LH., Zhang S., Li WJ. 2009b. *Sciscionella marina* gen. nov., sp. nov., a marine actinomycete isolated from a sediment in the northern South China Sea. *International Journal of Systematic and Evolutionary Microbiology* 59:222–228.

Traxler MF., Watrous JD., Alexandrov T., Dorrestein PC., Kolter R. 2013. Interspecies interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome. *mBio* 4.

Udwary DW., Zeigler L., Asolkar RN., Singan V., Lapidus A., Fenical W., Jensen PR., Moore BS. 2007. Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. *Proceedings of the National Academy of Sciences of the United States of America* 104:10376–81.

Ue H., Matsuo Y., Kasai H., Yokota A. 2011. *Miniimonas arenae* gen. nov., sp. nov., an actinobacterium isolated from sea sand. *International Journal of Systematic and Evolutionary Microbiology* 61:123–127.

Ugalde J a., Gallardo MJ., Belmar C., Muñoz P., Ruiz-Tagle N., Ferrada-Fuentes S., Espinoza C., Allen EE., Gallardo V a. 2013. Microbial life in a fjord: metagenomic analysis of a microbial mat in Chilean patagonia. *PloS one* 8:e71952.

Valliappan K., Sun W., Li Z. 2014. Marine actinobacteria associated with marine organisms and their potentials in producing pharmaceutical natural products. *Applied Microbiology and Biotechnology* 98:7365–7377.

Ventura M., Canchaya C., Tauch A., Chandra G., Fitzgerald GF., Chater KF., van Sinderen D. 2007. Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. *Microbiology and molecular biology reviews : MMBR* 71:495–548.

Vergeiner S., Schafferer L., Haas H., Müller T. 2013. Improved MALDI-TOF microbial mass spectrometry Imaging by application of a dispersed solid matrix. *Journal of The American Society for Mass Spectrometry*.

Vézina C., Kudelski A., Sehgal SN. 1975. Rapamycin, a new antifungal antibiotic. *Journal of Antibiotics*:721–726.

Vicente J., Stewart A., Song B., Hill RT., Wright JL. 2013. Biodiversity of actinomycetes associated with Caribbean sponges and their potential for natural product discovery. *Marine biotechnology (New York, N.Y.)* 15:413–24.

Walsh CT. 2004. Polyketide and nonribosomal peptide antibiotics: modularity and versatility. *Science* 303:1805–1810.

Ward AC., Bora N. 2006. Diversity and biogeography of marine actinobacteria. *Current opinion in microbiology* 9:279–86.

Watrous JD., Dorrestein PC. 2011. Imaging mass spectrometry in microbiology. *Nature reviews. Microbiology* 9:683–694.

Weber T., Welzel K., Pelzer S., Vente A., Wohlleben W. 2003. Exploiting the genetic potential of polyketide producing streptomycetes. *Journal of Biotechnology* 106:221–232.

Weber T., Blin K., Duddela S., Krug D., Kim HU., Bruccoleri R., Lee SY., Fischbach MA., Müller R., Wohlleben W., Breitling R., Takano E., Medema MH. 2015. antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic acids research* 43:W237-43.

Wietz M., Duncan K., Patin NV., Jensen PR. 2013. Antagonistic interactions mediated by marine bacteria: the role of small molecules. *Journal of chemical ecology* 39:879–91.

Wright GD., Molinas C., Arthur M., Courvalin P., Walsh CT. 1992. Characterization of VanY, a DD-carboxypeptidase from vancomycin-resistant *Enterococcus faecium* BM4147. *Antimicrobial Agents and Chemotherapy* 36:1514–1518.

Wu Z., Xie L., Xia G., Zhang J., Nie Y., Hu J., Wang S., Zhang R. 2005. A new tetrodotoxin-producing actinomycete, *Nocardiopsis dassonvillei*, isolated from the ovaries of puffer fish *Fugu rubripes*. *Toxicon: official journal of the International Society on Toxinology* 45:851–9.

Yagüe P., López-García MT., Rioseras B., Sánchez J., Manteca Á. 2013. Pre-sporulation stages of *Streptomyces* differentiation: State-of-the-art and future perspectives. *FEMS Microbiology Letters* 342:79–88.

Yang JY., Phelan V V., Simkovsky R., Watrous JD., Trial RM., Fleming TC., Wenter R., Moore BS., Golden SS., Pogliano K., Dorrestein PC. 2012. Primer on Agar-Based Microbial Imaging Mass Spectrometry. *Journal of Bacteriology* 194:6023–6028.

Yi H., Schumann P., Sohn K., Chun J. 2004. *Serinicoccus marinus* gen. nov., sp. nov., a novel actinomycete with L -ornithine and L -serine in the peptidoglycan. *International journal of systematic and evolutionary microbiology* 54:1585–1589.

Yi H., Schumann P., Chun J. 2007. *Demequina aestuarii* gen. nov., sp . nov ., a novel actinomycete of the suborder *Micrococcineae*, and reclassification of *Cellulomonas fermentans* Bagnara *et al*. 1985 as *Actinotalea fermentans*. *International Journal of Systematic and Evolutionary Microbiology* 57:151–156.

Yim G., Thaker MN., Koteva K., Wright G. 2014. Glycopeptide antibiotic biosynthesis. *The Journal of Antibiotics* 67:31–41.

Yuan M., Yu Y., Li H-R., Dong N., Zhang X-H. 2014. Phylogenetic diversity and biological activity of *Actinobacteria* isolated from the Chukchi Shelf marine sediments in the Arctic Ocean. *Marine drugs* 12:1281–97.

Zazopoulos E., Huang K., Staffa A., Liu W., Bachmann BO., Nonaka K., Ahlert J., Thorson JS., Shen B., Farnet CM. 2003. A genomics-guided approach for discovering and expressing cryptic metabolic pathways. *Nature biotechnology* 21:187–190.

Zhang L., An R., Wang J., Sun N., Zhang S., Hu J., Kuai J. 2005. Exploring novel bioactive compounds from marine microbes. *Current opinion in microbiology* 8:276–81.

Zhang H., Lee YK., Zhang W., Lee HK. 2006. Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Antonie van Leeuwenhoek* 90:159–69.

Zotchev SB. 2012. Marine actinomycetes as an emerging resource for the drug development pipelines. *Journal of biotechnology* 158:168–75.

Zotchev SB. 2014. Genomics-based insights into the evolution of secondary metabolite biosynthesis in actinomycete bacteria. In: Pontarotti P ed. *Evolutionary Biology: Genome Evolution, Speciation, Coevolution and Origin of Life*. Cham: Springer International Publishing, 35–46.