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Genomics approaches to exploit the biotechnological potential of marine sponge-derived *Streptomyces* spp. isolates

Thesis presented by

**Eduardo Leao de Almeida**

for the degree of

**Doctor of Philosophy**

University College Cork

School of Microbiology

Head of School: Prof Paul O’Ttoole

Supervisor: Prof Alan D.W. Dobson

2019
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Declaration

I, Eduardo Leao de Almeida, hereby certify that this thesis and the work presented in it are my own, and have not been submitted for any other degree, either at University College Cork or elsewhere.

__________________________________

Eduardo Leao de Almeida
“Two roads diverged in a wood, and I—

I took the one less traveled by,

And that has made all the difference.”

Excerpt from “The Road Not Taken,” by Robert Frost
Acknowledgements

It has been a long road to reach to this point, although it felt more like a roller coaster at times. The PhD itself took four years and a few months; however, in fact, all the hard work, choices, struggles and achievements in the course of my academic career have led me here; alongside with a number of amazing people that supported me throughout this journey, making everything possible.

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Thanks to my sponsor, the Brazilian National Council for Scientific and Technological Development (CNPq), for the unique opportunity awarded to me, which enabled all this to happen. Thanks also to the excellent staff at University College Cork and the School of Microbiology for all the assistance provided to me during this time.

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Abstract

Members of the *Streptomyces* genus are widely known for their capability in producing compounds of pharmacological, clinical, and biotechnological interest, being the source of approximately a third of all the antibiotics that have been identified to date. However, the discovery of natural products with antimicrobial activities has declined following the so-called “Golden Age of Antibiotics” (1940s-1950s), particularly due to the common re-discovery of previously known compounds. Thus, natural products discovery research has shifted towards investigating diverse environmental niches, such as marine ecosystems, mangroves, and symbiotic communities of insects and sponges, resulting in the discovery of a variety of previously unidentified compounds of pharmacological interest; including those isolated from marine-derived *Streptomyces* species. However, in despite of their relevance as producers of potentially novel bio-active molecules with pharmacological, clinical and biotechnological interest, marine-derived *Streptomyces* isolates are still rather underexplored and under-characterized, particularly those found in association with marine sponges.

In the studies presented in this thesis, various state-of-the-art methodologies related to genome mining and bioinformatics-based pipelines, together with molecular and synthetic biology, were employed and proved to be extremely useful in helping to uncover the biotechnological potential of marine sponge-derived *Streptomyces* isolates. These studies essentially aimed at a) genetically characterizing marine sponge-derived *Streptomyces* spp. isolates and their potential to produce novel secondary metabolites, as shown in Chapter 2; b) to *in silico* identify, isolate, and quantify a secondary metabolite produced by a marine sponge-derived *Streptomyces* isolate, together with genetically characterizing its genome-encoded biosynthetic gene cluster (BGC), as reported in Chapter 3; and c) to perform an *in silico* screening of a novel polyesterase from a marine sponge-derived *Streptomyces*
isolate, followed by heterologous protein expression in an *E. coli* host, as demonstrated in Chapter 4.

In Chapter 2, two of the first complete genomes from marine sponge-derived *Streptomyces* spp. isolates were determined, namely from *Streptomyces* sp. SM17 and *Streptomyces* sp. SM18. The high-quality data provided in this study allowed for a reliable prediction of secondary metabolites biosynthetic gene clusters (BGCs) in their genomes, which determined that these isolates possess a variety of BGCs potentially encoding for the production of known compounds, and also potentially new molecules. Differential growth assessment determined that the marine isolates SM17 and SM18 grew and differentiated better in the presence of salts in the culture medium, when compared to their phylogenetically determined closely-related terrestrial relatives, namely *S. albidoflavus* J1074 (referred to as *S. albus* J1074 in Chapter 2) and *S. pratensis* ATCC 33331, respectively. Comparative genomics allowed for the identification of a proposed environmental niche adaptations (ENA) gene pool, which included genes related to osmotic stress defence, transcriptional regulation; symbiotic interactions; antimicrobial compound production and resistance; ABC transporters; together with horizontal gene transfer and defence-related features. These results shed new light on some of the genetic traits possessed by these marine sponge-derived isolates, and on how these might be linked to secondary metabolites production, and further highlighted their importance for the discovery of potentially novel natural products.

In Chapter 3, the previously unreported capability of the *Streptomyces* sp. SM17 to produce surugamides has been described. Surugamides are a family of compounds that have been previously reported to possess antitumor and antifungal activities. This was performed employing genome mining, which allowed for the identification of the surugamides BGC (*sur* BGC) in the SM17 genome, and analytical chemistry techniques for compound isolation and quantification.
Abstract

Phylogenomics analyses provided novel insights with respect to the distribution and conservation of the sur BGC at a genetic level, and provided evidence that the sur BGC might have had a marine origin. Additionally, when comparing the surugamide A production capabilities of a marine isolate (strain SM17) with a terrestrial relative (strain J1074) employing a “One Strain Many Compounds” (OSMAC)-based cultivation approach, the *Streptomyces* sp. SM17 isolate was shown to produce higher levels of surugamide A in all the conditions tested for. These findings may provide important insights towards a better characterisation, improved production and industrial development of this family of compounds.

In Chapter 4, the capability of marine sponge-derived *Streptomyces* spp. isolates to degrade synthetic polyesters was investigated. This was based on the fact that these microorganisms might have developed mechanisms to assimilate components of micro-plastics, which are now believed to be ubiquitous in marine ecosystems and pose as one of the top environmental problems that society faces today. Using 15 known PET hydrolases (PETases) as references, including the *Ideonella sakaiensis* 201-F6 PETase, *in silico* screening was performed to determine the presence of homologs to these reference PETase enzymes in 52 *Streptomyces* genome sequences (of which 29 were derived from marine ecosystems). The best candidate identified, namely the SM14est protein from the marine sponge-derived *Streptomyces* sp. SM14, was *in silico* characterised with respect to its amino acid sequence and predicted three dimensional structure, and was subsequently heterologously expressed in an *E. coli* host. This allowed for the confirmation of the polyesterase activity possessed by the SM14est enzyme, via a polycaprolactone (PCL) plate-clearing assay. Better characterising, identifying sources, and determining methods for improved protein expression are essential steps towards the development of biotechnological applications and industrial processes employing this family of enzymes, such as new plastic waste processing technologies.
Chapter 1

Introduction
1. Marine *Streptomyces* isolates as reservoirs of compounds of biotechnological and clinical interest

1.1. Historical perspective of natural products

The discovery and applications of natural products (NPs) have been important in the development of pharmacology and medicine, and for the development of human society over many numbers of years (Cragg and Newman, 2013; Dias et al., 2012). NPs are also referred to as “secondary metabolites”, or “specialised metabolites”, and this class of molecule generally encompasses compounds that are usually not required for growth, development, or reproduction of the producing organism; and are thus rather considered to be involved in mechanisms required for adaptation to the environment, communication (e.g. quorum sensing), and/or defence (Bibb, 2005; Cragg and Newman, 2013; Dias et al., 2012; Luckner, 2014). In contrast, “primary metabolites” are those involved in the biosynthesis and breakdown of molecules which are considered essential to all living organisms, such as nucleic acids, proteins, carbohydrates, and lipids (Dias et al., 2012; Hodgson, 2004; Luckner, 2014). Therefore, whereas primary metabolism is found to be present in all varieties of macro- and microorganisms, the ability to produce different types of secondary metabolites is believed to be much more limited, hence certain types of secondary metabolites are only found in certain organisms from particular environmental niches (Bibb, 2005; Cragg and Newman, 2013; Dias et al., 2012; Luckner, 2014).

Plants-derived NPs, in particular, have been historically exploited for numerous applications in medicine, with the earliest records dating from 2,600 B.C., in Mesopotamia; with the use of around 1,000 of these plant-derived compounds having been documented, including oils from plants that are used even to this day, e.g. *Cupressus sempervirens* and *Commiphora* species, in the treatment of
inflammation, coughs, and colds (Cragg and Newman, 2013; Dias et al., 2012). The usage of plant-based compounds was also extended to other ancient civilisations; which have also been documented, for example, in the Egyptian pharmaceutical record “Ebers Papyrus” which dates from 1,500 B.C.; the Chinese Materia Media from 1,100 B.C.; the records of medicinal herbs by the Greek physician Dioscorides from 100 A.D.; while monasteries in Europe together with the Arabs were responsible for preserving much of our knowledge about medicinal plants throughout the 5th to 12th centuries, in the Dark and Middle Ages (Cragg and Newman, 2013; Dias et al., 2012). Among important drugs that are derived from plant NPs, there is aspirin (acetylsalicylic acid) (Figure 1A), which is derived from the salicin – a secondary metabolite isolated from Salix alba L. (willow tree) (Dias et al., 2012; Newman et al., 2000; Schröer, 2016). Morphine (Figure 1B) is also a classic example of a plant-derived NPs of commercial and clinical relevance, which was originally isolated from Papaver somniferum L. (opium poppy) (Dias et al., 2012; Newman et al., 2000).

Other than plant-derived NPs, important sources of bio-active compounds also include fungi, bacteria, and, more recently, marine organisms such as sponges and algae (Carroll et al., 2019; Cragg and Newman, 2013; Dias et al., 2012; Newman and Cragg, 2016). With respect to fungal-derived NPs, there is penicillin (Figure 1C), isolated from Penicillium notatum, which has an importance for modern medicine that cannot be overestimated and which has undoubtedly saved countless lives (Dias et al., 2012; Fleming, 1929, 1944; Gaynes, 2017; Newman et al., 2000). Among clinically relevant bacterial-derived NPs, there are the glycopeptide antibiotic vancomycin (Figure 1D) and the macrolide antibiotic erythromycin (Figure 1E) (Haight and Finland, 1952; McCormick et al., 1955). The former, which was isolated from Amycolatopsis orientalis, possesses bio-activity against Gram-positive and Gram-negative bacteria and also fungi; while the latter, which was isolated from
Saccharopolyspora erythraea, is commonly used to treat respiratory tract infections, and possesses bio-activity against Gram-positive bacilli and cocci (Dias et al., 2012). More recently, secondary metabolites derived from marine organisms have been reported to be particularly effective as anticancer agents (Carroll et al., 2019; Dias et al., 2012). The secondary metabolite plitidepsin (Figure 1F), for example, was isolated from the tunicate Aplidium albicans and has been shown to be effective in the treatment of various types of cancers (Bravo et al., 2005; Carroll et al., 2019; Dias et al., 2012; Mateos et al., 2005).

**Figure 1:** Chemical structure of the natural products A) acetylsalicylic acid (aspirin); B) morphine; C) penicillin G; D) vancomycin; E) erythromycin; F) plitidepsin. Structures obtained from https://www.drugbank.ca/ (Wishart et al., 2018).
1.2. The “Golden Age of Antibiotics” and Streptomyces-derived bio-active compounds

The discovery of perhaps the first and most important NP derived from a microorganism, namely the aforementioned penicillin from the fungus Penicillium notatum, which was discovered by Fleming in 1929 (Fleming, 1929, 1944; Gaynes, 2017), initiated a new era in modern medicine, in the so-called “Golden Age of Antibiotics” (1940s-1950s) (Figure 2) (Fernandes, 2006; Lewis, 2012; Mohr, 2016). This prompted the investigation of various microorganisms, in particular bacteria belonging to the Actinomycetales order, as potential sources of what later became extremely valuable bio-active secondary metabolites for clinical applications and for the pharmaceutical industry; such as those with antimicrobial, immunosuppressive, and anticancer activities (Carroll et al., 2019; Challinor and Bode, 2015; Lewis, 2012).

**Figure 2**: Timeline representing the discovery of antibiotics molecules, with a clear decrease in the discovery of new families of compounds after the 1960s (Lewis, 2012).

Among these bacteria which were shown to possess the capacity to produce bio-active compounds of clinical, commercial and pharmacological interest, members of the Streptomyces genus – which at that point had been isolated mainly from soil – were determined to be highly prolific producers of antibiotics, being the
source of up to two thirds of all known antibiotics to date (Mohr, 2016; Watve et al., 2001). The *Streptomyces* genus (Actinomycetales order) consists of Gram-positive, filamentous, aerobic, and mycelial bacteria, with a complex development, morphological differentiation, and multicellular behaviour (Figure 3) (Hoskisson and van Wezel, 2019; Law et al., 2018; McCormick and Flärdh, 2012). *Streptomyces* species possess long linear chromosomes (6-10 Mbp) with high G+C content, usually above 70%; which is considerably higher than *Bacillus* species, for example, which commonly possess around a 40% G+C content (Hoskisson and van Wezel, 2019; Lee et al., 2019; Muto and Osawa, 1987). Members of the *Streptomyces* genus are well-known for their exceptional capabilities of producing secondary metabolites with various bio-activities, particularly those with antimicrobial activity (Clardy et al., 2006; Watve et al., 2001). The first couple of antibiotics to be isolated from *Streptomyces* species were streptothricin, in 1942 (Waksman and Woodruff, 1942), and streptomycin, in 1944 (Schatz et al., 1944); the latter of which is considered to be responsible for triggering a more systematic screening approach for the discovery of novel bio-active compounds derived from this genus (Clardy et al., 2006; Watve et al., 2001). It has been estimated that around 3,000 antibiotics molecules have to date been identified that are produced by the Actinomycetales order, of which 90% are derived from *Streptomyces* species (Clardy et al., 2006; Watve et al., 2001). Additionally, in a conservative estimate, 150,000 compounds have been proposed to be produced by *Streptomyces* species; thus, only a very small fraction – around 1-3% – of the full potential number of compounds that may be produced by *Streptomyces* have been identified to date (Clardy et al., 2006; Watve et al., 2001).
Figure 3: Morphology of *Streptomyces coelicolor* (Hoskisson and van Wezel, 2019).

Among clinically and commercially relevant compounds derived from *Streptomyces* species, others include the antibiotics chloramphenicol, which was isolated from *Streptomyces venezuelae* (Bartz, 1948; Ehrlich et al., 1948); daptomycin, isolated from *Streptomyces roseosporus* (Raja et al., 2003); fosfomycin, isolated from *Streptomyces fradiae* (Hendlin et al., 1969); lincomycin, isolated from *Streptomyces lincolnensis* (Schaffer et al., 1963); neomycin, isolated from *Streptomyces fradiae* (Waksman and Lechevalier, 1949); the aforementioned streptomycin, isolated from *Streptomyces griseus* (Schatz et al., 1944); and tetracycline, isolated from *Streptomyces rimosus* and *Streptomyces aureofaciens* (Putnam et al., 1953). Other clinically relevant compounds that are also produced by *Streptomyces* species, include the anticancer compounds doxorubicin (Arcamone et al., 1969) and daunorubicin (Dubost et al., 1964), which were isolated from *Streptomyces peucetius*, and also streptozotocin, isolated from *Streptomyces achromogenes* (Vavra et al., 1959).
1.3. The importance of the discovery of new antibiotics

Although, as previously mentioned, numerous secondary metabolites were identified and ultimately commercialised in the course of the so-called “Golden Age of Antibiotics”, a steady decrease in the number of novel compounds identified was observed throughout subsequent years, mostly due to the re-discovery of previously known molecules (Fernandes, 2006; Lewis, 2012). This phenomenon led to a reluctance by the pharmaceutical industry, to invest in research aimed at the discovery of new bio-active NPs; and even to the belief that apparently all the antibiotics molecules may have been discovered (Fernandes, 2006). The latter, of course, has been proven not to be the case, particularly since 1) only a very small fraction of the microbial world has to date been explored since it has been estimated that only 1–2% of environmental bacteria are culturable (Nesme et al., 2016; Torsvik et al., 1990; Wade, 2002), an issue which could be addressed with the development of new culture-independent technologies, such as metagenomics (Chen et al., 2019); and 2) it has been estimated that the *Streptomyces* genus alone could potentially be able to produce tens of thousands of compounds that have not yet been discovered (Watve et al., 2001).

However, new and important challenges have recently arisen particularly in a clinical context, with respect to the treatment of bacterial infections. In the past few decades, an increase in bacterial resistance to broad spectrum antibiotics has been reported (Pendleton et al., 2013; Sirijan and Nitaya, 2016; Tommasi et al., 2015), which has consequently increased the necessity of finding novel antimicrobial compounds, and in particular those that possesses alternative mechanisms of bacterial growth inhibition; as one of the top priorities in contemporary scientific research. In 2009, it has been estimated that multidrug-resistant (MDR) bacterial infections caused 25,000 extra-deaths per year in Europe, while a more recent study estimated that MDR bacteria-related infections could lead to a number as high as
10 million deaths per year by 2050 (Abat et al., 2018; O’Neill, 2014). These MDR bacterial isolates are commonly responsible for life-threatening nosocomial infections, with a particular concern surrounding those belonging to a group of microorganisms which have been described as the ESKAPE pathogens – an acronym that refers to Gram-positive and Gram-negative bacterial species that include Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp. (Pendleton et al., 2013; Sirijan and Nitaya, 2016; Tommasi et al., 2015).

1.4. Exploring diverse environmental niches in the search for new bio-active molecules

The aforementioned decline in the discovery of novel bio-active compounds together with the increased need to find novel molecules – particularly those with antimicrobial activity due to the emergence of multi-drug resistant bacteria – has then led to a shift in efforts aimed at the discovery of novel NPs (Pendleton et al., 2013; Tommasi et al., 2015). Concerted efforts started to focus on the investigation of microorganisms derived from diverse environmental niches, which had previously been neglected when compared to soil isolates; such as those isolated from marine ecosystems, mangroves, and in particular those found in association with terrestrial and marine invertebrates, such as insects and marine sponges (Carroll et al., 2019; Chevrette et al., 2019; Dias et al., 2012; Hassan et al., 2017; Kemung et al., 2018; Manivasagan et al., 2014). These led to the successful identification and isolation of completely novel small molecules with antibacterial, antifungal, and anticancer activities; including those with inhibitory activity against multi-drug resistant ESKAPE pathogens (Figure 4) (Andryukov et al., 2019; Liu et al., 2019b; Schinke et al., 2017). From marine-derived Bacillus species, for example, the compounds bogorol A and loloatin B have been reported, which possess inhibitory activity against methicillin-resistant Staphylococcus aureus.
Chapter 1

(MRSA) and vancomycin-resistant enterococcal strains (VRE) (Barsby et al., 2001; Gerard et al., 1996). The compounds stachyin B, stachybacin A, stachybacin B, and ilicicolin – all of which were isolated from the marine fungus *Stachybotrys* sp. MF347 – have also been reported to have inhibitory activity against MRSA (Liu et al., 2019b; Wu et al., 2014). Hence, it became clear that microorganisms derived from marine ecosystems might provide a valuable resource in the treatment of multi-drug resistant infections.

![Graphical representation of 167 marine-derived natural products active against drug-resistant bacteria. A) Phylum-wise distribution. B) Time-wise distribution (adapted from Liu et al., 2019).](image)

Among marine-derived sources of novel bio-active small molecules, marine sponges have been reported to host several groups of microorganisms such as fungi and bacteria which are capable of producing quite unique secondary metabolites with a range of bio-activities, including some with antimicrobial and anticancer properties (Carroll et al., 2019). To date, sponge bacteria-derived antimicrobial compounds have been identified from 35 different genera, with *Streptomyces* being the most predominant genus; being the source of approximately 30% of the compounds identified to date (Indraningrat et al., 2016). Examples of compounds which have recently been isolated from marine sponge-
associated *Streptomyces* isolates (Table 1) that have been reported to inhibit some
of the ESKAPE pathogens include mayamycin, which has activity against
*Staphylococcus aureus*, and streptophenazines G and K, which have activity
against *Staphylococcus epidermis* and *Bacillus subtilis* (Table 1). Both of these
compounds are produced by *Streptomyces* sp. HB202 isolated from the sponge
*Halichondria panicea* (Table 1) (Schneemann et al., 2010); together with the
compound 2-pyrrolidone, which has activity against *Staphylococcus aureus*,
*Escherichia coli*, and *Klebsiella pneumoniae*, and was found to be produced by
*Streptomyces* sp. MAPS15 isolated from *Spongia officinalis* (Table 1)
(Sathiyanarayanan et al., 2014). Additionally, bio-activity screening followed by
genetic characterisation studies performed with 13 *Streptomyces* spp. isolates from
both shallow water and deep-sea sponges have reported antimicrobial activities
against several clinically relevant bacterial and yeast species, including MRSA
(Jackson et al., 2018; Kennedy et al., 2009). These isolates included *Streptomyces* sp.
SM17, which possesses inhibitory activity against *Escherichia coli* NCIMB 12210,
MRSA, and *Candida* species; and *Streptomyces* sp. SM18, which possesses
inhibitory activity against MRSA and *Bacillus subtilis* 1A40; bio-activities which
were determined using deferred antagonism assays (Jackson et al., 2018; Kennedy
et al., 2009). Although further investigation is required in order to isolate specific
bio-active compounds and ultimately commercialise them as novel pharmaceutical
drugs, it is clear that marine sponge-derived *Streptomyces* isolates possess the
potential to produce novel bio-active secondary metabolites with clinical and
biotechnological relevance; and these aforementioned recent studies provide clear
evidence that investigating bacteria such as *Streptomyces* species from these
marine-based environmental niches is a promising field of research.
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<th>Organism</th>
<th>Compound</th>
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<td><em>Spongia officinalis</em></td>
<td><em>Streptomyces</em> sp. MAPS15</td>
<td>2-pyrrolidone</td>
<td><em>Staphylococcus aureus</em> PC6; <em>Escherichia coli</em> PC1; <em>Klebsiella pneumoniae</em> PC7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MRSA; <em>Staphylococcus epidermidis</em>, <em>Brevibacterium epidermidis</em>, <em>Dermabacter hominis</em>, <em>Propionibacterium acnes</em>, <em>Xanthomonas campestris</em></td>
</tr>
<tr>
<td><em>Halichondria panicea</em></td>
<td><em>Streptomyces</em> sp. HB202</td>
<td>Mayamycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>Streptophenazine G</em></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td><em>Streptophenazine K</em></td>
</tr>
<tr>
<td><em>Haliclona simulans</em></td>
<td><em>Streptomyces</em> sp. SM8</td>
<td>Mixture Kitamycin A or B and Antimycin A3 or A7</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antimycin A2, A8, A11 or A17 Antimycin A3 or A7</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Antimycin A2, A8, A11 or A17 Antimycin A3 or A7</td>
<td></td>
</tr>
<tr>
<td><em>Dysidea tupha</em></td>
<td><em>Streptomyces</em> sp. RV15</td>
<td>Naphthacene glycoside SF2446A2</td>
<td><em>Chlamydia trachomatis</em></td>
</tr>
</tbody>
</table>

**Table 1:** Compounds with antimicrobial activity isolated from marine sponge-derived *Streptomyces* spp. (adapted from Indraningrat et al., 2016).

It has been proposed that environmental niche adaptations possessed by these marine sponge-derived *Streptomyces* species may play a role, at least in part, in their ability to produce a diverse range of secondary metabolites (Almeida et al., 2019; Bibb, 2005), as the abiotic conditions faced by these microorganisms such as differences in pressure, salinity, pH, light, temperature and oligotrophic conditions...
are likely to be linked to the biosynthesis of NPs (Abdelmohsen et al., 2014; van der Meij et al., 2017). In addition to these abiotic stresses, symbiotic relationships may also be particularly relevant for the production of secondary metabolites in these marine sponge-derived *Streptomyces* isolates (Chevrette et al., 2019; Kwan et al., 2014). It has been reported, for example, that the production of antimicrobial compounds was promoted when the sponge-associated *Streptomyces* ACT-52A was exposed to the sponge *Aplysilla rosea* (Mehbub et al., 2016). However, the mechanisms that regulate the production of secondary metabolites in marine sponge-associated *Streptomyces* species is likely to be quite complex and to date remains for the most part unresolved. Thus, it is of crucial importance to better characterise these microorganisms morphologically, phenotypically and genetically.
2. Strategies to identify novel compounds and enzymes from marine *Streptomyces* isolates

2.1. The genetics basis for the production of secondary metabolites in bacterial species

The biosynthesis of secondary metabolites is a complex process that, at a genetic level, is performed by a physical clustering of two or more genes that together encode for the enzymes involved in the biosynthetic pathways required for the production of these secondary metabolites (Chen et al., 2019; Medema et al., 2015). These group of genes are commonly referred to as secondary metabolite biosynthetic gene clusters (BGCs). In addition to genes encoding enzymes that are required for the production and assembly of secondary metabolites, BGCs also contain regulatory, resistance, and transport-related genes (Chen et al., 2019).

The main families of compounds produced by BGCs consist of polyketides, nonribosomal peptides, terpenoids, ribosomally synthesised and post-translationally modified peptides (RiPPs), saccharides, and alkaloids (Medema et al., 2015). Of these, the polyketides and nonribosomal peptides are of major research interest as they commonly comprise of natural products with antibacterial, antifungal, and immunosuppressant activities (Chen et al., 2019). Polyketides are synthesised by polyketide synthases (PKSs), while nonribosomal peptide synthases (NRPSs) are responsible for synthesising nonribosomal peptides, and these BGCs often possess complex structure and distinct mechanisms of biosynthesis.

To date, three main types of bacterial PKSs have been described (Figure 5) (Robbins et al., 2016; Shen, 2003). Type I PKSs are modular, non-iterative, multifunctional enzymes, for which each module is responsible for a different elongation step of the polyketide chain (Figure 5a) (Shen, 2003). The type I PKS 6-deoxyerythronolide B synthase (DEBS), for example, is involved in the synthesis of
the macrolide antibiotic erythromycin (Khosla et al., 2007; Staunton and Wilkinson, 1997; Weber et al., 1991). Type II PKSs, on the other hand, are complexes of multiple small, discrete enzymes with iterative activities and particular functions (Figure 5b) (Shen, 2003; Yu et al., 2012). The anthracycline antibiotic tetracenomycin C is an example of a compound that is produced by a type II PKS (Hutchinson, 1997).

Finally, type III PKSs comprise of self-contained homodimeric enzymes that perform iterative reactions to assemble polyketide products (Figure 5c) (Shen, 2003; Yu et al., 2012). In bacterial species, it has been reported that type III PKSs produce compounds of biological relevance and also provide precursors for the biosynthesis of other secondary metabolites (Katsuyama and Ohnishi, 2012; Shimizu et al., 2017). For example, the type III PKS SrsA from S. griseus is involved in the biosynthesis of alkyquinone, which has been reported to confer penicillin resistance to this microorganism (Funabashi et al., 2008; Katsuyama and Ohnishi, 2012). Additionally, the type III PKS PhlD from Pseudomonas is involved in the biosynthesis of 2,4-diacetylphloroglucinol, which has been reported to possess biocontrol activity against soil borne fungal plant pathogens (Bangera and Thomashow, 1999; Katsuyama and Ohnishi, 2012). Besides the clear differences which exist between the different types of PKSs, all of them possess a ketoacyl synthase (KS) in the form of a domain(s), for type I PKSs, or as a subunit, for type II and III PKSs (Figure 5) (Shen, 2003).
Figure 5: Examples of (a) type I, (b) type II, and (c) type III PKS structures and mechanisms of biosynthesis. AT: acyl transferase; ACP: acyl carrier protein; KS: ketoacyl synthase; KR: ketoreductase; DH: dehydratase (adapted from Shen, 2003).
In contrast, NRPSs are a family of mega-enzymes that contain multiple domains (adenylation, peptidyl carrier protein, condensation, and thioesterase domains) that form modules (initiation, elongation and termination modules); functioning together to incorporate amino acids into the peptide chain (Figure 6) (Miller and Gulick, 2016; Reimer et al., 2018). Adenylation (A) domains are responsible for adding the substrate to the nascent peptide chain and usually consist of approximately 500 amino acid residues; condensation (C) domains catalyse the bond formation between two substrates and usually consist of approximately 450 residues; peptidyl carrier protein (PCP) domains are the smallest NRPS domains and usually consist of 70–90 amino acid residues, and are responsible for the transfer of substrate and peptide intermediates between different domains; and finally the thioesterase (Te) domains, which usually consist of 230–270 amino acid residues, are responsible for releasing the peptide and freeing the NRPS enzyme (Miller and Gulick, 2016). Initiation modules commonly consist of A and PCP domains; while elongation modules encompass C domains together with A and PCP domains; and termination modules possess the Te domain. Hence, A–PCP-(C–A–PCP)$_n$–Te is considered to be the canonical organisation of a basic NRPS (Figure 6) (Reimer et al., 2018). Examples of NRPS-encoded secondary metabolites of clinical and biotechnological relevance include the antibiotics gramicidin A, daptomycin, vancomycin and bacitracin A; the immunosuppressant cyclosporin A; and the compound surfactin, which can be employed for the remediation of oil-contaminated soils (Felnagle et al., 2008; Martínez-Núñez and López, 2016; Reimer et al., 2018).
Figure 6: Canonical domain and module organisation of a generic NRPS enzyme. Although represented in the figure, the tailoring domain that formylates (F) the N-terminal amino acid is not present in all NRPSs (adapted from Reimer et al., 2018).

2.2. Genome mining and the state-of-the-art of bioinformatics-assisted compounds discovery

Traditionally, in the pre-omics era, the discovery of natural products were typically performed based on culture-dependent techniques and bio-activity screening of plants, fungi and bacteria, coupled with chemistry-based methods such as high-performance liquid chromatography (HPLC); mass spectrometry, and nuclear magnetic resonance (NMR) for the isolation and characterisation of compounds (Chen et al., 2019; Lee et al., 2019; Ziemert et al., 2016). For a number of reasons, the use of these approaches alone proved to be relatively limited after some time, which resulted at least in part to a decline in the number of novel compounds identified following the so-called “Golden Age of Antibiotics” (Fernandes, 2006; Lewis, 2012). Technical aspects such as costs and the time required for the isolation of compounds; the common rediscovery of previously known compounds; together with the fact that culturing environmental microorganisms in the laboratory can be very challenging, since most bacteria are considered to be “unculturable”; resulted in helping to limit the discovery of novel
bio-active compounds when employing strictly in vitro-based approaches (Chen et al., 2019).

However, with advances in DNA sequencing, genomics, bioinformatics, analytical chemistry and synthetic biology, together with the first complete genome sequence of a Streptomyces isolate, namely Streptomyces coelicolor, in 2002 (Bentley et al., 2002), a new revolution in the discovery of novel bio-active compounds began, employing the concept of “genome mining” (Ziemert et al., 2016). Essentially, genome mining consists on the prediction and isolation of natural products based on genetic information, even without a chemical structure being available (Ziemert et al., 2016). As more genetic information regarding secondary metabolite biosynthetic pathways and their regulation began to be elucidated, it became increasingly apparent that even previously well-studied Streptomyces isolates could potentially produce different and quite novel compounds; other than those that had previously been identified and characterised using chemical-based methods (Ziemert et al., 2016). Since then, our knowledge with respect to the genetic basis for the production of secondary metabolites – with the discovery and characterisation of BGCs – has vastly improved (Ziemert et al., 2016). This has led to the development of a number of in silico tools and bioinformatics-based pipelines, together with synthetic biology and high-throughput biochemical methods, which has helped to optimise the discovery of potentially novel and unique compounds; many of which would have been virtually impossible to uncover if not for these new approaches (Figure 7) (Chen et al., 2019; Lee et al., 2019; Ziemert et al., 2016).
Figure 7: The evolution of genome mining throughout the omics era (adapted from Ziemert et al., 2016).

Classical “genome mining”, which is based on the search for genes encoding enzymes such as PKSs or NRPSs, which are known to be involved in the biosynthesis of secondary metabolites, is among the most broadly used genomics-based approach currently employed in small molecule/natural products discovery. In its basic form, classical genome mining utilises sequence- or profile-based comparison tools, such as BLAST (Altschul et al., 1990; Camacho et al., 2009) and HMMer (Zhang and Wood, 2003), respectively; while more advanced, specialised, and automated tools have also been developed, including the antiSMASH, BAGEL, and the ClusterFinder programs (Blin et al., 2019; Cimermancic et al., 2014; de Jong et al., 2006). These more sophisticated tools are also employed for comparative genome mining, which is a method that employs the analysis and comparison of complete genomes or genomic regions, in a search for partial or complete BGCs, instead of single genes and enzymes. Among these tools, antiSMASH is one of the most prominently employed programs for the prediction of BGCs in the genomes.
of bacterial isolates (Blin et al., 2017, 2019). This program allows for the comparison
of genome sequences with other known BGC-encoding sequences present in other
microorganisms and in the Minimum Information about a Biosynthetic Gene
cluster (MIBiG) curated database (Epstein et al., 2018; Medema et al., 2015). This
allows for the identification of known and also potentially new BGCs, and is
particularly useful for *Streptomyces* species. In fact, around a third of all the
curated BGCs available in the MIBiG database to date belong to *Streptomyces*
species, which further highlights the importance of the *Streptomyces* genus in the
discovery of secondary metabolites. Comparative genome mining methods can be
further strengthened when coupled with other *in silico* pipelines such as sequence
similarity network analysis, for example by using programs such as the BiG-
SCAPE (Navarro-Muñoz et al., 2018), which clusters predicted BGCs by
antiSMASH into gene cluster families according to sequence similarity. Such an
approach has proven to be very useful in assessing the novelty of potential BGCs
and in prioritising isolates and compounds for further investigation (Blin et al.,
2017; Ziemert et al., 2016).

Additional interesting and innovative modern methods of genome mining include
phylogeny-based mining, which can be performed using tools such as the EvoMining
program (Sélem-Mojica et al., 2018); and resistance gene-based mining, which can be
performed using the Antibiotic Resistant Target Seeker (ARTS) tool (Alanjary et al., 2017).
Additionally, updated information regarding the latest bioinformatics tools for the
discovery of secondary metabolites can be found in The Secondary Metabolite

The aforementioned bioinformatics-based approaches have proven to be
extremely useful in the discovery and activation of the so-called “silent” or
“cryptic” BGCs, i.e. those predicted BGCs that are apparently not being expressed
under standard laboratory culture conditions. One such example is *Streptomyces*
albidoflavus J1074, where the use of chemical stress elicitors, i.e. the cytotoxins etoposide and ivermectin, promoted the production of compounds previously unknown to be produced by this strain, namely surugamides molecules and their derivatives, which were determined to be encoded by a BGC which until that point was considered to be transcriptionally silent in this isolate (Xu et al., 2017).

Omics-based approaches have also allowed the investigation of the potential for the production of secondary metabolites by uncultured organisms present in different microbiomes, through genome and metagenome sequencing (Blin et al., 2017; Chen et al., 2019; Cragg and Newman, 2013; Lee et al., 2019; Ziemert et al., 2016). For example, in a recent study, functional screening of a human microbiome metagenomic library has led to the identification of a BGC encoding the bacteriocin colicin V with inhibitory activity against E. coli, which may be involved in the ecology of human-associated bacteria, and could also possess a therapeutic role (Cohen et al., 2018).

2.3. The “One Strain Many Compounds” (OSMAC) approach combined with genome mining

The “One Strain Many Compounds” (OSMAC) approach, which was proposed and conceptualised by Zeeck and collaborators, is based on more classical approaches employing chemical, physical and culture-dependent methods in the discovery of novel secondary metabolites (Bode et al., 2002). Essentially, this method is based on observations that the production of secondary metabolites can be promoted through exposing the producing strains to stressful culture conditions; thus systematic changes in simple cultivation parameters, such as the media composition, culture aeration, and the type of culture vessel, may promote the production of an increased number of compounds from a single microorganism; with this approach being particularly useful for Streptomyces species (Bibb, 2005;
Bode et al., 2002; Chiang et al., 2011; Goodfellow and Fiedler, 2010; Pan et al., 2019; Romano et al., 2018). The OSMAC approach is a method that is relatively simple to implement but which can result very useful outcomes, particularly with respect to the activation of the previously mentioned “silent” BGCs, and hence promoting the production of compounds which were formerly not produced in some microbial isolates – even those well-described strains (Chiang et al., 2011; Pan et al., 2019; Romano et al., 2018; Xu et al., 2017).

Typical parameters that can be systematically manipulated in OSMAC-based experiments include: 1) changes in the nutrients available in the culture medium, such as changes in carbon, nitrogen, sulphur, and phosphorus sources, and trace elements; 2) changes in physical parameters, such as temperature, salinity, pH, aeration and shaking conditions, and culture vessel types; 3) applying chemical elicitors to the culture medium; and 4) the use of co-cultivation regimes, in an attempt to simulate biotic interactions found in nature, such as co-culturing microbial isolates with pathogenic bacteria, or with eukaryotic hosts, in the case of symbiotic isolates (Figure 8) (Bode et al., 2002; Chiang et al., 2011; Pan et al., 2019; Romano et al., 2018).
Figure 8: Basic implementation of the OSMAC principle, combined with omics approaches. A, B, C, and D represent the employment of different nutrient regimes, physical parameters, co-cultivation, and chemical elicitors, respectively (adapted from Romano et al., 2018).

The OSMAC approach employing different culture media has proven to be particularly useful in investigating novel secondary metabolites produced by marine-derived Streptomyces species (Goodfellow and Fiedler, 2010). For example, a significant variation in the metabolic profile of Streptomyces sp. YB104, which was isolated from deep-sea sediment, was observed when the isolate was grown in different complex media, which led to the identification of a novel bio-active compound, namely inthomycin B, which belongs to a family of compounds with antimicrobial, herbicidal and anticancer activities (Wu et al., 2018). Also, considerable variations in the levels of the antibiotic SBR-22 being produced by the marine sediment isolate Streptomyces psammoticus BT-408, which possesses inhibitory activity against MRSA, has been reported when different carbon sources were present in the culture media (Sujatha et al., 2005). Additionally, levels of
oxygen in the culture have been reported to influence the secondary metabolites
production profile in the marine-derived *Streptomyces* sp. CNQ-525, for which
hypoxic conditions shifted the production of the antibiotic napyradiomycin
towards its intermediate 8-aminoflavioin, a compound which may potentially
function as an endogenous extracellular electron shuttle (Gallagher et al., 2017).

Ultimately, the OSMAC principle can further strengthen genome mining-
based natural products discovery, and vice-versa, since it may provide novel
insights in metabolic pathways and regulatory mechanisms which can be linked to
the genetic information which is available regarding the producing strain. At the
same time, prior information on the BGC present in the producing strain –
provided by genome mining approaches – can serve as a “blueprint” describing the
types of compounds that may potentially be produced by the subject
microorganism, and hence provide insights into which culture conditions that may
induce production of certain compounds.

2.4. The discovery and characterisation of the surugamides family of compounds

The recently discovered surugamides family of compounds is a very good
example of novel secondary metabolites of clinical relevance, whose discovery
involved the application of chemistry-based and culture-dependent methods,
Together with genomics-based and state-of-the-art synthetic biology approaches
(Matsuda et al., 2019b; Ninomiya et al., 2016; Takada et al., 2013; Xu et al., 2017).
Surugamides A–E are cyclic octapeptides which have been reported to possess
cathepsin B inhibitory activity – an anticancer target – and that were originally
identified, isolated and characterised from the marine-derived *Streptomyces* sp.
JAMM992 via culture-dependent methods, coupled with liquid chromatography–
mass spectrometry (LC-MS) analyses (Figure 9) (Takada et al., 2013). Subsequent
research (Ninomiya et al., 2016), which involved genome sequencing and genome
mining for BGCs using antiSMASH (Blin et al., 2019), together with genetic 
manipulation generating knockout strains, revealed not only the main biosynthetic 
genes involved in the biosynthesis of surugamides (namely the NRPS genes 
surABCD) (Figure 10), but also allowed for the identification of the new compound 
surugamide F, a linear decapeptide. In fact, this study determined an 
unprecedented organisation of a BGC, in which the genes surAD were involved in 
the production of surugamides A–E, while the surBC genes were involved in the 
production of surugamide F – findings which were only possible due to the 
combination of in vitro and in silico methodologies.

Figure 9: Surugamides chemical structures, where 2–5 indicate the 
surugamides B–E, respectively. X = H, Y = Me, p = position (Matsuda et 
al., 2019b).
Figure 10: The sur BGC and the biosynthesis of surugamides, where 1 indicates the products surugamides A–E, encoded by the surAD genes; 6 the cyclosurugamide F, and 7 the surugamide F, both which are encoded by the surBC genes (Matsuda et al., 2019b).

Interestingly, similar BGCs to the previously described surugamides BGC (sur BGC) were also identified in other Streptomyces isolates, including the well-described Streptomyces albidoflavus J1074 (Xu et al., 2017), however, this BGC was considered to be “silent” in this strain. Genomics analyses, coupled with high-throughput screening employing chemical stress elicitors, allowed for the induction of production of surugamides by the isolate, and also for the identification of completely novel compounds also produced by the sur BGC, such as the surugamide I, which also possesses cathepsin B inhibitory activity, and acyl-surugamide A, which possesses antifungal activity. This study also employed molecular biology and synthetic biology methodologies that allowed for a better characterisation of metabolic pathways and genes involved in the production of surugamides, identifying the transcriptional repressor surR which was reported to play an important role in the regulation of production of surugamides.
More recent studies which further investigated the biosynthesis of surugamides also applied chemistry-based approaches coupled with genomics and synthetic biology in order to better characterise genes, enzymes, and metabolic pathways involved in the production of these molecules. These studies revealed, for example, another gene which is also involved in the tailoring of these compounds, namely \textit{surE}, which encodes a thioesterase involved in the cyclisation of the cyclic surugamides molecules (Kuranaga et al., 2018; Matsuda et al., 2019b, 2019a; Thankachan et al., 2019; Zhou et al., 2019).

Taken together, these studies highlight the power of genomics-based approaches coupled with culture-dependent, chemistry and synthetic biology methods, employed to identify completely novel molecules of clinical interest, such as the surugamides, even when applied to well characterised isolates, such as \textit{Streptomyces albidoflavus} J1074.
Chapter 1

3. PETases and the enzymatic hydrolysis of synthetic polyesters

3.1. Plastic pollution in terrestrial and marine environments

Synthetic polyesters, or plastics, such as polyethylene terephthalate (PET), are widely used in our society due to their advantageous physico-chemical properties such as their bio-inertia, together with their low production cost. These characteristics are particularly useful for the packaging industry, a sector that contributes to around 40% of the plastics demand in Europe (Figure 11) (Lebreton and Andrady, 2019; PlasticsEurope, 2018). However, as a consequence of these advantageous properties, many synthetic polyesters are highly recalcitrant to biodegradation, and in turn became an enormous problem not only to the environment but to our society as a whole (Wei and Zimmermann, 2017a).

Figure 11: Plastics converter demand in Europe in 2017 (adapted from PlasticsEurope, 2018).
It has been estimated that, in 2017 alone, worldwide plastics production was around 348 million tonnes; and that at the current rate, it is estimated that plastics production could double in the next 20 years (Lebreton and Andrady, 2019; PlasticsEurope, 2018). Although increased efforts in improving plastic waste management have helped to decrease the problem of plastic pollution, there is still a considerable amount of mismanaged plastic waste that ends up in the environment. In 2015 alone, it has been estimated that 60-90 million metric tonnes of mismanaged plastic waste were produced globally, and that this amount could triple by 2060 (Lebreton and Andrady, 2019).

Plastic pollution becomes particularly concerning when it reaches the marine environment, for which it has been estimated that, in 2010 alone, 4.8-12.7 metric tonnes of plastic waste was disposed of in the oceans. Given that these levels only considered coastal countries, this value could in fact be much larger (Jambeck et al., 2015). Following recent research, additional issues must be taken into account when considering plastic pollution in the oceans. It has been reported that, for example, plastic debris undergoes fragmentation into micro-plastics (< 5mm), due to weathering effects such as sunlight, and these are believed to be ubiquitous in many soil and aquatic environments (Geyer et al., 2017; Lebreton and Andrady, 2019). These micro-plastics have been reported to be commonly ingested by animals, which are believed to transfer pollutants along the food chain, including to seafood species consumed by humans (Santillo et al., 2017).

Mechanical recycling, which involves collection, sorting, washing and grinding, is currently the most commonly applied method for plastic waste recycling (Ragaert et al., 2017). A huge challenge for mechanical recycling is, however, the presence of impurities in the post-consumer plastic waste (Drzyzga and Prieto, 2019). Alternatively, chemical recycling has also been applied to improve plastic waste management processes, with the advantage that the plastic
polymers can also be converted into raw materials for the synthesis of chemicals, fuels, and virgin plastics (Drzyzga and Prieto, 2019). However, these chemical processes can be very costly and usually require the use of high temperatures and toxic chemicals (Awaja and Pavel, 2005; Wei and Zimmermann, 2017b).

3.2. PET hydrolases sources, characterisation and mechanisms

More recently, a potential alternative for a more efficient and environment-friendly approach for the recycling of post-consumer plastic waste has been proposed, namely the enzymatic hydrolysis of plastics (Drzyzga and Prieto, 2019; Wei and Zimmermann, 2017a, 2017b). A few bacterial-derived enzymes with the capacity to degrade synthetic polyesters, including PET, have been identified in the past decade (Kawai et al., 2019; Wei and Zimmermann, 2017a). These were mainly identified in thermophilic actinomycetes, particularly in the genus Thermobifida, and the enzymes involved commonly comprise of members of the cutinases, lipases, and esterases families (Silva et al., 2011; Wei and Zimmermann, 2017a). A ground-breaking discovery in the field occurred when, in 2016, Yoshida and co-workers isolated a bacterium, namely Ideonella sakaiensis 201-F6, which was capable of degrading and assimilating PET as its major energy and carbon source, that was isolated from a bottle recycling plant in Sakai, Japan (Yoshida et al., 2016). The enzyme responsible for the hydrolysis of PET, namely ISF6_4831, was classified as a PETase (or PET hydrolase, EC 3.1.1.101), and it has been reported to possess higher enzymatic activity and substrate specificity for PET than any other previously described PET hydrolase, being able to degrade PET even at moderate temperatures of around 30°C (Joo et al., 2018; Yoshida et al., 2016). These findings prompted research groups worldwide to further characterise this class of enzymes, together with the metabolic and biochemical processes and pathways involved in the degradation of PET and other synthetic polyesters (Chen et al., 2018; Han et al., 2017; Joo et al., 2018; Liu et al., 2018). It has been proposed that PETases act by
catalysing the hydrolytic cleavage of PET, resulting in the production of smaller monomeric molecules of ethylene glycol (EG), terephthalate (TPA), mono(2-hydroxyethyl) terephthalate (MHET), and bis(2-hydroxyethyl) terephthalate (BHET) (Figure 12) (Chen et al., 2018; Han et al., 2017; Kawai et al., 2019).

Figure 12: General mechanisms of PET hydrolysis by PETase (Kawai et al., 2019).

The crystal structure of the *Ideonella sakaiensis* 201-F6 PETase ISF6_4831 (IsPETase) has determined (Figure 13) (Austin et al., 2018; Han et al., 2017; Joo et al., 2018; Liu et al., 2018, 2019a). These studies were aimed at elucidating and gaining further insights into the structural characteristics that conferred the efficiency and affinity that was observed with respect to the degradation of PET by the IsPETase. The enzyme was determined to belong to the α/β hydrolase superfamily (Hotelier...
et al., 2004; Nardini and Dijkstra, 1999; Ollis et al., 1992), with a conserved serine hydrolase motif Gly-x1-Ser-x2-Gly (Gly158-Trp159-Ser160-Met161-Gly162), and to be arranged in 9 β-sheets and 7 α-helices (Figure 13). By analysing the three-dimensional structure of this enzyme together with employing molecular docking experiments and site-directed mutagenesis assays, some features that could explain the superior hydrolysis of PET by the IsPETase have been proposed (Joo et al., 2018). Firstly, it has been proposed that the IsPETase possesses two sub-sites that facilitate a superior binding to the PET moieties when compared to others PET hydrolases from other microorganisms. Sub-site I (consisting of Tyr87, Met161, Trp185, Ile208), and which seems to be conserved in other PET hydrolases, has been suggested to bind to the first PET MHET moiety, and to be linked to stabilisation of the ligand. On the other hand, the sub-site II (consisting of Thr88, Ala89, Trp159, Ile232, Asn233, Ser236, Ser238, Asn241, Asn244, Ser245, Asn246, Arg280) has been proposed to be involved in accommodating the three MHET moieties of PET, and seems to be a more uncommon structural feature possessed by the IsPETase, therefore being partially responsible for the superior enzymatic activity. Secondly, the IsPETase possesses an extended loop connecting β8 and α6, which has been proposed to provide a conformation that allows improved accommodation of the substrate. Finally, an extra disulfide bond (between Cys203 and Cys239) in the proximity of the catalytic triad (Ser160, Asp206, His237) is present in the IsPETase (Figure 13), which could also be related to a superior catalytic activity of the enzyme when compared to other PETase enzymes (Joo et al., 2018).
Figure 13: Three-dimensional structure of the IsPETase, highlighting the two disulfide bonds; the catalytic triad (Ser160, Asp206, His237); and a simulated 2-HE(MHET)$_4$ molecule mimicking the structure of PET as the binding substrate at the active site (adapted from Joo et al., 2018).

Although also consisting of members of the Actinomycetales order, very little is currently known about the potential of *Streptomyces* species to degrade synthetic polyesters, unlike the previously mentioned *Thermobifida* genus. Some research efforts have previously been employed to investigate the ability of some soil-derived isolates to degrade synthetic polyesters; for example, *Streptomyces* sp. strain MG (Calabia and Tokiwa, 2004; Tokiwa and Calabia, 2004) and *Streptomyces thermoviolaceus* (Chua et al., 2013) have been reported to being able to degrade polycaprolactone (PCL); and *Streptomyces bangladeshensis* 77T-4 which has been shown to degrade poly(D-3-hydroxybutyrate) (PHB) (Hsu et al., 2012). Given the fact that marine-derived *Streptomyces* species, which are well-adapted to diverse environments and nutrient sources – particularly those found in association with marine sponges – are likely to be exposed to micro-plastics which are ubiquitous in the oceans, and hence may have developed mechanisms to degrade and assimilate...
these synthetic polymer molecules; therefore, it is perhaps reasonable to presume that enzymes with synthetic polyesters-degrading activity may be present in these isolates. Thus, further investigation of marine sponge-derived *Streptomyces* isolates, employing genome mining together with culture-dependent screening assays and synthetic biology approaches, may prove useful in providing new insights into marine-derived PETase enzymes and how widespread these enzymes may be in nature. These PETase enzymes could be extremely useful in the development of alternative and more environment-friendly processes of plastic waste recycling and/or bioremediation, thus, helping to alleviate at least partially the alarming issue of plastic pollution in terrestrial and aquatic environments.
Chapter 1

4. References


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Chapter 1


Clusters in the Genomes of Marine Sponge Derived *Streptomyces* spp. Isolates. 


Chapter 2

Comparative genomics of marine sponge-derived *Streptomyces* spp. isolates SM17 and SM18 with their closest terrestrial relatives provides novel insights into environmental niche adaptations and secondary metabolite biosynthesis potential

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1. **Abstract**

The emergence of antibiotic resistant microorganisms has led to an increased need for the discovery and development of novel antimicrobial compounds. Frequent rediscovery of the same natural products (NPs) continues to decrease the likelihood of the discovery of new compounds from soil bacteria. Thus, efforts have shifted toward investigating microorganisms and their secondary metabolite biosynthesis potential, from diverse niche environments, such as those isolated from marine sponges. Here we investigated at the genomic level two *Streptomyces* spp. strains, namely SM17 and SM18, isolated from the marine sponge *Haliclona simulans*, with previously reported antimicrobial activity against clinically relevant pathogens; using single molecule real-time (SMRT) sequencing. We performed a series of comparative genomic analyses on SM17 and SM18 with their closest terrestrial relatives, namely *S. albus* J1074 and *S. pratensis* ATCC 33331 respectively; in an effort to provide further insights into potential environmental niche adaptations (ENAs) of marine sponge-associated *Streptomyces*, and on how these adaptations might be linked to their secondary metabolite biosynthesis potential. Prediction of secondary metabolite biosynthetic gene clusters (smBGCs) indicated that, even though the marine isolates are closely related to their terrestrial counterparts at a genomic level; they potentially produce different compounds. SM17 and SM18 displayed a better ability to grow in high salinity medium when compared to their terrestrial counterparts, and further analysis of their genomes indicated that they possess a pool of 29 potential ENA genes that are absent in *S. albus* J1074 and *S. pratensis* ATCC 33331. This ENA gene pool included functional categories of genes that are likely to be related to niche adaptations and which could be grouped based on potential biological functions such as osmotic stress defense; transcriptional regulation; symbiotic interactions; antimicrobial compound production and resistance; ABC transporters; together with horizontal gene transfer and defense-related features.
2. **Introduction**

With the emergence and rapid spread of antibiotic resistant microorganisms, displaying resistance to many currently available antibiotics, a concerted effort continues to be needed to discover novel antimicrobial agents (Thabit et al., 2015; Rolain et al., 2016). Members of the *Streptomyces* genus are also known to produce a broad range of other natural products (NPs) which possess immunosuppressant, anti-fungal, anti-cancer, anti-parasitic and anti-thrombotic activities (Hwang et al., 2014; Ser et al., 2017). However, the frequent re-discovery of previously characterized bioactive compounds from terrestrial *Streptomyces*, has somewhat limited the interest of researchers in terrestrial ecosystems as potential reservoirs for novel biomolecules (Yagüe et al., 2012; Dalisay et al., 2013; Paulus et al., 2017). Instead, interest has begun to focus on the isolation of *Streptomyces* from other environmental niches; with *Streptomyces* involved in symbiotic relationships or associated with plants, insects, fungi, lichens, sea-cucumbers, seaweeds and marine sponges also attracting increased attention as potential reservoirs for these types of bioactive molecules (Motohashi et al., 2010; Seipke et al., 2012; van der Meij et al., 2017). The ability of these *Streptomyces* to colonize such a wide variety of hosts is due in part to their ability to produce useful NPs, such as antimicrobials which help their hosts defend themselves against predators or pathogenic bacteria and fungi (Adnani et al., 2017; van der Meij et al., 2017).

Marine ecosystems are attracting particular attention, where extreme and rapidly changing environmental conditions such as differences in pressure, salinity, pH, light intensity, temperature and oligotrophic conditions are believed to be linked to secondary metabolites production (Abdelmohsen et al., 2014; van der Meij et al., 2017). In this respect, marine ecosystems have been a particularly fruitful source of *Streptomyces* strains which have the potential to produce new bioactive NPs (Hassan et al., 2017; Jin et al., 2018; Xu et al., 2018), with marine
**Streptomyces** being isolated from seashores, coastal waters, bottom sediments, fishes, molluscs, sponges, seaweeds and mangroves (Manivasagan et al., 2014; Ser et al., 2017).

Marine sponges (phylum *Porifera*) in particular are known to be a rich source of bioactive compounds, many of which are produced by the bacteria which reside within the sponge host (Abdelmohsen et al., 2014; Fuerst, 2014). Many of these bioactives have antimicrobial activities, making these sponge-associated microbial and fungal communities a potentially valuable source of novel antimicrobials (Baker et al., 2009; Flemer et al., 2012; Hoppers et al., 2015; Indraningrat et al., 2016; Jackson et al., 2018). While sponge bacteria-derived antimicrobial compounds have to date been identified from 35 different genera, the most predominant producing genera include *Streptomyces*, *Pseudovibrio* and *Bacillus* strains (Indraningrat et al., 2016). Of these, *Streptomyces* are the predominant genus, producing around 30% of the compounds identified to date (Indraningrat et al., 2016). Good examples of bioactive compounds produced from *Streptomyces* associated with marine sponges include: mayamycin, produced by *Streptomyces* sp. HB202 isolated from *Halichondria panicea* (Schneemann et al., 2010); the naphthacene glycoside SF2446A2, produced by *Streptomyces* sp. RV15 isolated from *Dysidea tupha* (Reimer et al., 2015); and Petrocidin A, produced by *Streptomyces* sp. SBT348 isolated from *Petrosia ficiformis* (Cheng et al., 2017).

As previously mentioned, in addition to marine sponges, many *Streptomyces* strains have also evolved symbiotic relationships with plants, fungi, and insects, amongst others; and there is increasing evidence that the host may control which metabolic pathways are activated within their symbionts, such as in the tunicate *Lissoclinum patella* and the squid *Euprymna scolopes* (Kwan et al., 2014; Gromek et al., 2016). In *Streptomyces* spp., it is clear that not only do they benefit from the resources of the hosts they interact with, but that these interactions
control the expression of secondary metabolite biosynthetic gene clusters (smBGCs); thereby promoting the high degree of chemical diversity observed in the secondary metabolites being produced by these organisms (van der Meij et al., 2017). An example is the recent report that exposure of the endosymbiont *Streptomyces ACT-52A* to *Aplysilla rosea* promoted production of bioactive compounds with antibacterial activity (Mehbub et al., 2016). The factors involved in controlling the expression of these smBGCs are likely to be quite diverse, given the large degree of variability in the habitats and potential hosts, and how they are presumably influencing the secondary metabolite biosynthetic potential of *Streptomyces* symbionts (Adnani et al., 2017). Thus, it is clear that an increased knowledge of the genetics underpinning the interactions and signaling between the sponge host and the symbiont is required, through identification of smBGCs in the genomes of these sponge associated *Streptomyces* strains, coupled with identification of potential environmental “triggers” from the sponge, from other sponge endosymbionts, and/or from the surrounding marine environment that may regulate transcription of these smBGCs (Mehbub et al., 2016; Adnani et al., 2017; van der Meij et al., 2017).

To this end, we recently sequenced the genomes of 13 *Streptomyces* spp. isolated from both shallow water and deep-sea sponges, that displayed antimicrobial activities against a number of clinically relevant bacterial and yeast species (Kennedy et al., 2009; Jackson et al., 2018). Using the antiSMASH (antibiotics and Secondary Metabolite Analysis Shell) software (Blin et al., 2017), the strains were found to host abundant smBGCs which potentially encode polyketides, non-ribosomal peptide synthases (NRPS), siderophores, lantipeptides, and bacteriocins (Jackson et al., 2018). Thus, these strains appear to be a promising source of novel bioactive secondary metabolites, as the abundance and diversity of smBGCs displayed high degrees of novelty. In addition, the strains were enriched for genes potentially involved in the biosynthesis and transport of compatible solutes and for heat-shock proteins, genes
which are typically associated with marine adaptations (Penn and Jensen, 2012; Tian et al., 2016).

Around sixty marine adaptation genes (MAGs) have previously been proposed for the obligate marine actinomycete genus *Salinispora*, with the function of these genes being associated with electron transport, sodium and ABC transporters, together with channels and pores (Penn and Jensen, 2012). Even though sponge-associated *Streptomyces* are marine bacteria, the environmental niche occupied by these organisms differs quite markedly from *Salinispora*, thus the genetic adaptions may not necessarily be similar. This was confirmed by the Zotchev group, when the draft genome of two sponge associated *Streptomyces* strains were analyzed for MAGs, revealing the presence of only seven of the *Salinispora* MAG gene pool (Ian et al., 2014). They suggested that specific marine sponge genetic adaptations may exist, given that different genes were identified in these sponge-associated *Streptomyces* which were absent in their soil counterparts (Ian et al., 2014). However, drawing conclusions for these genetic adaptations is quite difficult due to the limited number of sponge-associated *Streptomyces* genomes that are currently available. To this end, we sequenced the genomes of *Streptomyces* strains SM17 and SM18, two of the aforementioned 13 sponge-derived *Streptomyces* spp. that had displayed antimicrobial activity, using the PacBio RSII Single Molecule, Real-Time (SMRT) sequencing platform. This allowed us to study not only the smBGCs that these bacteria possess, but also other genetic characteristics that may be involved in their life cycle; such as for example adaptation to the marine environment and symbiosis. By employing comparative genomics, we compared the genomes of these strains with their most closely related terrestrial type-strain relatives, with complete genomes available in the GenBank database (namely *S. albus* J1074 for SM17 and *S. pratensis* ATCC 33331 for SM18), in an attempt to identify genes potentially associated with ENA, together with genes encoding potentially novel smBGCs.
3. **Material and methods**

3.1. **Bacterial strains, maintenance and differential growth assessment**

The SM17 and SM18 strains were isolated from the marine sponge *Haliclona simulans* (Kilkieran Bay, Galway, Ireland), as previously described (Kennedy et al., 2009). The *S. albus* J1074 strain was provided by Dr. Andriy Luzhetskyy (Helmholtz Institute for Pharmaceutical Research Saarland, Germany), while *S. flavogriseus/S. pratensis* ATCC 33331 was obtained from the American Type Culture Collection (ATCC Inc., United States). SM17, SM18, *S. albus* J1074 and *S. flavogriseus/S. pratensis* ATCC 33331 spores were propagated on mannitol-soya (MS) agar medium at 28°C for 8–10 days and stored in 20% glycerol at −80°C. Strains were cultivated on ISP2 and ISP2 plus artificial sea water (ASW) medium when indicated, for differential growth analysis. The ASW was obtained by adding 3% Instant Ocean® Sea Salt (Instant Ocean Inc., United States) to the medium. It is important to note that the ATCC 33331 strain, due to a more recent taxonomy classification (Rong et al., 2013), is described with two different names: in GenBank as *S. pratensis* ATCC 33331 (new classification), and in the ATCC® culture collection as *S. flavogriseus* ATCC 33331 (old classification). From now on, the ATCC 33331 isolate will be referred to as *S. pratensis* ATCC 33331.

3.2. **Genome sequencing, assembly and annotation**

Biomass from the SM17 and SM18 strains was obtained after cultivation on TSB medium for 3 days at 28°C and 220 rpm. Genomic DNA from SM17 was isolated using the DNeasy Blood & Cell Culture DNA Midi Kit (Qiagen Inc.); and by using the phenol-chloroform-isoamyl alcohol extraction method for SM18 (Wilson, 2001). Genome sequencing was performed by Macrogen (Seoul, South Korea), using the PacBio RSII sequencing platform.
The PacBio raw reads were processed and quality filtered using the BamTools toolkit v2.4.1 (subread length >1000, subread quality >0.75) (Barnett et al., 2011). The genome assemblies were performed using the Canu v1.7 software (Koren et al., 2017), followed by assembly polishing using Quiver v2.1.0 (Pacific Biosciences Inc). The assembly coverage check was performed using the BBMap program v37.90 (available at https://sourceforge.net/projects/bbmap/). Genome assembly statistics were calculated using the QUAST v4.6.3 program (Gurevich et al., 2013). Genome annotation was performed using the Prokka v1.12 program for this study’s analyses (Seemann, 2014), and with the NCBI Prokaryotic Genome Annotation Pipeline for data submission on the GenBank database (Tatusova et al., 2016; Benson et al., 2018). Prediction of smBGCs was performed using the antiSMASH 4 software (Blin et al., 2017). Similarity clustering of smBGCs families was performed using the Biosynthetic Genes Similarity Clustering and Prospecting Engine (BiG-SCAPE, version 2018100) (Navarro-Muñoz et al., 2018) and Cytoscape (v3.7.1) (Shannon et al., 2003), with annotations based on the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository (v1.4) (Medema et al., 2015). Genome maps were generated using the Artemis v17.0.1 and the DNAPlotter v17.0.1 programs (Rutherford et al., 2000; Carver et al., 2009). Proteins of interest were manually annotated using the NCBI BLAST tool; the GenBank database; and the Conserved Domain Database (CDD) (Johnson et al., 2008; Marchler-Bauer et al., 2015; Benson et al., 2018).

3.3. Comparative genomics

The closest reference strains for the sponge-derived isolates SM17 and SM18 were determined by employing a phylogenetic analysis performed in two steps: (1) based on the 16S rRNA sequence of the SM17 and SM18 isolates, we picked the top 30 most similar Streptomyces species to each of the isolates (for a total of 60 genomes from the database), with complete genome available in GenBank (Benson
et al., 2018), using the NCBI BLAST tool (Johnson et al., 2008) (2) we then
performed a phylogenetic analysis employing concatenated sequences (Gadagkar
et al., 2005) of the 16S rRNA and the housekeeping genes *atpD* (ATP synthase
subunit beta), *gyrB* (DNA gyrase subunit B), *recA* (recombinase RecA), *rpoB*
(DNA-directed RNA polymerase subunit beta), and *trpB* (tryptophan synthase beta
chain), of the SM17 and SM18 strains, plus the previously determined top 60 most
similar *Streptomyces* species. Alignment of the concatenated sequences was
performed using the MAFFT program (Katoh and Standley, 2013), and phylogeny
was determined using the MrBayes program (Ronquist et al., 2012), applying the
General Time Reversible (GTR) model of nucleotide substitution with gamma-
distributed rates across sites with a proportion of invariable sites (Waddell and
Steel, 1997), and an average standard deviation of split frequencies cut off of 0.01.
The final condensed tree, with a posterior probability cut off of 95%, was generated
using MEGA X (Kumar et al., 2018) and Inkscape (available at https://inkscape.org).

To further support genomic similarities between the SM17 and SM18 strain and
their closest type-strain terrestrial relative determined with the phylogeny analysis,
alignments of the individual housekeeping genes were performed and sequence
similarity was determined, using the NCBI BLAST tool (Johnson et al., 2008); and
whole genome nucleotide alignments were performed using the MUMmer 3.0
program (Kurtz et al., 2004). Plasmids sequences were determined by similarity
searches in the GenBank database (Benson et al., 2018). Orthologous gene analysis
was performed using the Roary v3.12.0 program, with an identity cut-off set to 50%
(Page et al., 2015). The Roary outputs were processed using the R software
environment in the RStudio IDE (Racine, 2012; RStudio Team, 2015; R Core Team,
2018), with data frame handling using the plyr package (Wickham, 2011); and Venn
diagrams generated using the venn package (Dusa, 2018).
3.4. Accession numbers

The complete genome sequences of SM17, SM18, and the SM17 plasmid sequences pSM17A, pSM17B, pSM17C, have been deposited in GenBank under the accession numbers CP029338, CP029342, CP029339, CP029340, and CP029341, respectively. The closest reference genomes used in this study for comparative purposes were *S. albus* J1074 (accession no. CP004370.1) and *S. pratensis* ATCC 33331 (accession no. CP002475.1).
4. Results and discussion

4.1. Genome sequencing and assembly

The genomes of the marine sponge-derived Streptomyces spp. isolates SM17 and SM18 were sequenced using the PacBio RSII sequencing platform, which generated a total of 140,538 and 87,756 subreads respectively, after adapter removal and quality/length filtering (Table 1A). The PacBio sequencing provided long read lengths, averaging 9,702 and 8,923 bp for SM17 and SM18, respectively. Combining the large number of reads and their long length, an approximate sequencing coverage of 194× and 101× was obtained for SM17 and SM18, respectively.

<table>
<thead>
<tr>
<th></th>
<th>SM17</th>
<th>SM18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome size (bp)</td>
<td>7,179,914*</td>
<td>7,703,166</td>
</tr>
<tr>
<td>Number of subreads</td>
<td>140,538</td>
<td>87,756</td>
</tr>
<tr>
<td>Average subread length (bp)</td>
<td>9,702</td>
<td>8,923</td>
</tr>
<tr>
<td>Approximate average sequencing coverage (fold)</td>
<td>194</td>
<td>101</td>
</tr>
<tr>
<td>GC content (%)</td>
<td>73.35</td>
<td>71.84</td>
</tr>
<tr>
<td>Number of contigs</td>
<td>4**</td>
<td>1</td>
</tr>
<tr>
<td>N50</td>
<td>6,975,788</td>
<td>7,703,166</td>
</tr>
<tr>
<td>L50</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of coding sequences</td>
<td>6,181</td>
<td>6,670</td>
</tr>
<tr>
<td>Number of rRNAs</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Number of tRNAs</td>
<td>78</td>
<td>82</td>
</tr>
<tr>
<td>Number of tmRNAs</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1A: General characteristics of the SM17 and the SM18 genomes.

For SM17, the statistics include the sequence of the chromosome in addition to the sequences determined to represent three plasmids, hence the * > 7 Mb genome size and the **total of 4 contigs. No plasmids were identified in the SM18 strain, so the statistics represented above are for the chromosome sequence. No gaps or ambiguous bases (Ns) are present in the final genome assemblies.
Chapter 2

The genome assemblies for both isolates were of a very high quality, resulting in single contig assemblies of the chromosomes, without gaps or ambiguous bases (Ns), with a total genome size comprising of 7,179,914 bp (including plasmids sequences, with 6,975,788 bp for the chromosome alone) for SM17; and 7,703,166 bp (without plasmids) for SM18 (Table 1A). High quality genome assemblies are highly advantageous for determining the core genome; identifying genome sequence and structure variants; analyzing gene acquisition and duplication; together with exploring the potential presence of smBGCs at a genetic level, which is particularly relevant for studies on the *Streptomyces* genus (Bentley et al., 2002; Schmid et al., 2018). Although a few marine *Streptomyces* spp. isolates have recently had their genomes sequenced, the majority of these consist of considerably fragmented sequences due to the complexity of the genome assemblies; which to a large extent hinders an in-depth analysis of these organisms at a genomic level, particularly with respect to analyzing the presence of smBGCs (Gomez-Escribano et al., 2015; Jackson et al., 2018). To our knowledge, this is one of the first studies to report the complete genome sequence of marine sponge-derived *Streptomyces* spp. isolates.

The sequencing approach employed allowed the identification of plasmids in the SM17 isolate – pSM17A, pSM17B, and pSM17C (Table 1B). A series of factors led to their classification as plasmids, instead of simply fragments of the chromosome. Firstly, the contigs were much smaller than the super contig determined to be the chromosome: 153,923 bp, 28,056 bp, and 22,147 bp, respectively, when compared to 6,975,788 bp for the chromosome. In addition, their GC content varied from that of the chromosome, which is characteristic of exogenous and plasmid DNA (Nishida, 2012). The approximate sequencing coverage of the sequences was also varied, which is an indicator of differences in the copy number of the plasmid molecules, with pSM17B having a considerably
larger coverage of 548\times, as opposed to 170\times for pSM17A and 95\times for pSM17C (Rasko et al., 2007). Finally, they were determined to share high sequence identity to other plasmids from *Streptomyces* spp. deposited in the GenBank database, as shown in Table 1B (Guo et al., 2011; Wang et al., 2012; Liu et al., 2016).

<table>
<thead>
<tr>
<th></th>
<th>SM17 chromosome</th>
<th>Plasmid pSM17A</th>
<th>Plasmid pSM17B</th>
<th>Plasmid pSM17C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (bp)</td>
<td>6,975,788</td>
<td>153,923</td>
<td>28,056</td>
<td>22,147</td>
</tr>
<tr>
<td>Approximate coverage (fold)</td>
<td>148</td>
<td>170</td>
<td>548</td>
<td>95</td>
</tr>
<tr>
<td>GC content (%)</td>
<td>73.43</td>
<td>69.9</td>
<td>72.68</td>
<td>74.33</td>
</tr>
<tr>
<td>Number of coding sequences (hypothetical proteins)</td>
<td>5,972 (2,465)</td>
<td>170 (145)</td>
<td>30 (24)</td>
<td>24 (21)</td>
</tr>
<tr>
<td>Top BLASTN hit</td>
<td><em>Streptomyces</em> sp. HK1 plasmid pSHK1 (accession no. EU372836.1)</td>
<td><em>Streptomyces</em> sp. Y27 plasmid pWTY27 (accession no. GU226194.2)</td>
<td><em>Streptomyces</em> sp. FR-008 plasmid pSSFR2 (accession no. CP009804.1)</td>
<td></td>
</tr>
</tbody>
</table>

*Table 1B*: General characteristics of the SM17 chromosome and the three linear plasmids detected in the genome assembly.

Potential Terminal Inverted Repeats (TIRs) with an estimated size of approximately 13.4 kb and 14.6 kb were identified in both the SM17 and SM18 chromosomes respectively, using a reciprocal BLASTN approach at the ends of the chromosome sequences (Gomez-Escribano et al., 2015). The *Streptomyces* genus is known to possess linear chromosomes with TIRs, with lengths varying among species; ranging from 14 bp in *Streptomyces hygroscopicus* 5008 to over 1 Mbp in *S. coelicolor* (Weaver et al., 2004; Wu et al., 2012). Although TIRs are commonly encountered in *Streptomyces* spp., their function has not yet been definitively proven, with suggested roles been proposed including chromosome stability, replication and recombination; and genome plasticity (Volff et al., 1997; Goshi et al., 2001; Wu et al., 2012).
The main genomic features of SM17 and the three plasmids, and SM18 (number of base pairs, coding sequences (CDSs), GC% content, and the TIRs regions) are presented in the genome maps in Figure 1.

**Figure 1:** Genome maps of the SM17 and the SM18 chromosomes (A,B), and the SM17 plasmids pSM17A (C), pSM17B (D), pSM17C (E), generated using the Artemis and DNAplotter programs. All the molecules were *in silico*-determined to be linear, although they are represented in a circular fashion, and the sizes are not representative of the scale. The following are represented from the outer to the inner circles: the nucleotide position; coding sequences (CDSs) in the forward strand (in blue); CDSs in the reverse strand (in cyan); regions of putative secondary metabolite biosynthetic gene clusters (smBGCs, in orange); tRNA and rRNA genes (in gray and green, respectively); GC% plot on default settings (above average in olive and below average in purple). In (A,B), detailed in red are the regions determined to be the terminal inverted repeats (TIRs).
4.2. Determining the closest terrestrial type-strain relative for the marine sponge-derived isolates

In order to analyze possible niche adaptations in the marine sponge-derived SM17 and SM18 isolates, phylogenetic and whole-genome alignment analyses were performed to identify the closest terrestrial type-strain relative, with the complete genome sequence available in GenBank, of each isolate; with a view to performing subsequent phenotypic, morphological and genomic comparisons once these relatives had been determined.

Phylogenetic analysis was performed using the 16S rRNA and other housekeeping aforementioned genes, which allowed us to determine that *S. albus* J1074 and *S. pratensis* ATCC 33331 were the closest type-strain relative to the SM17 and SM18 strains, respectively (Figure 2). Notably, SM17 and J1074 – a derivative of the soil isolate *Streptomyces albus* G (Chater and Wilde, 1976, 1980) – are included in the same sub-clade, while SM18 and ATCC 33331 are not, indicating that the latter pair are more distantly related than the former. Nevertheless, further analyses were performed with the ATCC 33331 strain, as it was the type-strain included in the SM18 clade that was readily available in culture collections. Also, it is important to note that the ATCC 33331 strain is the only soil-derived isolate present in the SM18 clade (NCBI BioSample: SAMN00191232), while SirexAA-E was isolated from an insect/microbe symbiotic community (Bianchetti et al., 2013); PAMC26508 was isolated in association with the Antarctic lichen *Cladonia borealis* (Shin et al., 2013); and S501 was isolated from the sediment from a seaside wetland (NCBI BioSample: SAMN10144670). Thus, for these aforementioned reasons (being a type-strain with its complete genome available on GenBank, isolated from soil, and available from culture collections), the ATCC 33331 strain was determined to be the most suitable isolate identified in the SM18 clade for the purposes of this study.
Figure 2: Phylogenetic tree of the concatenated nucleotide sequence of the 16S rRNA gene, plus the housekeeping genes *atpD, gyrB, recA, rpoB*, and *trpB*. Including in this analysis are the SM17 and SM18 isolates, plus 60 *Streptomyces* isolates with complete genomes available in the GenBank database. Generated using MrBayes and MEGA X, with a posterior probability cut off of 95%.
To further support the similarities between our marine strains, SM17 and SM18, and their closest terrestrial counterparts, J1074 and ATCC 33331, alignments of the individual 16S rRNA and the other housekeeping genes were performed with NCBI BLASTN and BLASTX (Table 2). The high identity values determined by the analysis allowed further comparisons to be determined between the related pairs, and also between all four Streptomyces strains. Notably, the identities for the SM17- *S. albus* J1074 pair are higher (>99% for all the genes analyzed) than those for the SM18 – *S. pratensis* ATCC 33331 pair; (91% to 99% identity depending on the gene using BLASTN, and >95% using BLASTX). This further indicates that SM17 and *S. albus* J1074 are very closely related organisms – possibly even belonging to the same species, while the SM18 and *S. pratensis* ATCC 33331 are more distantly related.
**Table 2:** 16S rRNA and housekeeping gene alignment comparisons using the NCBI BLAST tool, between the pairs SM17 and *S. albus* J1074 (second column: SM17-J1074); and SM18 and *S. pratensis* ATCC 33331 (third column: SM18-ATCC 33331). For the housekeeping genes, the first values presented are the BLASTN (nucleotide-nucleotide) alignment identities, while the second values below are the BLASTX (translated nucleotide-protein) alignment identities. For the 16S rRNA analysis only the BLASTN alignment was performed.

Following the 16S rRNA and housekeeping genes analyses, *S. albus* J1074 and *S. pratensis* ATCC 33331 were selected for subsequent similarity analysis using a whole-genome alignment approach with the MUMmer program (Supplementary Figure S1). Large sections of the genomes are quite well conserved between the marine sponge-derived isolates and their closest relative organism, particularly when comparing SM17 with *S. albus* J1074 (Supplementary Figure S1A). This result further confirms previous analyses, and further supports *S. albus* J1074 and *S. pratensis* ATCC 33331 as suitable terrestrial relatives, for comparative purposes.
Interestingly, previous studies also reported *Streptomyces* spp. marine sponge-derived isolates that were determined to be closely related to *S. albus* J1074 (Ian et al., 2014; Iniyan et al., 2016; Almeida et al., 2018). Some of these strains, namely PVA 94-07; GBA 94-10; and *Streptomyces albus* ICN33; were isolated from completely different sample types and geographic locations than those of the current study. While SM17, which based on the aforementioned comparative analysis appears to be closely related to *S. albus* J1074, was isolated from the sponge *Haliclona simulans* from Kilkieran Bay (Galway, Ireland), at a depth of 15 m; the strains PVA 94-07 and GBA 94-10 were isolated from the sponges *Phakellia ventilabrum* and *Geodia barretti*, respectively; from the Tautra ridge (Trondheim fjord, Norway), at a depth of 121 m (Ian et al., 2014), while *Streptomyces albus* ICN33 was isolated from the sponge *Acanthella elongata*, from the Colachel coast (Kanyakumari District, Tamil Nadu), at an unspecified depth (Iniyan et al., 2016). This raises the possibility that “albus-like” *Streptomyces* strains may be ubiquitously associated with marine sponges.

### 4.3. Phenotype, morphology, and differential growth assessment

Members of the *Streptomyces* genus are known to be capable of colonizing a wide variety of different ecosystems, including soil, rhizosphere, lake and marine sediments, and have also been reported to be associated with insects, lichen, and sponges (Goodfellow and Fiedler, 2010; Bianchetti et al., 2013; Rashad et al., 2015; Liu et al., 2017; Ay et al., 2018; Jackson et al., 2018). Thus, it is reasonable to assume that these organisms possess a genetic plasticity and capability that facilitates their adaptation to such varied environmental niches (Hoff et al., 2018). Interestingly, previous studies have reported that *Streptomyces* spp. isolated from marine environments often possess the capacity of growing independently of the presence of sea salts in the growth medium (Goodfellow and Fiedler, 2010; Ian et al., 2014). In fact, many marine isolates often display very active metabolic profiles under
such conditions (Goodfellow and Fiedler, 2010). To assess whether the SM17 and SM18 isolates had phenotypical and/or morphological differences with respect to their ability to grow under different conditions, they were cultured in ISP2 medium with and without the presence of ASW and compared with their terrestrial relatives (Figure 3), in a similar fashion to work previously conducted by the Zotchev group (Ian et al., 2014).

**Figure 3:** Differential growth assessment of marine and terrestrial *Streptomyces* strains. From left to right, (A) *S. albus* J1074 and SM17 on ISP2 agar medium; (B) *S. albus* J1074 and SM17 on ISP2 + ASW agar medium; (C) *S. pratensis* ATCC 33331 and SM18 on ISP2 agar medium; (D) *S. pratensis* ATCC 33331 and SM18 on ISP2 + ASW agar medium, following 3 days growth.

All the pair-wise comparisons showed clear morphological differences between the marine sponge-derived isolates and their respective terrestrial counterparts (Figure 3). All the isolates grew effectively in the ISP2 medium
without ASW (Figures 3A,C), even though there were slight differences regarding growth and sporulation; with the SM17 isolate being able to grow and sporulate more rapidly in comparison to *S. albus* J1074 (Figure 3A). There was no clear difference in the growth of SM18 and *S. pratensis* ATCC 33331 on the ISP2 growth medium without ASW, although they clearly displayed very different morphological features (Figure 3C). On the other hand, when grown on the ISP2 medium with ASW, *S. albus* J1074 was clearly less capable of growing in the presence of sea salts, while SM17 thrived (Figure 3B). This result is particularly interesting, since, as previously shown (Figure 2, Table 2, and Supplementary Figure S1), these two organisms are genetically very similar. In contrast, there were less marked differences in the ability of both SM18 and *S. pratensis* ATCC 33331 to grow in the presence of sea salts (Figure 3D). While SM18 appeared to grow better, nevertheless *S. pratensis* ATCC 33331 was still able to grow in the ISP2 medium containing sea salts albeit more slowly than SM18; and indeed, more slowly than when *S. pratensis* ATCC 33331 was cultured in the absence of ASW (Figure 3C). From these observations, it became clear that a more thorough analysis of the SM17 and SM18 genomes might provide some interesting insights regarding potential genome-wide adaptations that may have occurred in these marine isolates, which may have resulted in them being able to grow more efficiently in the ISP2 medium supplemented with ASW; relative to their terrestrial counterparts.

4.4. Prediction of secondary metabolite biosynthetic gene clusters (smBGCs)

Members of the Actinomycetales order are historically known to produce a broad range of bioactive compounds of biotechnological and clinical interest, and among them, the *Streptomyces* genus excels, with over 10,000 bioactive compounds produced by members of the genus being discovered to date (Hwang et al., 2014; Ziemert et al., 2016; Kamjam et al., 2017; Lee et al., 2018). The marine sponge-derived SM17 and SM18 strains have previously been reported to possess
antimicrobial activity against gram-negative and gram-positive bacteria – including the methicillin-resistant *S. aureus* (MRSA), and yeasts (Kennedy et al., 2009; Jackson et al., 2018). To provide insights at a genomic level regarding which compounds might be responsible for the previously observed antimicrobial activity, we employed the antiSMASH program in an attempt to predict the presence of putative smBGCs, based on homology to known smBGCs deposited in the databases (Blin et al., 2017). Several gene clusters were predicted to be present in both SM17 and SM18 (Supplementary Tables S1, S2), with a total of 20 potential smBGCs in SM17, and 26 in SM18; with a variety of cluster types being assigned, including: type I polyketide synthases (T1pks), type II polyketide synthases (T2pks), type III polyketide synthases (T3pks), non-ribosomal peptide synthetases (NRPS), lantipeptides, bacteriocins, and terpenes. These types of clusters are known to produce a variety of compounds with antimicrobial activity, including: erythromycin (T1pks); tetracenomycin (T2pks); germicidin (T3pks); daptomycin (NRPS); nisin (lantipeptide/lantibiotic/bacteriocin); and pentalenolactone (terpene) (Shen, 2003; Tetzlaff et al., 2006; Robbel and Marahiel, 2010; Shi et al., 2011; Yamada et al., 2015; Čihák et al., 2017).

The antiSMASH predictions were also further analyzed using the BiG-SCAPE program (Navarro-Muñoz et al., 2018), which allowed us to cluster the predicted smBGCs into gene cluster families (GCFs) based on their sequences and Pfam protein families similarities (El-Gebali et al., 2019), and also to compare them to known smBGCs available from the latest version of the MIBiG repository (version 1.4) (Medema et al., 2015), which can also assist in improving the annotations of the predicted smBGCs. Based on their similarity to known smBGCs, some of the bioactive compounds predicted to be encoded by these smBGCs may be compatible with the previously determined antimicrobial capabilities of the SM17 and SM18 isolates (Figure 4 and Supplementary Tables S1, S2). For example,
SM17 appears to possess a candididin, an antimycin, and a polycyclic tetramate macrolactam cluster (SGR PTMs) (Figure 4 and Supplementary Table S1), with similarity to the candididin, antimycin and tetramate macrolactam sequences from *Streptomyces* sp. FR-008, *Streptomyces* sp. S4 and *Streptomyces griseus* in the database, respectively, and which are known to have anti-fungal properties (Campelo and Gil, 2002; Chen et al., 2003; Seipke et al., 2011; Luo et al., 2013). SM17 also contains clusters that may potentially encode for the production of surugamides (Figure 4) and the glycopeptide antibiotic mannopeptimycin (Supplementary Table S1), with the former possessing gene similarity with the surugamide A/D sequence from *Streptomyces albus* in the database (Ninomiya et al., 2016), and the latter sharing similarity to the mannopeptimycin sequence from *Streptomyces hygroscopicus* in the database, with the main biosynthetic genes being present in the predicted smBGC (Singh et al., 2003; Magarvey et al., 2006).

SM18 appears to possess a cluster encoding the anti-bacterial compound bafilomycin (Figure 4 and Supplementary Table S2), with similarity to the bafilomycin sequence from *Streptomyces lohii* (Bowman et al., 1988; Zhang et al., 2013; Nara et al., 2017); as well other clusters with similarity to known smBGCs that encode anti-fungal and anti-bacterial compounds such as SGR PTMs, curamycin, and caboxamycin (Figure 4), from *Streptomyces griseus* (Luo et al., 2013), *Streptomyces curacoï* (Galmarini and Deulofeu, 1961), and *Streptomyces* sp. NTK 937 (Hohmann et al., 2009; Losada et al., 2017), respectively.
Figure 4: Gene clusters families (GCFs) analysis using antiSMASH (version 4), BiG-SCAPE (version 20181005), MIBiG database (version 1.4), and Cytoscape. Each node represents a smBGC predicted in the respective organism (labeled in different colors), and the interactions represent cluster similarity. Annotations of the MIBiG database smBGCs are labeled accordingly. Singletons, i.e., smBGCs without similarities with the smBGCs in the MIBiG database, or without similarities with the smBGCs predicted in the other genomes analyzed in this study, are not included in this figure.

We also performed the antiSMASH and BiG-SCAPE analysis on *S. albus* J1074 and *S. pratensis* ATCC 33331 genomes, in an effort to determine to what extent the marine sponge-derived isolates SM17 and SM18 may potentially produce similar and/or unique compounds when compared to their terrestrial counterparts (Figure 4 and Supplementary Tables S3, S4). Based on the BiG-SCAPE similarity clustering, a Venn diagram was generated, representing the presence/absence of GCFs in the SM17, SM18, *S. albus* J1074, and *S. pratensis* ATCC
33331 genomes (Figure 5). In keeping with the phylogeny results which indicated that SM17 and *S. albus* J1074 were very closely related organisms, the smBGCs predictions and similarity clustering results were also strikingly similar (Figures 4, 5). Among a total of 42 predicted smBGCs in both genomes (22 in *S. albus* J1074 and 20 in SM17), 10 seem to be unique (6 in *S. albus* J1074, and 4 in SM17) (Figure 5). In contrast, there was a much larger number of predicted unique smBGCs between SM18 and *S. pratensis* ATCC 33331, where amongst a total of 53 predicted clusters (27 in *S. pratensis* ATCC 33331 and 26 in SM18), only 6 appear to be present in both genomes; with the majority being potentially unique (20 in *S. pratensis* ATCC 33331 and 20 in SM18) (Figure 5). Also, a total of 4 smBGCs were shared among all of the strains analyzed (Figure 5), and these were determined to be: hopene; SGR PTMs family of smBGCs, ectoine, and a predicted siderophore smBGC without significant similarity to sequences in the MIBiG database (Figures 4, 5).

**Figure 5:** Venn diagram representation of GCFs presence/absence analysis using BiG-SCAPE.
Notably, smBGCs encoding the production of desferrioxamines, which are hydroxamate siderophores, while present in *S. albus* J1074, *S. pratensis* ATCC 33331 and SM17 (Figures 4, 5), are absent in the SM18 genome (Supplementary Table S2 and Figure 4). Siderophores are specialized metabolites that function to scavenge Fe$^{3+}$, and hence are crucial for sessile organisms to assimilate iron (Hider and Kong, 2010). Genes involved in desferrioxamines production, in particular, are widely conserved in marine microorganisms, and are believed to be present in all *Streptomyces* species (Tierrafría et al., 2011; Cruz-Morales et al., 2017). Thus, this may be the first report of a *Streptomyces* isolate that does not possess a smBGC that encodes for the production of desferrioxamines. The SM18 isolate does, however, possess smBGCs encoding other siderophores, such as coelichelin, and mirubactin (Supplementary Table S2 and Figure 4), which may circumvent for the lack of production of desferrioxamines with respect to iron acquisition in the strain.

During processing of the data for this study, a newer version of the antiSMASH webserver (version 5) was released (Blin et al., 2019). Using this new version of antiSMASH did not result in any major differences being detected in the data being analyzed, it did however result in the identification of a smBGC encoding mycemycin in the SM18 genome. Mycemycin is a relatively newly identified compound, from marine and soil *Streptomyces* isolates, belonging to the dibenzoxazepinone (DBP) family, which possesses HIV-1 reverse transcriptase inhibitory activity (Liu et al., 2015; Song et al., 2018). Production of the DBP family of compounds appear to date to be rare in the microbial world, and these compounds possess a broad range of interesting activities, including anti-HIV and anti-tumor activities (Zhang et al., 2018). Thus, pursuing the identification of new members of this family of compounds may be worthwhile, and it is interesting to report the potential presence of a smBGC encoding the production of mycemycin in another *Streptomyces* isolate.
Nevertheless, it is clear that further analysis would need to be undertaken to confirm that these compounds are in fact being produced by the SM17 and SM18 isolates, as some of these smBGCs are likely to be cryptic and the compounds may not be produced under certain culture conditions (Rutledge and Challis, 2015; Rigali et al., 2018). Given that SM17 and *S. albus* J1074 are genetically very similar, it is perhaps reasonable to expect that regulation of secondary metabolite production may to some extent be similar in both strains. Therefore, it may be possible to use what is currently known about the better studied *S. albus* J1074 isolate to gain a better understanding regarding the expression of certain smBGCs and the metabolic pathways involved in SM17 (Hoz et al., 2017; Kallifidas et al., 2018; Nguyen et al., 2018).

4.5. **Comparative genomics**

A series of comparative genomics analyses was then performed in order to further characterize the marine sponge-derived isolates SM17 and SM18 at the genome level, and in particular to compare them to their respective closest terrestrial relative.

4.5.1. **Analysis of orthologous genes**

The Roary program was used to determine the pan-genome; the core genome; the accessory genome; and the strain-specific genome (the genes that are uniquely present in only one of the isolates), in the marine sponge-derived isolates *Streptomyces* sp. strain SM17 and *Streptomyces* sp. strain SM18, and their respective closest terrestrial relatives *S. albus* J1074 and *S. pratensis* ATCCC 33331 (Figure 6) (Page et al., 2015). The pan-genome was determined to consist of 11,305 genes; while the core genome consisted of 3,303 genes (~29% of the pan-genome); and the accessory genome consisted of 8,002 genes (~71% of the pan-genome). For the strain-specific genomes, SM17 had 485 unique genes; SM18 had 1,860 unique
genes; *S. albus* J1074 had 258 unique genes; and *S. pratensis* ATCC 33331 had 1,874 unique genes. This is a combined total of 4,477 unique genes (~39% of the pan-genome, and ~56% of the accessory genome). Notably, in keeping with what we had previously observed with the phylogeny and whole-genome alignment analyses, the SM17 and J1074 strains shared a very large number of orthologous genes (a total of 5,515 shared genes, or ~89% and ~94% of the SM17 and the J1074 total number of CDSs, respectively), further indicating that they are very closely related organisms. In contrast, SM18 and *S. pratensis* ATCC 33331 shared a much lower proportion of their genes: 4,469 genes (or ~67% and ~66% for the SM18 and ATCC 33331 total number of CDSs, respectively). A total of 64 orthologous genes were found to be commonly present in the marine sponge-derived isolates SM17 and SM18, while absent in their terrestrial counterparts J1074 and ATCC 33331 (Figure 6). Given that they are absent in both terrestrial relatives, we undertook further analyses of these genes to assess their potential function(s) in an effort to provide insights into potential ENAs in both these sponge-derived isolates.
Figure 6: Venn diagram representing the presence/absence of orthologous genes in the SM17, SM18, *S. albus* J1074, and the *S. pratensis* ATCC 33331 genomes. Orthologous genes that are present commonly in the marine sponge-derived isolates SM17 and SM18, while absent in their terrestrial counterparts J1074 and ATCC 33331, are circled in red.

4.5.2. Orthology analysis of smBGC-associated genes

The genes previously determined to be associated with smBGCs, using the antiSMASH program, were subsequently analyzed using the Roary program, to identify smBGCs-associated genes which were shared or unique between the four genomes (Supplementary Figure S2) (Page et al., 2015; Blin et al., 2017). With respect to potential smBGCs-associated genes, very few genes appeared to be conserved in all the organisms (a total of 58 genes, corresponding to 2.8% of the total smBGCs-associated gene pool, or 0.017% of the core genome) (Supplementary Figure S2). The largest number of unique smBGCs-associated genes is present in
the SM18 isolate (623 genes), followed by *S. pratensis* ATCC 33331 (485 genes)
which may be indicative of a greater potential to produce diverse secondary
metabolites in these isolates. In contrast, SM17 and *S. albus* J1074 appear to possess
a lower quantity of unique smBGCs-associated genes; with 132 and 150 unique
genes, respectively (Supplementary Figure S2).

Interestingly, when comparing the Venn diagrams from Figure 6 and
Supplementary Figure S2, it appears that a large portion of the unique genes
present in the isolates are potentially related to the production of secondary
metabolites. For SM17, ~27% (132 out of 485) of the unique genes are potentially
smBGCs-associated genes, while for SM18 this percentage is ~33% (623 out of 1860);
~58% (150 out of 258) for *S. albus* J1074; and ~26% (485 out of 1874) for *S. pratensis*
ATCC 33331. Taken together, these results indicate that, even for closely related
*Streptomyces* spp. isolates (particularly when considering the pair SM17 and *S.
*albus* J1074), there is still potential to discover different secondary metabolites from
these strains, with potentially unique characteristics. Previous reports have also
indicated that the use of closely related *Streptomyces* strains to identify new
smBGCs is useful for the identification of novel specialized biosynthetic pathways
(Antony-Babu et al., 2017; Vicente et al., 2018).

4.5.3. Groups of orthologous genes commonly present in the marine sponge-
derived isolates

Given that the SM17 and SM18 were isolated from a marine sponge and
have been shown to be more adapted to higher salinity medium (Figure 3), it is
likely that the identification of genes that are commonly present in SM17 and SM18
but not in their terrestrial relatives J1074 and ATCC 33331 may help in the
identification of potential ENAs that these strains might possess, at a genetic level.
The previous analysis of orthologous genes allowed us to determine which groups
of orthologous genes are present commonly in the marine sponge-derived isolates
SM17 and SM18, while absent in their terrestrial counterparts J1074 and ATCC 33331, as highlighted in Figure 6. This was performed by taking into account sequence homology and gene synteny (e.g., splitting paralogous genes with the Roary program); hence different copies of a gene can belong to different orthologous group due to potentially different evolutionary events such as gene duplication or lateral gene transfer occurring in the *Streptomyces* genomes (Zhou et al., 2012; Page et al., 2015). Thus, from here on the orthologous genes will be referred to simply as “genes.” In doing this we identified a potential ENA gene pool which consisted of 64 genes (Table 3). These were then manually annotated using the NCBI GenBank, CDD, UniProt, and the InterPro databases (Johnson et al., 2008; Marchler-Bauer et al., 2011; UniProt Consortium, 2015; Benson et al., 2018; Mitchell et al., 2018), and hypothetical proteins were removed, resulting in a final total of 57 genes (Supplementary Table S5). The ENA gene pool included functional categories of genes that are likely to be related to niche adaptations in the marine sponge-derived isolates, and included a total of 29 genes that could be grouped based on potential biological functions such as osmotic stress defense; transcriptional regulation; symbiotic interactions; antimicrobial compounds production and resistance; ABC transporters; together with horizontal gene transfer and defense-related features (Table 3).
### Environmental niche adaptation

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Osmotic stress defence</strong></td>
<td></td>
</tr>
<tr>
<td><em>nuoA</em></td>
<td>NADH-quinone oxidoreductase subunit A</td>
</tr>
<tr>
<td><em>nuoH</em></td>
<td>NADH-quinone oxidoreductase subunit H</td>
</tr>
<tr>
<td><em>nuoJ</em></td>
<td>NADH-quinone oxidoreductase subunit J</td>
</tr>
<tr>
<td><em>nuoK</em></td>
<td>NADH-quinone oxidoreductase subunit K</td>
</tr>
<tr>
<td><em>nuoL</em></td>
<td>NADH-quinone oxidoreductase subunit L</td>
</tr>
<tr>
<td><em>nuoM</em></td>
<td>NADH-quinone oxidoreductase subunit M</td>
</tr>
<tr>
<td><em>nuoN</em></td>
<td>NADH-quinone oxidoreductase subunit N</td>
</tr>
<tr>
<td><em>proP</em></td>
<td>Proline/betaine transporter</td>
</tr>
</tbody>
</table>

### Transcriptional regulation

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>bepr^</strong>*</td>
<td>HTH-type transcriptional repressor Bepr / TetR family transcriptional regulator</td>
</tr>
<tr>
<td><em>cynR</em></td>
<td>HTH-type transcriptional regulator CynR / LysR family transcriptional regulator</td>
</tr>
<tr>
<td><em>degU</em></td>
<td>Transcriptional regulatory protein DegU / DNA-binding response regulator</td>
</tr>
<tr>
<td><em>group_5796</em></td>
<td>Transcriptional regulator, IclR family</td>
</tr>
<tr>
<td><em>group_5819</em></td>
<td>Transcriptional regulator PadR-like family protein</td>
</tr>
<tr>
<td><em>rhmR</em></td>
<td>HTH-type transcriptional regulator KipR / MarR family transcriptional regulator</td>
</tr>
<tr>
<td><em>tcrA</em></td>
<td>Transcriptional regulatory protein CutR / DNA-binding response regulator</td>
</tr>
</tbody>
</table>

### Symbiotic interactions

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<th>Genes</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>group_5772</td>
<td>Tetratricopeptide repeat protein</td>
</tr>
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</table>

### Antimicrobial compounds production and resistance

<table>
<thead>
<tr>
<th>Genes</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>aprx^</strong>*</td>
<td>Serine protease AprX / Subtilase family protein / Peptidase S8</td>
</tr>
<tr>
<td><em>group_5198</em></td>
<td>Aminoglycoside phosphotransferase</td>
</tr>
<tr>
<td><em>group_5385</em></td>
<td>Aminoglycoside phosphotransferase</td>
</tr>
<tr>
<td>Group</td>
<td>Annotation</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>group_5818</td>
<td>Acyltransferase 3</td>
</tr>
<tr>
<td>group_5836</td>
<td>Acyltransferase</td>
</tr>
<tr>
<td>liaS</td>
<td>HPK7 family sensor histidine kinase LiaS</td>
</tr>
</tbody>
</table>

**ABC transporters**

<table>
<thead>
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<th>Group</th>
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</tr>
</thead>
<tbody>
<tr>
<td>group_5821</td>
<td>ABC transporter permease</td>
</tr>
<tr>
<td>tauB</td>
<td>Aliphatic sulfonates import ATP-binding protein SsuB / ABC transporter ATP-binding protein</td>
</tr>
<tr>
<td>yknY</td>
<td>Uncharacterised ABC transporter ATP-binding protein YknY</td>
</tr>
</tbody>
</table>

**Horizontal gene transfer and defence-related features**

<table>
<thead>
<tr>
<th>Group</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>group_1044</td>
<td>Integrase core domain / IS3 family transposase</td>
</tr>
<tr>
<td>group_1272</td>
<td>Toxin-antitoxin system, RelE family</td>
</tr>
<tr>
<td>group_1944</td>
<td>Restriction endonuclease</td>
</tr>
<tr>
<td>group_1945</td>
<td>IS3 family transposase</td>
</tr>
</tbody>
</table>

Table 3: Groups of orthologous genes and their respective annotations (excluding hypothetical proteins), which are present commonly in the sponge-derived isolates SM17 and SM18, while absent in their terrestrial counterparts J1074 and ATCC 33331. When the gene name was not determined, a generic unique name was given (e.g., group_5796) by the Roary program. *genes without multiple copies or paralogs in the terrestrial isolates’ genomes, taking into consideration only those with a defined gene name.
4.5.3.1. Resistance to osmotic stress

For bacteria to survive in marine environments where salinity levels of approximately 3.5% exist, they must be able to simultaneously overcome stresses due to both high osmotic pressure and high Na+ concentrations (Yaakop et al., 2016); together with other stresses including pressure, temperature and oligotrophic conditions (Xie et al., 2018). Bacteria typically respond to variations in external osmotic pressure by accumulating or releasing solutes, thereby attenuating water fluxes and maintaining cellular homeostasis (Wood, 2015). The marine sponge-derived isolates SM17 and SM18 appeared to grow and differentiate more rapidly when grown on media containing artificial seawater, when compared to their closely related terrestrial counterparts (Figure 3), thus indicating a potential increased fitness to higher salinity environments, as also previously described in other marine Streptomyces isolates (Ian et al., 2014). Previous studies with marine Actinomycetes, specifically with the genera Salinispora, Streptomyces, and Kocuria, have proposed that the NADH-quinone oxidoreductases nuoAHJKLMN genes, which encode a proton pump, could be classified as potential MAGs (Penn and Jensen, 2012; Ian et al., 2014; Sun et al., 2018). This proton pump is believed to create a proton-motive force which generates ATP, helping to maintain a proton gradient in seawater (Penn and Jensen, 2012; Ian et al., 2014; Sun et al., 2018). We identified the nuoAHJKLMN genes in the ENA gene pool in both SM17 and SM18 (Table 3). Further analysis indicated that both isolates possessed one extra copy of these genes when compared to their terrestrial counterparts, and that these genes were organized in an operon-like structure, similar to that previously reported in Salinispora arenicola CNS-205 and in Kocuria flava S43 (Sun et al., 2018). Furthermore, the same gene synteny for the partial nuo-operon was present in Streptomyces sp. SM17, Streptomyces sp. SM18, Salinispora arenicola CNS-205, Salinispora tropica CNB-440, and Kocuria flava S43
(Figure 7); with nuoA, followed by a hypothetical protein, and then followed by nuoH, nuoJ, nuoK, nuoL, nuoM, and nuoN. It is important, however, to note that differences in sequence identity and reading frames are present (Supplementary Table S6 and Figure 7), which may indicate that different evolutionary events may have occurred in the aforementioned genomes. The presence of this partial nuo-operon in the sponge derived SM17 and SM18 isolates and in the other marine actinomycetes (Salinispora arenicola CNS-205, Salinispora tropica CNB-440, and Kocuria flava S43), which are absent in their terrestrial counterparts J1074 and ATCC 33331, may explain, at least in part, the increased tolerance to salinity we observed in SM17 and SM18 relative to J1074 and ATCC 33331; which although still able to grow in the presence of ASW, grew much more slowly (Figure 3). Another important mechanism which bacteria employ as a defense mechanism against osmotic stress is both the synthesis and the uptake of compatible solutes, such as proline, glycine, betaine and ectoine, in order to maintain membrane turgor pressure (Krämer, 2010; Lim and Lee, 2015). Extra copies of the proP gene, which encodes a potential proline/betaine transporter (ProP), were found in both the SM17 and SM18 strains. It has been shown in E. coli that the ProP transporter acts both as an osmoregulator and as an osmosensor; and is capable of transporting proline, glycine betaine, proline betaine, carnitine, ectoine and other compounds (MacMillan et al., 1999; Roessler and Muller, 2001; Burg and Ferraris, 2008). Therefore, the proP genes may also be related to the increased capacity of the SM17 and SM18 strains to tolerate hyperosmotic environments, as evidenced by their growth on the ASW medium (Figure 3).
4.5.3.2. Antimicrobial compounds production and resistance

For many years the main ecological function of antibiotics production in bacteria in natural environments was believed to be inhibition of the growth of other microorganisms, thereby conferring a selective advantage on the producing strain with respect to colonization of particular environmental niches (Linares et al.,
In this respect, antibiotic production may be employed as a defense mechanism for the *Streptomyces* spp. isolates SM17 and SM18 — and other members of the symbiotic community — against other competitor microorganisms in the marine sponge host; as has been previously reported to be the case with other antibiotic producing microorganisms, such as *Streptomyces* spp. which have been isolated from different hosts including plants and insects (Bondarev et al., 2013; van der Meij et al., 2017; Ceapă et al., 2018; Engl et al., 2018). Furthermore, antibiotics may also play an important role in the overall defense of the sponge host itself by protecting it against pathogens, in a biological interaction defined as defensive symbiosis (Clay, 2014), which has been reported in a number of systems, including beewolf wasps and antibiotic-producing *Streptomyces* bacteria (Engl et al., 2018). Nevertheless, more recently it has been proposed that in natural environments antibiotics may also act as small molecules with signaling functions, functioning in a similar fashion to quorum sensing molecules; acting for example to alter the expression of genes; to induce biofilm formation; or to modulate colony morphology — all of which may be important in coordinated communication within symbiotic communities (Romero et al., 2011). Thus, antibiotics may have a number of roles in a niche environment such as in marine sponges, which may include both defense-related and signaling roles (Linares et al., 2006; Romero et al., 2011). The presence of a wide variety of predicted smBGCs in both SM17 and SM18 — many of which are potentially involved in the production of antimicrobial compounds (Supplementary Tables S1, S2), coupled with the previously reported antimicrobial activities in these strains (Kennedy et al., 2009; Jackson et al., 2018); supports a possible role for these two *Streptomyces* spp. isolates in defensive symbiosis in *Haliclona simulans*, from which they were isolated. In this respect two acyltransferase genes potentially involved in the biosynthesis of type II PKS antibiotics, or type I PKSs that require discrete acyltransferase enzymes, were present in the ENA gene pool (Table 3) (Cheng et al., 2003; Zhang et al., 2017).
addition, a subtilase-like serine protease gene (aprX) was also identified in the ENA gene pool (Table 3), which belongs to a family of proteins that are known to play a number of different biological roles, including involvement in the biosynthesis of antimicrobial peptides, with some possessing algicidal properties, which could potentially be relevant from a sponge defense perspective (Lee et al., 2000; Barra et al., 2017; Montalbán-López et al., 2018). Protease producing marine bacteria are known to be important in the degradation of organic nitrogen which is essential for nitrogen recycling in marine sediments (Zhang et al., 2015). Marine bacterial proteases are also known to play a role in sponge host nitrogen metabolism, which may also explain the presence of this specific protease in the marine isolates SM17 and SM18, and its absence in their terrestrial counterparts (Li et al., 2016; Kiran et al., 2018). Further work on these specific proteases might also be relevant from an industrial perspective, given the interest in proteases of marine origin which are typically cold adapted, salt tolerant, with broad optimal pH values (Li et al., 2016) and which are particularly suited for a number of biotechnological applications, including laundry detergents, food processing, the leather and textile industries, and in waste water treatment applications (Li et al., 2013; Salwan and Sharma, 2018).

A LiaS-encoding gene was also present in the ENA pool, which has previously been reported to be part of the two-component LiaS/LiaR regulatory system, a stress-sensing module that is conserved in Firmicutes bacteria and which is involved in the response to a subset of cell wall-active antibiotics such as bacitracin and vancomycin in Bacillus subtilis, while also being involved in response to cationic antimicrobial peptides and secretion stress (Mascher et al., 2004). In Listeria monocytogenes, the LiaS/R system also plays an important role in resistance to the food preservative nisin (Collins et al., 2012). The presence of antibiotic resistance-related genes in our two sponge-derived isolates may be
significant from two perspectives. Firstly, they may function as part of a self-resistance mechanism in these strains, allowing them to be protected from the antimicrobial compounds that they themselves are producing; and/or secondly, as a resistance mechanism to protect themselves from the antimicrobial compounds produced by other microorganisms within the sponge symbiotic community (Wright, 2005, 2012).

4.5.3.3. ABC transporters

ATP-binding cassette (ABC) transporters are ATP-dependent protein complexes that are widespread in all forms of life and which are vital in mediating the transport of both organic and inorganic molecules across cell membranes (ter Beek et al., 2014; Wilkens, 2015). In bacteria, they confer resistance to antibiotics and to other toxic compounds through efflux/transport mechanisms (Greene et al., 2018); and are also involved in nutrient acquisition and in helping to maintain osmotic balance in the cell (Wood, 2007; Fan et al., 2013; Teichmann et al., 2018). The ENA gene pool includes an yknY-like ABC transporter (Table 3), which has been reported to be involved in the efflux of the sporulation-delaying protein (SDP) in *Bacillus* spp., although it is still poorly characterized in other genera, such as in *Streptomyces* (González-Pastor et al., 2003; Xu et al., 2016; Greene et al., 2018). The SDP protein is a killing factor exported by cells that have started the sporulation process, therefore inducing the lysis of sister cells, making more nutrients available, and ultimately delaying the sporulation process and maintaining regular cell growth (González-Pastor et al., 2003). Thus, it is reasonable to assume that the bacterial members of the sponge symbiotic community may employ similar mechanisms and resistance genes targeting these potentially harmful proteins, which may be the case in both SM17 and SM18. A tauB/ssuB-like ABC transporter was also present in the ENA gene pool, which may be responsible in allowing more
versatile nutrient acquisition and cycling – specifically for nitrate and sulfonate – for the marine *Streptomyces* isolates SM17 and SM18, as it has been previously suggested to be the case for marine sponge symbiotic communities, through metagenome binning analysis (Karimi et al., 2018).

### 4.5.3.4. Transcriptional regulation

Being able to efficiently respond to changes in their environment is crucial in helping bacteria adapt to and survive within these environments (Feklistov et al., 2014; Daniel-Ivad et al., 2018); and, as previously mentioned, it is particularly important for the sponge-derived bacteria to be able to react appropriately to osmotic and other environmental stresses such as the presence of antibiotics and other potentially harmful compounds; the lack of nutrients; or allowing cell-to-cell communication through quorum sensing.

Transcriptional regulators play a crucial role in allowing bacteria to respond appropriately to numerous environmental stimuli and are believed to be intrinsically linked to lifestyle and environmental adaptation in bacteria (Stock et al., 1990; Feklistov et al., 2014; Daniel-Ivad et al., 2018). Since the SM17 and SM18 isolates inhabit the same niche environment and are subsequently exposed to similar conditions, it is likely that they employ similar adaptive mechanisms in response to those conditions. The ENA gene pool does include a range of transcriptional regulators (Table 3), further indicating that the marine sponge-derived isolates SM17 and SM18 share signal transduction mechanisms that are absent in their terrestrial counterparts, which may account for important niche adaptations that have been acquired. Notably, the TetR, LysR, DegU, IclR, PadR, and CutR families of transcriptional regulators are present in the ENA gene pool. These are commonly associated with mechanisms that could also be potential adaptations employed by the sponge-derived isolates SM17 and SM18, such as for
example: antibiotics production (TetR, DegU); antibiotics resistance (TetR, LysR); multidrug resistance (IclR, PadR), quorum sensing (TetR, LysR, IclR); sporulation (IclR); detoxification (PadR); salt stress response (DegU); and copper stress response (CutR) (Huillet et al., 2006; Molina-Henares et al., 2006; Maddocks and Oyston, 2008; Fibriansah et al., 2012; Rademacher and Masepohl, 2012; Cuthbertson and Nodwell, 2013; Rodríguez et al., 2013; Tian et al., 2014; Hoffmann and Bremer, 2016).

For example, a gene encoding a LysR family transcriptional regulator, that is present uniquely in the SM18 genome, is located upstream of a gene which appears to encode a Beta-lactamase enzyme family protein, which are enzymes that provide mechanisms of resistance to β-lactam antibiotics (Majiduddin et al., 2002; Naas et al., 2017). In addition, a gene encoding a IclR family transcriptional regulator that is present in both the SM17 and SM18 genomes, is located upstream of a proP gene, which potentially encodes a proline/betaine transporter and, which previously mentioned, could be related to osmotic regulation in these organisms (MacMillan et al., 1999; Roessler and Muller, 2001; Burg and Ferraris, 2008). While a gene encoding a transcriptional regulator PadR-like family protein which is present in both the SM17 and SM18 genomes; is located upstream of a gene coding an ABC transporter, which as previously mentioned, could be involved with nutrient acquisition, resistance to toxic molecules, or in maintaining osmotic balance in these isolates (Wood, 2007; Fan et al., 2013; Greene et al., 2018; Teichmann et al., 2018).

4.5.3.5. Genomic evolution through horizontal gene transfer

Horizontal gene transfer (HGT) is an important mechanism in bacterial genome evolution, and commonly involves the acquisition of mobile genetic elements (MGEs) (Bellanger et al., 2014). Previous studies have reported that the
genomes of symbiotic bacteria – including sponge symbionts – possess a higher number of MGEs than those of free-living microorganisms (Thomas et al., 2010; Fan et al., 2012, 2013). It has been proposed that MGEs play a crucial role in co-evolution with the host and convergent evolution of marine sponge symbiotic communities in a number of ways, such as enabling the members of the symbiotic community to share important traits for niche adaptation (Fan et al., 2012), such as for example genes related to stress tolerance, antibiotics resistance, and nutrient acquisition. In addition, the MGEs can function in the deactivation or removal of non-essential genes, such as those that are only required by free-living bacteria, or those related to functions that are already being performed by other members of the symbiotic community (Fan et al., 2012). Two genes encoding transposases were found in the ENA gene pool (Table 3), indicating that they may be involved in HGT events and co-evolution between the marine sponge isolates SM17 and SM18. Also, the three plasmids that were identified in SM17 (Table 1B and Figure 1), which are absent in its terrestrial counterpart S. albus J1074, provide additional evidence of potential genomic evolution through transferable elements occurring within the marine sponge microbiota.

The high filter feeding rates of sponges mean that they are likely to be exposed to phage attack from the plankton, and that bacterial sponge symbionts may be subjected to phage-mediated transduction which can lead to cell lysis (Thomas et al., 2010). Therefore, it might be expected that sponge bacterial symbiotic communities would require defense mechanisms to protect themselves from foreign DNA, such as restriction modification (R-M) systems and toxin-antitoxin (T-A) systems (Fan et al., 2012; Horn et al., 2016; Slaby et al., 2017). R-M systems are also linked to MGEs in that they can be transferred via the MGEs, or they can act as MGEs in transposon-like structures (Furuta and Kobayashi, 2013). In the ENA gene pool, we identified one restriction endonuclease that could be part
of a transferrable R-M system and one T-A system gene from the RelE family in SM17 and SM18 (Table 3). This further highlights the possibility that HGT events may be occurring between the sponge-derived isolates and the possibility of shared niche adaptations between them, and also the requirement for defense mechanisms against foreign DNA in the symbiotic bacteria. Importantly, T-A systems have also been proposed to provide mechanisms to cope with stress – such as nutrient stress – by either programmed cell death or by inducing bacteriostasis, which may be another important role played by the T-A systems in symbiotic communities in oligotrophic environments (Van Melderen, 2010; de Goeij et al., 2013).

4.5.3.6. Eukaryotic-like proteins and potential host interaction

Metagenomic and genomic studies have reported that bacterial symbionts contain a large number of genes encoding for eukaryotic-like proteins (ELPs) (Reynolds and Thomas, 2016). ELPs contain repeat domains that are commonly found in eukaryotic proteins, such as tetratricopeptide repeats (TPRs), and are believed to play an important role in symbiotic relationships, by mediating protein-protein interactions for a range of cellular proteins (Thomas et al., 2010; Li et al., 2015; Reynolds and Thomas, 2016). These ELPs may have a broader function in mediating bacterial-sponge interactions and may modulate the host’s behavior (Li et al., 2015; Reynolds and Thomas, 2016). The ENA gene pool contained a tetratricopeptide repeat-containing protein, which is a class of ELP that has been proposed to function as a means for symbiotic bacteria to avoid digestion, or as a mechanism for the sponge to distinguish between food and symbionts (Thomas et al., 2010). The fact that the relatively phylogenetically distant SM17 and SM18 isolates possess orthologs of the same TPR, while their closest terrestrial relatives do not; suggests that this protein may indeed play a role in the symbiotic interactions between these bacteria and their sponge host *Haliclona simulans.*
4.5.4. ENA gene pool genes commonly present in other environmental Streptomyces isolates

In a similar fashion to the aforementioned analysis of orthologous genes, an additional analysis was performed, including the genomes from the other isolates previously determined to belong to the SM17 and SM18 phylogenetic clades (Figure 2). The aim was to assess whether genes present in the SM17 and SM18’s ENA gene pool are also present in other closely related relatives derived from other diverse environments, given the possibility that they may possess adaptations to their particular environmental niches that overlap with those identified in our marine sponge-associated SM17 and SM18 strains.

In the previously identified SM17 clade (Figure 2), in addition to its closely related terrestrial type-strain J1074, the clade also included the environmental isolates *Streptomyces albiflavus* SM254, which was isolated from copper-rich subsurface fluids within an iron mine (Badalamenti et al., 2016); *Streptomyces sampsonii* KJ40, which was isolated from rhizosphere soil in a poplar plantation (Li et al., 2018); *Streptomyces koyangensis* VK-A60T, which was isolated from rhizosphere soil in a radish plantation (Lee et al., 2005); and *Streptomyces* sp. CLI2509, which is a fungus-derived isolate (Wyche et al., 2017). It is important to note that the SM17 clade also included the *Streptomyces* sp. FR-008 strain, however, this strain was not included in the analysis since it does not appear to be an environmental isolate, and it is a product of protoplast breeding of strains with little information in the literature regarding their isolation source (NCBI BioSample: SAMN03120580). The SM18 clade (Figure 2), in addition to its closely related terrestrial type-strain ATCC 33331, also included the environmental isolates *Streptomyces* sp. PAMC26508, which is an endosymbiotic bacterium isolated from the Antarctic lichen *Cladonia borealis* (Shin et al., 2013); *Streptomyces* sp. S501, isolated in sediment from a seaside wetland (NCBI BioSample: SAMN10144670);
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and *Streptomyces* sp. SirexAA-E, isolated from an insect/microbe symbiotic community (Bianchetti et al., 2013).

Interestingly, the majority of the genes present in the ENA gene pool were also present in the genomes of the other isolates. This is perhaps not surprising given the potential similarity in environmental stresses that these isolates may encounter, as the marine sponge-associated SM17 and SM18 strains; since they were all isolated from either (1) symbiotic communities, (2) high osmotic pressure environments and/or (3) aquatic environments. For example, the aforementioned *nuo* operon genes (Figure 7); potentially involved in adaptation to osmotic stress, are also present in the KJ40, the VK-A60T, and SM254 strains from the SM17 clade (Figure 2). It is well documented that osmoadaptation is an important trait possessed by rhizosphere-derived bacteria, since water uptake and exclusion of solutes such as Na$^+$ and Cl$^-$ by plants roots are likely to induce changes in osmolarity (Miller and Wood, 1996; Qurashi and Sabri, 2011), and for that reason salt-tolerant bacterium are commonly isolated from plant rhizospheres (Yuwono, 2005; Qurashi and Sabri, 2011). Thus, it is reasonable to assume that the presence of the *nuo* operon genes in the KJ40 and in the VK-A60T strains, both rhizosphere-derived isolates, may also be related to an increased resistance to osmotic stress, as it also seems to be the case to our marine sponge-derived isolates. Likewise, it is also possible that the SM254 strain, isolated from copper-rich subsurface fluids in an iron mine, will be exposed to osmotic stress and hence require appropriate adaptations to these conditions. Hence, it is plausible that the genes encoded in the *nuo* operon are not an adaptive response that is exclusively employed by some marine bacteria, as previously suggested (Penn and Jensen, 2012; Ian et al., 2014; Sun et al., 2018), but rather a more general mechanism of osmoadaptation that may be employed by bacteria in other environments as well.
Similarly, proP gene homologs were also present in all of the other genomes analyzed, with exception to the fungus-derived CLI2905 strain from the SM17 clade (Figure 2). Thus, given as has been previously discussed, that ProP acts both as an osmoregulator and as an osmosensor, together with transporting compatible solutes in *E. coli*, it may also be related to osmoadaptation in these isolates. These observations further highlight the potential adaptations which have been proposed in the ENA gene pool, that may be present in these other closely related relatives derived from other diverse environments, which may overlap with those identified in our marine sponge-associated SM17 and SM18 strains.
5. Conclusion

The *Streptomyces* genus is exceptionally important when it comes to the identification and production of bioactive molecules, but those derived from the marine environment are currently particularly not well characterized. This study provides novel insights into possible ENAs employed by *Streptomyces* spp. isolated from marine sponges, and how these are potentially linked to diverse secondary metabolite biosynthesis. By providing high quality genomic information for the SM17 and SM18 strains isolated from *Haliclona simulans*, which have been previously shown to have antimicrobial activity against important pathogens, we were able to perform several comparative analyses with their terrestrial counterparts *S. albus* J1074 and *S. pratensis* ATCC 33331. The genomic analyses identified a diversity of putative smBGCs, which could potentially explain the previously determined antimicrobial activities reported for these marine isolates, such as smBGCs potentially encoding the production of candicidin, antimycin, SGR PTMs, surugamides, and mannopeptimycin, in SM17; and smBGCs potentially encoding the production of bafilomycin, SGR PTMs, curamycin, and caboxamycin, in SM18. Several smBGCs appear to be unique in the marine isolates in comparison to their terrestrial counterparts, which is particularly true in the case of the *Streptomyces* sp. SM18 isolate, when compared to *S. pratensis* ATCC 33331. Interestingly, while SM18 contains smBGCs encoding the production of siderophores such as coelichelin and mirubactin, it lacks the smBGC encoding the production of desferrioxamines; which is to our knowledge the first report of a *Streptomyces* isolate lacking this capacity. Comparative genomics analysis allowed us to identify genes that could be involved in mechanisms that may be relevant for their adaptation to their particular environmental niche, including resistance to osmotic stress; transcriptional regulation; symbiotic interactions; antimicrobial compounds production and resistance; ABC transporters; and HGT and other
potential defense-related features. Expanding on the genetic knowledge of these organisms and their underlying mechanisms of adaptability is important, in not only allowing us to gain a better understanding of marine bacteria and their evolution, but also in helping with the discovery of potential new bioactive small molecules and in how to potentially manipulate and optimize their production.
6. References


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doi:10.1021/acs.orglett.8b03373.


### Supplementary material

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**Table S1**: Putative smBGCs predicted to be present in the SM17 genome using the antiSMASH program.
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<td>Hopene biosynthetic gene cluster (69% of genes show similarity)</td>
</tr>
</tbody>
</table>
Cluster 23 Nrps 6978302 7029194  Coelichelin biosynthetic gene cluster (100% of genes show similarity)
Cluster 24 T1pks-Nrps 7030460 7111803  Herboxidiene biosynthetic gene cluster (16% of genes show similarity)
Cluster 25 T1pks 7248399 7346791  Bafilomycin biosynthetic gene cluster (100% of genes show similarity)
Cluster 26 Nrps 7527142 7577107  Mirubactin biosynthetic gene cluster (50% of genes show similarity)

Table S2: Putative smBGCs predicted to be present in the SM18 genome using the antiSMASH program.
<table>
<thead>
<tr>
<th>Cluster</th>
<th>Type</th>
<th>From</th>
<th>To</th>
<th>Most similar known cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>T1pks-Nrps</td>
<td>3011</td>
<td>61711</td>
<td></td>
</tr>
<tr>
<td>Cluster 2</td>
<td>T1pks-Nrps</td>
<td>224752</td>
<td>274162</td>
<td>SGR PTMs biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 3</td>
<td>Terpene</td>
<td>308626</td>
<td>335190</td>
<td>Hopene biosynthetic gene cluster (76% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 4</td>
<td>Bacteriocin</td>
<td>415688</td>
<td>425903</td>
<td></td>
</tr>
<tr>
<td>Cluster 5</td>
<td>Otherks</td>
<td>669953</td>
<td>711002</td>
<td>Avermectin biosynthetic gene cluster (66% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 6</td>
<td>Bacteriocin</td>
<td>879961</td>
<td>891289</td>
<td></td>
</tr>
<tr>
<td>Cluster 7</td>
<td>Nrps</td>
<td>1136316</td>
<td>1199422</td>
<td>Tetronasin biosynthetic gene cluster (9% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 8</td>
<td>Siderophore</td>
<td>1268164</td>
<td>1283196</td>
<td></td>
</tr>
<tr>
<td>Cluster 9</td>
<td>Terpene</td>
<td>1531774</td>
<td>1554059</td>
<td>Kanamycin biosynthetic gene cluster (1% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 10</td>
<td>Terpene</td>
<td>1865241</td>
<td>1886215</td>
<td>Albaflavenone biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 11</td>
<td>Thiopptide</td>
<td>2376688</td>
<td>2409159</td>
<td></td>
</tr>
<tr>
<td>Cluster 12</td>
<td>Bacteriocin</td>
<td>2560714</td>
<td>2571226</td>
<td>Goadsporin biosynthetic gene cluster (12% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 13</td>
<td>Lantipeptide</td>
<td>2694944</td>
<td>2735867</td>
<td>SAL-2242 biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 14</td>
<td>Nrps</td>
<td>3553726</td>
<td>3604015</td>
<td>Scabichelin biosynthetic gene cluster (40% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 15</td>
<td>Nrps</td>
<td>3877105</td>
<td>3982777</td>
<td>Mannopeptimycin biosynthetic gene cluster (7% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 16</td>
<td>Nrps</td>
<td>4469477</td>
<td>4513826</td>
<td></td>
</tr>
<tr>
<td>Cluster 17</td>
<td>Siderophore</td>
<td>4740450</td>
<td>4752270</td>
<td>Desferrioxamine B biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 18</td>
<td>Ectoine</td>
<td>5635346</td>
<td>5645744</td>
<td>Ectoine biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 19</td>
<td>Other</td>
<td>6337383</td>
<td>6381213</td>
<td>Indigoidine biosynthetic gene cluster (80% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 20</td>
<td>Bacteriocin-Terpene</td>
<td>6396161</td>
<td>6430146</td>
<td>Carotenoid biosynthetic gene cluster (54% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 21</td>
<td>T3pks</td>
<td>6520374</td>
<td>6561471</td>
<td>Herboxidiene biosynthetic gene cluster (12% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 22</td>
<td>Lantipeptide-T1pks-Nrps</td>
<td>6566423</td>
<td>6838639</td>
<td>Candididin biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
</tbody>
</table>

Table S3: Putative smBGCs predicted to be present in the *S. albus* J1074 genome using the antiSMASH program.
<table>
<thead>
<tr>
<th>Cluster</th>
<th>Type</th>
<th>From</th>
<th>To</th>
<th>Most similar known cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
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<td>70873</td>
<td>221343</td>
<td>Carbapenem MM 4550 biosynthetic gene cluster (65% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>Nrps</td>
<td>327693</td>
<td>378596</td>
<td>Coelicelin biosynthetic gene cluster (90% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 3</td>
<td>Terpene</td>
<td>393138</td>
<td>418630</td>
<td>Isorenieratene biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 4</td>
<td>Bacteriocin</td>
<td>557521</td>
<td>568045</td>
<td></td>
</tr>
<tr>
<td>Cluster 5</td>
<td>Blactam</td>
<td>634715</td>
<td>658209</td>
<td>Clavulanic acid biosynthetic gene cluster (20% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 6</td>
<td>Terpene</td>
<td>738599</td>
<td>765184</td>
<td>Hopene biosynthetic gene cluster (69% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 7</td>
<td>T1pks</td>
<td>1104244</td>
<td>1198984</td>
<td>Vicenistatin biosynthetic gene cluster (60% of genes show similarity)</td>
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<tr>
<td>Cluster 8</td>
<td>Bacteriocin</td>
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<td>1420548</td>
<td></td>
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<tr>
<td>Cluster 9</td>
<td>Nrps</td>
<td>1560425</td>
<td>1622443</td>
<td>Arylomycin biosynthetic gene cluster (22% of genes show similarity)</td>
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<tr>
<td>Cluster 10</td>
<td>Siderophore</td>
<td>1894420</td>
<td>1909004</td>
<td></td>
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<td>Cluster 11</td>
<td>Terpene</td>
<td>1973589</td>
<td>1994659</td>
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<tr>
<td>Cluster 12</td>
<td>Bacteriocin</td>
<td>2151745</td>
<td>2161966</td>
<td></td>
</tr>
<tr>
<td>Cluster 13</td>
<td>Butyrolactone</td>
<td>3819031</td>
<td>3829957</td>
<td>Lactonamycin biosynthetic gene cluster (3% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 14</td>
<td>T1pks-Nrps</td>
<td>4023172</td>
<td>4080102</td>
<td>Istamycin biosynthetic gene cluster (11% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 15</td>
<td>Siderophore</td>
<td>4727646</td>
<td>4739427</td>
<td>Desferrioxamine B biosynthetic gene cluster (83% of genes show similarity)</td>
</tr>
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<td>Cluster 16</td>
<td>Lantipeptide</td>
<td>4792335</td>
<td>4815403</td>
<td></td>
</tr>
<tr>
<td>Cluster 17</td>
<td>Terpene</td>
<td>5226078</td>
<td>5247109</td>
<td></td>
</tr>
<tr>
<td>Cluster 18</td>
<td>Ectoine</td>
<td>5705549</td>
<td>5715947</td>
<td>Ectoine biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 19</td>
<td>T2pks-Otherks</td>
<td>6079056</td>
<td>6136319</td>
<td>Cinerubin B biosynthetic gene cluster (28% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 20</td>
<td>Terpene</td>
<td>6181067</td>
<td>6202113</td>
<td>Steffimycin biosynthetic gene cluster (19% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 21</td>
<td>Ectoine-Terpene</td>
<td>6482662</td>
<td>6509101</td>
<td>Ectoine biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster 22</td>
<td>Bacteriocin</td>
<td>6532212</td>
<td>6542439</td>
<td></td>
</tr>
<tr>
<td>Cluster 23</td>
<td>T3pks</td>
<td>6695745</td>
<td>6736803</td>
<td>Tetronasin biosynthetic gene cluster (11% of genes show similarity)</td>
</tr>
<tr>
<td>Cluster</td>
<td>Type</td>
<td>Genes</td>
<td>Genes</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>---------------</td>
<td>-------</td>
<td>-------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>24</td>
<td>Melanin</td>
<td>6830784</td>
<td>6841248</td>
<td>Melanin biosynthetic gene cluster (100% of genes show similarity)</td>
</tr>
<tr>
<td>25</td>
<td>T2pk- Terpene</td>
<td>6882032</td>
<td>6931236</td>
<td>Spore pigment biosynthetic gene cluster (83% of genes show similarity)</td>
</tr>
<tr>
<td>26</td>
<td>Nrps</td>
<td>7082613</td>
<td>7132319</td>
<td>Zorbamycin biosynthetic gene cluster (6% of genes show similarity)</td>
</tr>
<tr>
<td>27</td>
<td>Butyrolactone</td>
<td>7211688</td>
<td>7222623</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table S4:** Putative smBGCs predicted to be present in the *S. pratensis* ATCC 33331 genome using the antiSMASH program.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Annotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>adhD</td>
<td>NDMA-dependent alcohol dehydrogenase / Zinc-binding alcohol dehydrogenase</td>
</tr>
<tr>
<td>abcY</td>
<td>Adenosylhomocysteinase</td>
</tr>
<tr>
<td>aprX</td>
<td>Serine protease AprX / Subtilase family protein / Peptidase S8</td>
</tr>
<tr>
<td>bepR</td>
<td>HTH-type transcriptional repressor BepR / TetR family transcriptional regulator</td>
</tr>
<tr>
<td>bioC</td>
<td>Malonyl-[acyl-carrier protein] O-methyltransferase / Class I SAM-dependent methyltransferase</td>
</tr>
<tr>
<td>cpnA</td>
<td>Cyclopentanol dehydrogenase / SDR family oxidoreductase</td>
</tr>
<tr>
<td>cynR</td>
<td>HTH-type transcriptional regulator CynR / LysR family transcriptional regulator</td>
</tr>
<tr>
<td>degU</td>
<td>Transcriptional regulatory protein DegU / DNA-binding response regulator</td>
</tr>
<tr>
<td>fccA</td>
<td>Fumarate reductase flavoprotein subunit / FAD-dependent oxidoreductase</td>
</tr>
<tr>
<td>folQ</td>
<td>Putative DHNTP pyrophosphohydrolase / NUDIX hydrolase</td>
</tr>
<tr>
<td>group_1044</td>
<td>Integrase core domain / IS3 family transposase</td>
</tr>
<tr>
<td>group_1217</td>
<td>Fumarylacetoacetase</td>
</tr>
<tr>
<td>group_1272</td>
<td>Toxin-antitoxin system, RelE family</td>
</tr>
<tr>
<td>group_1944</td>
<td>Restriction endonuclease</td>
</tr>
<tr>
<td>group_1945</td>
<td>IS3 family transposase</td>
</tr>
<tr>
<td>group_5008</td>
<td>Alpha-ketoglutaric semialdehyde dehydrogenase / NADP-dependent aldehyde dehydrogenase</td>
</tr>
<tr>
<td>group_5198</td>
<td>Aminoglycoside phosphotransferase</td>
</tr>
<tr>
<td>group_5212</td>
<td>NADH:flavin oxidoreductase</td>
</tr>
<tr>
<td>group_5385</td>
<td>Aminoglycoside phosphotransferase</td>
</tr>
<tr>
<td>group_5540</td>
<td>DUF3307 domain-containing protein</td>
</tr>
<tr>
<td>group_5542</td>
<td>Phosphohydrolase</td>
</tr>
<tr>
<td>group_5643</td>
<td>ATP/GTP-binding protein</td>
</tr>
<tr>
<td>group_5772</td>
<td>Tetratricopeptide repeat protein</td>
</tr>
<tr>
<td>group_5776</td>
<td>Radical SAM protein</td>
</tr>
<tr>
<td>group_5789</td>
<td>Darcynin</td>
</tr>
<tr>
<td>group_5793</td>
<td>Amine oxidase, flavin-containing</td>
</tr>
<tr>
<td>group_5796</td>
<td>Transcriptional regulator, IclR family</td>
</tr>
<tr>
<td>group_5798</td>
<td>Nuclear transport factor 2 family protein</td>
</tr>
<tr>
<td>group_5803</td>
<td>Nuclear transport factor 2 family protein</td>
</tr>
<tr>
<td>group_5818</td>
<td>Acyltransferase 3</td>
</tr>
<tr>
<td>group_5819</td>
<td>Transcriptional regulator PadR-like family protein</td>
</tr>
<tr>
<td>group_5821</td>
<td>ABC transporter permease</td>
</tr>
<tr>
<td>group_5836</td>
<td>Acyltransferase</td>
</tr>
<tr>
<td>hmgA</td>
<td>Homogentisate 1,2-dioxygenase</td>
</tr>
</tbody>
</table>
hsdA<sup>*</sup> 3-alpha-hydroxysteroid dehydrogenase/carbonyl reductase / SDR family oxidoreductase
htpG Chaperone protein HtpG / heat shock protein 90
liaS HPK7 family sensor histidine kinase LiaS
mitC<sup>b</sup> Putative mycofactocin radical SAM maturase MitC / radical SAM protein
ndx1<sup>*</sup> NUDIX hydrolase
nuoA<sup>a</sup> NADH-quinone oxidoreductase subunit A
nuoH<sup>a</sup> NADH-quinone oxidoreductase subunit H
nuoJ<sup>a</sup> NADH-quinone oxidoreductase subunit J
nuoM<sup>a</sup> NADH-quinone oxidoreductase subunit M
nuoN<sup>a</sup> NADH-quinone oxidoreductase subunit N
proP Proline/betaine transporter
ptsG PTS system glucose-specific EIICB component
rhmR<sup>a</sup> HTH-type transcriptional regulator KipR / MarR family transcriptional regulator
scoA<sup>*</sup> 3-oxoacid CoA-transferase, A subunit
scoB<sup>a</sup> 3-oxoacid CoA-transferase, B subunit
ssuE<sup>b</sup> FMN reductase (NADPH)
tauB Aliphatic sulfonates import ATP-binding protein SsuB / ABC transporter ATP-binding protein
tcrA<sup>a</sup> Transcriptional regulatory protein CutR / DNA-binding response regulator
xeCD<sup>a</sup> SDR family oxidoreductase
yihX<sup>*</sup> Alpha-D-glucose 1-phosphate phosphatase YihX / HAD family phosphatase
yknY<sup>a</sup> Uncharacterised ABC transporter ATP-binding protein YknY

Table S5: List of orthologous genes and their respective annotations (excluding hypothetical proteins), which are commonly present in the sponge-derived isolates SM17 and SM18, while absent in their terrestrial counterparts J1074 and ATCC 33331. When the gene name was not determined, a generic unique name was given (group_XXXX) by the Roary program. "<sup>*</sup>" - genes without multiple copies or paralogs in the terrestrial isolates’ genomes, considering only the ones with a defined gene name; "<sup>a</sup>" - genes for which both sponge-derived isolates presented a higher copy number in comparison to their terrestrial counterparts; "<sup>b</sup>" - genes for which the SM17 isolate had a higher copy number in comparison to its terrestrial counterpart <i>S. albus</i> J1074; "<sup>c</sup>" - genes for which the SM18 isolate had a higher copy number in comparison to its terrestrial counterpart <i>S. pratensis</i> ATCC 33331.
Table S6: Nucleotide sequence identity comparison between the partial *nuo*-operon present in the marine isolates *Streptomyces* sp. SM17, *Streptomyces* sp. SM18, *Salinispora arenicola* CNS-205, *Salinispora tropica* CNB-440, and *Kocuria flava* S43, obtained using discontiguous MegaBLAST alignments.

<table>
<thead>
<tr>
<th></th>
<th><em>Streptomyces</em> sp. SM17</th>
<th><em>Streptomyces</em> sp. SM18</th>
<th><em>Salinispora arenicola</em> CNS-205</th>
<th><em>Salinispora tropica</em> CNB-440</th>
<th><em>Kocuria flava</em> S43</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces</em> sp. SM17</td>
<td>-</td>
<td>70%</td>
<td>69%</td>
<td>69%</td>
<td>70%</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. SM18</td>
<td>70%</td>
<td>-</td>
<td>68%</td>
<td>69%</td>
<td>68%</td>
</tr>
<tr>
<td><em>Salinispora arenicola</em> CNS-205</td>
<td>69%</td>
<td>68%</td>
<td>-</td>
<td>86%</td>
<td>73%</td>
</tr>
<tr>
<td><em>Salinispora tropica</em> CNB-440</td>
<td>69%</td>
<td>69%</td>
<td>86%</td>
<td>-</td>
<td>73%</td>
</tr>
<tr>
<td><em>Kocuria flava</em> S43</td>
<td>70%</td>
<td>68%</td>
<td>73%</td>
<td>73%</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure S1: Whole genomes nucleotide alignments performed using the MUMmer 3 program. A) Alignment between the *S. albus* J1074 chromosome (x axis) and the SM17 chromosome (y axis) sequences. B) Alignment between the *S. pratensis* ATCC 33331 chromosome (x axis) and the SM18 chromosome (y axis) sequences.
**Figure S2:** Venn diagram representing the presence/absence of orthologous genes in the genome regions predicted to contain smBGCs in the SM17, SM18, *S. albus* J1074, and the *S. pratensis* ATCC 33331 genomes.
Chapter 3

Genome mining coupled with OSMAC-based cultivation reveal differential production of surugamide A by the marine sponge isolate *Streptomyces* sp. SM17 when compared to its terrestrial relative *S. albido flavus* J1074

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2Marine Biodiscovery, School of Chemistry and Ryan Institute, National University of Ireland Galway (NUI Galway), University Road, H91 TK33 Galway

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1. Abstract

Much recent interest has arisen in investigating *Streptomyces* isolates derived from the marine environment in the search for new bioactive compounds, particularly those found in association with marine invertebrates, such as sponges. Among these new compounds recently identified from marine *Streptomyces* isolates are the octapeptidic surugamides, which have been shown to possess anticancer and antifungal activities. In this study, based on genome mining followed by an OSMAC-based approach, we identified the previously unreported capability of a marine sponge-derived isolate, namely *Streptomyces* sp. SM17, to produce surugamide A. Phylogenomics analyses provided novel insights with respect to the distribution and conservation of the surugamides biosynthetic gene cluster (*sur* BGC) at a genetic level. We observed differential production of surugamide A when comparing the closely related marine and terrestrial isolates, namely *Streptomyces* sp. SM17 and *Streptomyces albidoflavus* J1074. SM17 produced higher levels of surugamide A than *S. albidoflavus* J1074 under all conditions tested, and in particular producing >13-fold higher levels when grown in YD and 3-fold higher levels in SYP-NaCl medium. In addition, surugamide A production was repressed in TSB and YD medium, suggesting that carbon catabolite repression (CCR) may influence the production of surugamides in these strains.
2. Introduction

Members of the *Streptomyces* genus are widely known to be prolific producers of natural products. Many of these compounds have found widespread use in the pharmaceutical industry as antibiotics, immunosuppressant, antifungal, anticancer and anti-parasitic drugs (Hwang et al., 2014). However, there continues to be an urgent need to discover new bioactive compounds, and especially antibiotics; primarily due to the emergence of antibiotic resistance in clinically important bacterial pathogens (Thabit et al., 2015; Tommasi et al., 2015). In particular, the increase in multi-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) has focused research efforts to develop new antibiotics to treat these priority antibiotic-resistant bacteria (Demers et al., 2018).

Up until relatively recently, marine ecosystems had largely been neglected as a potential source for the discovery of novel bioactive compounds, in comparison to terrestrial environments; primarily due to issues of accessibility (Indraningrat et al., 2016). Marine sponges are known to host a variety of different bacteria and fungi which produce a diverse range of natural products, including compounds with antiviral, antifungal, antiprotozoal, antibacterial, and anticancer activities (Calcabrini et al., 2017; Indraningrat et al., 2016). Marine sponge-associated *Streptomyces* spp. are a particularly important source of bioactive compounds, with examples including *Streptomyces* sp. HB202, isolated from the sponge *Halichondria panicea* which produces mayamycin, a compound with activity against *Staphylococcus aureus* (Schneemann et al., 2010); and streptophenazines G and K, with activity against *Bacillus subtilis* (Kunz et al., 2014); together with *Streptomyces* sp. MAPS15, which was isolated from *Spongia officinalis* which produces 2-pyrrolidine, with activity against *Klebsiella*...
pneumoniae (Sathiyanarayanan et al., 2014). Additionally, our group has reported the production of antimycins from *Streptomyces* sp. SM8 isolated from the sponge *Haliclona simulans*, with antifungal and antibacterial activities (Almeida et al., 2018; Viegelmann et al., 2014). In further work we genetically characterised 13 *Streptomyces* spp. that were isolated from both shallow and deep-sea sponges, which displayed antimicrobial activities against a number of clinically relevant bacterial and yeast species (Jackson et al., 2018; Kennedy et al., 2009). Amongst these strains, the *Streptomyces* sp. SM17 demonstrated an ability to inhibit the growth of *E. coli* NCIMB 12210, methicillin resistant *S. aureus* (MRSA) and *Candida* spp., when employing deferred antagonism assays (Jackson et al., 2018; Kennedy et al., 2009).

Among other clinically relevant natural products derived from marine *Streptomyces* isolates are the recently identified surugamides family of molecules. The cyclic octapeptide surugamide A and its derivatives were originally identified in the marine-derived *Streptomyces* sp. JAMM992 (Takada et al., 2013), and have been shown to belong to a particularly interesting family of compounds due not only to their relevant bioactivity, but also due to their unusual metabolic pathway involving d-amino acids (Matsuda et al., 2019b; Takada et al., 2013; Xu et al., 2017). Since their discovery, concerted efforts have been employed in order to chemically characterise these compounds and determine the genetic mechanisms involved in their production (Kuranaga et al., 2018; Matsuda et al., 2019b; Ninomiya et al., 2016; Takada et al., 2013; Thankachan et al., 2019; Zhou et al., 2019). The surugamides and their derivatives have been shown to possess a number of bioactivities, with the surugamides A-E and the surugamides G-J being shown to possess anticancer activity by inhibiting bovine cathepsin B, a cysteine protease reported to be involved in the invasion of metastatic tumour cells (Takada et al., 2013; Xu et al., 2017); while another derivative, namely acyl-surugamide A, has been shown to
possess anti-fungal activity (Xu et al., 2017). It has been determined that the non-ribosomal peptide synthase-encoding \textit{surABCD} genes are the main biosynthetic genes involved in the biosynthesis of surugamides and their derivatives (Ninomiya et al., 2016), with these genes being involved in the production of at least 20 different compounds (Xu et al., 2017). Surugamides A-E have been reported to be produced by the \textit{surA} and \textit{surD} genes, while the linear decapeptide surugamide F has been shown to be produced by the \textit{surB} and \textit{surC} genes, involving a unique pattern of intercalation of the biosynthetic genes (Ninomiya et al., 2016). Further metabolic pathways studies have reported that the expression of the \textit{surABCD} gene cluster is strongly regulated by the \textit{surR} transcriptional repressor (Xu et al., 2017), while the cyclisation of the cyclic surugamides has been shown to involve a penicillin binding protein (PBP)-like thioesterase encoded by the \textit{surE} gene (Matsuda et al., 2019a; Thankachan et al., 2019; Zhou et al., 2019).

Although apparently widespread in marine-derived \textit{Streptomyces} isolates (Ninomiya et al., 2016; Zhou et al., 2019), the production of surugamides has also been reported in the \textit{S. albidoflavus} strain J1074 (Koshla et al., 2019; Xu et al., 2017), a derivative of the soil isolate \textit{S. albus} G (Chater and Wilde, 1976, 1980). The \textit{S. albidoflavus} strain J1074 is a well characterised \textit{Streptomyces} isolates which is frequently used as a model for the genus, and has commonly been successfully employed in the heterologous expression of biosynthetic gene clusters (BGCs) (Bilyk et al., 2016; Huang et al., 2019; Jiang et al., 2018; Myronovskyi et al., 2018; Zaburannyi et al., 2014). This strain was originally classified as an \textit{S. albus} isolate, however, due to more recent taxonomy studies, it has been re-classified as a \textit{S. albidoflavus} species isolate (Labeda et al., 2014, 2017). Interestingly, surugamides and their derivatives have been shown to only be produced by \textit{S. albidoflavus} J1074 under specific conditions, such as when employing chemical stress elicitors.
(Xu et al., 2017), and more recently when cultivating the strain in a soytone-based liquid based medium SG2(Koshla et al., 2019).

In a previous study (Almeida et al., 2019), we reported that the *S. albidoflavus* J1074 and *Streptomyces* sp. SM17 possessed morphological and genetic similarities. Differences were observed, however, when both strains were exposed to high salt concentrations using culture media, such as TSB or ISP2, in which the marine sponge-derived strain SM17 grew and differentiated more rapidly in comparison with the soil strain *S. albidoflavus* J1074, which appeared to have trouble growing and differentiating when salts were present in the growth medium (Almeida et al., 2019). Genome mining based on the prediction of secondary metabolites BGCs also showed many similarities between the two strains (Almeida et al., 2019). Among these predicted BGCs, both the *S. albidoflavus* J1074 and *Streptomyces* sp. SM17 isolates appeared to possess the *sur* BGC, encoding for the production of surugamides A/D; which had previously been identified in other marine-derived *Streptomyces* isolates (Ninomiya et al., 2016; Zhou et al., 2019), and which as has been previously mentioned is produced by the terrestrial isolate *S. albidoflavus* J1074 under specific culture conditions (Koshla et al., 2019; Xu et al., 2017). This prompted us to further investigate other isolates that shared genetic similarities to *S. albidoflavus* J1074, with respect to potential similarities in their genomes and between the metabolic profiles of the marine *Streptomyces* sp. SM17 and terrestrial *S. albidoflavus* isolates; particularly with respect to production of the well characterised *sur* BGC natural product surugamide A. To this end we employed an "One Strain Many Compounds" (OSMAC)-based approach, which has been shown to be a useful strategy in eliciting production of natural products from silent gene clusters by employing different culture conditions (Pan et al., 2019; Romano et al., 2018); to monitor production of surugamide A in both *S. albidoflavus* J1074 and *Streptomyces* sp. SM17.
3. Results and discussion

3.1. Multi-locus sequence analysis and taxonomy assignment of the *Streptomyces* sp. SM17 isolate

In order to taxonomically characterise the *Streptomyces* sp. SM17 isolate based on genetic evidence, multi-locus sequence analysis (MLSA) (Glaeser and Kämpfer, 2015) employing the 16S rRNA sequence, in addition to five housekeeping genes, namely *atpD* (ATP synthase subunit beta), *gyrB* (DNA gyrase subunit B), *recA* (recombinase RecA), *rpoB* (DNA-directed RNA polymerase subunit beta), and *trpB* (tryptophan synthase beta chain) was performed, in a similar manner to a previous report (Almeida et al., 2019). A similarity search was performed in the GenBank database (Benson et al., 2018), using the NCBI BLASTN tool (Camacho et al., 2009; Johnson et al., 2008), based on the 16S rRNA nucleotide sequence of the SM17 isolate. The top 30 most similar *Streptomyces* species for which complete genome sequences were available in GenBank were selected for further phylogenetic analysis.

The concatenated nucleotide sequences (Gadagkar et al., 2005; Glaeser and Kämpfer, 2015) of the 16S rRNA and the aforementioned five housekeeping genes, were first aligned using the MAFFT program (Katoh and Standley, 2013), and the phylogeny analysis was performed using the MrBayes program (Ronquist et al., 2012). The General Time Reversible (GTR) model of nucleotide substitution with gamma-distributed rates across sites with a proportion of invariable sites was applied (Waddell and Steel, 1997), with 1 million generations sampled every 100 generations. The final phylogenetic tree was then processed using MEGA X (Kumar et al., 2018), with a posterior probability cut off of 95% (Figure 1).
**Figure 1**: Phylogenetic tree of the concatenated sequences of the 16S rRNA and the housekeeping genes *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*, from the *Streptomyces* sp. SM17 together with 30 *Streptomyces* isolates for which complete genome sequences were available in the GenBank database. Analysis was performed using MrBayes, with a posterior probability cut off of 95%. 1) *albidoflavus* phylogroup. 2) clade including the neighbour isolate *Streptomyces koyangensis* strain VK-A60T. The strains SM17 and J1074 are indicated with asterisks.
The resulting phylogenetic tree clearly indicates the presence of a clade that includes the isolates *Streptomyces albidoflavus* strain J1074; *Streptomyces* sp. SM17; *Streptomyces albidoflavus* strain SM254; *Streptomyces sampsonii* strain KJ40; *Streptomyces* sp. FR-008; and *Streptomyces koyangensis* strain VK-A60T (clade 2 in Figure 1). In addition, this larger clade contains a sub-clade (clade 1 in Figure 1) that includes *Streptomyces* isolates similar to the type-strain *Streptomyces albidoflavus* J1074. The J1074 strain is a well-studied *Streptomyces* isolate widely used as a model for the genus and for various biotechnological applications, including the heterologous expression of secondary metabolites biosynthetic gene clusters (BGCs) (Bilyk et al., 2016; Huang et al., 2019; Jiang et al., 2018; Myronovskyi et al., 2018; Zaburannyi et al., 2014). This isolate was originally classified as “*Streptomyces albus* J1074”, but due to recent taxonomy data, it has been re-classified as *Streptomyces albidoflavus* J1074 (Labeda et al., 2014, 2017). Hence, in this study, this strain will be referred to as *Streptomyces albidoflavus* J1074, and this clade will from now on be referred to as the *albidoflavus* phylogroup (Figure 1).

Interestingly, members of the *albidoflavus* phylogroup were all isolated from quite different environments. The *Streptomyces albidoflavus* strain J1074 stems from the soil isolate *Streptomyces albus* G (Chater and Wilde, 1976, 1980). The *Streptomyces sampsonii* strain KJ40 was isolated from rhizosphere soil in a poplar plantation (Li et al., 2018). The *Streptomyces* sp. strain FR-008 is a random protoplast fusion derivative of two *Streptomyces hygroscopicus* isolates (Liu et al., 2016). On the other hand, two of these strains were isolated from aquatic saline environments, with *Streptomyces* sp. SM17 being isolated from the marine sponge *Haliclona simulans* (Kennedy et al., 2009); while the *Streptomyces albidoflavus* strain SM254 strain was isolated from copper-rich subsurface fluids within an iron mine, following growth on artificial sea water (ASW) (Badalamenti et al., 2016). The
fact that these isolates, although derived from quite distinct environmental niches, simultaneously share significant genetic similarities is interesting, and raises questions about their potential evolutionary relatedness.

3.2. Analysis of groups of orthologous genes in the *albidoflavus* phylogroup

In an attempt to provide further genetic evidence with respect to the similarities shared among the members of the *albidoflavus* phylogroup (Figure 1), a pan-genome analysis was performed to determine the number of core genes, accessory genes, and unique genes present in this group of isolates. The Roary program was employed for this objective (Page et al., 2015), which allowed the identification of groups of orthologous and paralogous genes (which from now on will be referred to simply as “genes”) present in the set of *albidoflavus* genomes, with a protein identity cut off of 95%, which is the identity value recommended by the Roary program manual when analysing organisms belonging to the same species.

A total of 7,565 genes were identified in the *albidoflavus* pan-genome, and among these a total of 5,177 were determined to be shared among all the *albidoflavus* isolates (i.e. the core genome) (Figure 2). This represents a remarkably high proportion of genes which appear to be highly conserved between all the isolates, representing approximately 68.4% of the pan-genome. Additionally, when considering the genomes individually (Table S1), the core genome accounts for approximately 84.5% of the FR-008 genome; 88.5% of J1074; 85.5% of KJ40; 86.7% of SM17; and 83.7% of the SM254 genome. On the other hand, the accessory genome (i.e. genes present in at least two isolates) was determined to consist of 1,055 genes (or ~13.9% of the pan-genome); while the unique genome (i.e. genes present in only one isolate) was determined to comprise of 1,333 genes (or ~17.6% of the pan-genome). This strikingly high conservation of genes present in their genomes,
together with the previous multi-locus phylogeny analysis, are very strong indicators that these microorganisms may belong to the same species.

**Figure 2**: Venn diagram representing the presence/absence of groups of orthologous genes in the organisms belonging to the *albidoflavus* phylogroup.

An additional pan-genome analysis similar to the aforementioned analysis was also performed including the *Streptomyces koyangensis* strain VK-A60T in the dataset (Figure S1), which was an isolate shown to be a closely related neighbour to the *albidoflavus* phylogroup (Figure 1, clade 2). When compared to the previous analysis, the pan-genome analysis including the VK-A60T isolate showed significant changes in the values representing the core genome, which changed from 5,177 genes (Figure 2) to 3,912 genes (Figure S1), with an additional 1,273 genes also shared among all of the *albidoflavus* isolates (Figure S1). The results also showed a much larger number of genes uniquely present in the VK-A60T genome.
than in the other genomes, with 2,059 unique genes identified from a total of 6,245 CDSs present in the VK-A60T genome in total, or approximately a third of its total number of genes (Figure S1). This proportion of unique genes present in the VK-A60T genome is considerably higher than the proportions of unique genes observed in the other *albidoflavus* phylotype genomes (Figure 2), which accounted for approximately only 2.5% of the total number of genes in SM17; 4.2% in J1074; 4.9% in KJ40; 5% in FR-008; and 5.1% in SM254. Taken together, these results further demonstrate the similarities between the isolates belonging to the *albidoflavus* phylogroup, while the VK-A60T isolate is clearly more distantly related.

Thus, from previous studies (Labeda et al., 2014, 2017), and in light of the phylogeny analysis and further genomic evidence presented in this study, it is likely that all the isolates belonging to the *albidoflavus* phylogroup are in fact members of the same species. It is reasonable to infer that, for example, the isolates in the *albidoflavus* phylogroup that possess no species assignment thus far (i.e. strains SM17 and FR-008) are indeed members of the *albidoflavus* species. Also, it is possible that the *Streptomyces sampsonii* KJ40 has been misassigned, and possibly requires re-classification as an *albidoflavus* isolate.

Misassignment and re-classification of *Streptomyces* species is a common issue, and an increase in the quantity and the quality of available data from these organisms (e.g. better quality genomes available in the databases) will provide better support for taxonomy claims, or correction of these when new information becomes available (Labeda et al., 2017; Li et al., 2019; Rong et al., 2013; Ward and Allenby, 2018).
3.3. Prediction of secondary metabolites biosynthetic gene clusters in the *albidoflavus* phylogroup

Isolates belonging to the *albidoflavus* phylogroup have been reported to produce bioactive compounds of pharmacological relevance, such as antibiotics. As mentioned previously, the *Streptomyces albidoflavus* strain J1074 is the best described member of the *albidoflavus* phylogroup to date. As such, several of secondary metabolites produced by this isolate have been identified, including acyl-surugamides and surugamides with antifungal and anticancer activities, respectively (Xu et al., 2017); together with paulomycin derivatives with antibacterial activity (Hoz et al., 2017). The *Streptomyces* sp. FR-008 isolate has been shown to produce the antimicrobial compound FR-008/candicidin (Chen et al., 2003; Zhao et al., 2015); while the *Streptomyces sampsonii* KJ40 isolate has been shown to produce a chitinase that possesses anti-fungal activity against plant pathogens (Li et al., 2018). On the other hand, although no bioactive compound have been characterised from *Streptomyces albidoflavus* SM254, this isolate has been shown to possess anti-fungal activity, specifically against the fungal bat pathogen *Pseudogymnoascus destructans*, which is responsible for the White-nose Syndrome (Badalamenti et al., 2016; Hamm et al., 2017). The *Streptomyces* sp. SM17 isolate has also previously been shown to possess antibacterial and antifungal activities against clinically relevant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) (Kennedy et al., 2009). However, no natural products derived from this strain have been identified and isolated until now.

In order to further *in silico* assess the potential of these *albidoflavus* phylogroup isolates to produce secondary metabolites, and also to determine how potentially similar or diverse they are within this phylogroup, prediction of secondary metabolites biosynthetic gene clusters (BGCs) was performed using the
antSMASH (version 5) program (Blin et al., 2019). The antSMASH prediction was processed using the BiG-SCAPE program (Navarro-Muñoz et al., 2018), in order to cluster the BGCs into gene cluster families (GCFs), based on sequence and Pfam (El-Gebali et al., 2019) protein families similarity, and also by comparing them to the BGCs available from the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository (Medema et al., 2015) (Figure 3). When compared to known BGCs from the MIBiG database, a significant number of BGCs predicted to be present in the albidoflavus phylogroup genomes could potentially encode for the production of novel compounds, including those belonging to the non-ribosomal peptide synthetase (NRPS) and bacteriocin families of compounds (Figure 3). The presence/absence of homologous BGCs in the albidoflavus isolates’ genomes was determined using BiG-SCAPE and is represented in Figure 4. Interestingly, the vast majority of the BGCs predicted in the albidoflavus phylogroup are shared among all of its members (15 BGCs); while another large portion (8 BGCs) are present in at least two isolates (Figure 4). Among the five members of the albidoflavus phylogroup, only the J1074 strain and the SM17 strain appeared to possess unique BGCs when compared to the other strains. Three unique BGCs were predicted to be present in the J1074 genome: a predicted type I polyketide synthase (T1PKS)/NRPS without significant similarity to the BGCs from the MIBiG database; a predicted bacteriocin which also did not show any significant similarity to the BGCs from the MIBiG database; and a BGC predicted to encode for the production of the antibiotic paulomycin, with similarity to the paulomycin-encoding BGCs from Streptomyces paulus and Streptomyces sp. YN86 (Li et al., 2015), which has also been experimentally shown to be produced by the J1074 strain (Hoz et al., 2017). One BGC predicted to encode a type III polyketide synthase (T3PKS) – with no significant similarity to the BGCs from the MIBiG database – was also identified as being unique to the SM17 genome.
Figure 3: BGCs similarity clustering using BiG-SCAPE. Singletons, i.e. BGCs without significant similarity with the BGCs from the MIBiG database or with the BGCs predicted in other genomes, are not represented.

Figure 4: Venn diagram representing BGCs presence/absence in the genomes of the members of the *albidoflavus* phylogroup, determined using antiSMASH and BiG-SCAPE.
Importantly, BGCs with similarity to the surugamide A/D BGC from "Streptomyces albus J1074" (now classified as *S. albidoflavus*) from the MIBiG database (Xu et al., 2017) were identified in all the other genomes of the members of the *albidoflavus* phylogroup. This raises the possibility that this BGC may be commonly present in *albidoflavus* species isolates. However, as only a few complete genomes of isolates belonging to this phylogroup are currently available, further data will be required to support this hypothesis. Nevertheless, these results further highlight the genetic similarities of the isolates belonging to the *albidoflavus* phylogroup, even with respect to their potential to produce secondary metabolites.

### 3.4. Phylogeny and gene synteny analysis of sur BGC homologs

In parallel to the previous phylogenomics analysis performed with the *albidoflavus* phylogroup isolates, sequence similarity and phylogenetic analyses were performed, using the previously described and experimentally characterised *Streptomyces albidoflavus* LHW3101 surugamides biosynthetic gene cluster (*sur* BGC, GenBank accession number: MH070261) as a reference (Zhou et al., 2019). The aim was to assess how widespread in nature the *sur* BGC might be, and the degree of genetic variation, if any; that might be present in *sur* BGCs belonging to different microorganisms.

Nucleotides sequence similarity to the *sur* BGC was performed in the GenBank database (Benson et al., 2018), using the NCBI BLASTN tool (Camacho et al., 2009; Johnson et al., 2008). It is important to note that, since the quality of the data is crucial for sequence similarity, homology and phylogeny inquiries; only complete genome sequences were employed in this analysis. For this reason, for example, the marine *Streptomyces* isolate in which surugamides and derivatives were originally identified, namely *Streptomyces* sp. JAMM992 (Takada et al., 2013),
was not included, since its complete genome is not available in the GenBank database.

The sequence similarity analysis identified 5 microorganisms that possessed homologs to the sur BGC and had their complete genome sequences available in the GenBank database: *Streptomyces* sp. SM17; *Streptomyces albidosflavus* SM254; *Streptomyces* sp. FR-008; *Streptomyces albidosflavus* J1074; and *Streptomyces sampsonii* KJ40. Notably, these results overlapped with the isolates belonging to the previously discussed *albidosflavus* phylogroup (Figure 1), further highlighting the possibility that the sur BGC may be commonly present in and potentially exclusive to the *albidosflavus* species.

Phylogenetic analysis was performed in the genomic regions determined to be homologs to the *Streptomyces albidosflavus* LHW3101 sur BGC, using the MrBayes program (Ronquist et al., 2012) (Figure 5). Although a larger number of sequences should ideally be employed in this type of analysis, these results suggest the possibility of a clade with aquatic saline environment-derived sur BGCs (Figure 5). Thus, these aquatic saline environment-derived sur BGCs are likely to share more genetic similarities amongst each other, rather than with those derived from terrestrial environments. Since this analysis took into consideration the whole genome regions that contained the sur BGCs of each isolate, it is likely that the similarities and differences present in these regions involve not only coding sequences (CDSs) for biosynthetic genes and/or transcriptional regulators, but also could include promoter regions and other intergenic sequences.
**Figure 5:** Consensus phylogenetic tree of the *sur* BGC region of the *S. albidoflavus* LHW3101 reference *sur* BGC sequence, plus 5 *Streptomyces* isolates determined to have *sur* BGC homologs, generated using MrBayes and Mega X, with a 95% posterior probability cut off. Aquatic saline environment-derived isolates are highlighted in cyan.

With this in mind, the genomic regions previously determined to share homology with the *sur* BGC from *S. albidoflavus* LHW3101 were further analysed, with respect to the genes present in the surrounding region, the organisation of the BGCs, together with the overall gene synteny (Figure 6). Translated CDSs predicted in the region were manually annotated using the NCBI BLASTP tool (Camacho et al., 2009; Johnson et al., 2008), together with GenBank (Benson et al., 2018) and the CDD (Marchler-Bauer et al., 2015) databases. These included the main biosynthetic genes, namely *surABCD*, the transcriptional regulator SurR, and the thioesterase SurE – all of which had previously been reported to have roles in the biosynthesis of surugamides and their derivatives (Matsuda et al., 2019b; Ninomiya et al., 2016; Thankachan et al., 2019; Xu et al., 2017; Zhou et al., 2019) (Figure 6).
Figure 6: Gene synteny of the *sur* BGC region, including the reference
*sur* BGC nucleotide sequence (LHW3101) and each of the *albidoflavus* phylogroup genomes. Arrows at different positions represent genes transcribed in different reading frames.

Interestingly, this result indicated that the gene synteny of the biosynthetic genes as well as the flanking genes is highly conserved, with the exception to the 3’ flanking region of the BGC from *S. sampsonii* KJ40. Notably, even the reading frames of the *surE* gene and the *surABCD* genes are conserved amongst all the genomes. As indicated by the numbers in Figure 6, the 5’ region in all the genomic regions consisted of: 1) a MbtH-like protein, which have been reported to be involved in the synthesis of non-ribosomal peptides, antibiotics and siderophores, in *Streptomyces* species (Lautru et al., 2007; Quadri et al., 1998); 2) a putative ABC transporter, which is a family of proteins with varied biological functions, including conferring resistance to drugs and other toxic compounds (Glavinas et al., 2004; Polgar and Bates, 2005); 3) a BcrA family ABC transporter, which is a family commonly involved in peptide antibiotics resistance (Ohki et al., 2003; Podlesek et
al., 1995); 4) a hypothetical protein; followed by 5) the transcriptional repressor SurR, which has been experimentally demonstrated to repress the production of surugamides (Xu et al., 2017); 6) a hypothetical membrane protein; 7) the thioesterase SurE, which is homologous to the penicillin binding protein, reported to be responsible for the cyclisation of surugamides molecules (Matsuda et al., 2019a); and finally 8-11) the main surugamides biosynthetic genes \textit{surABCD}, all of which encode non-ribosomal peptide synthetase (NRPS) proteins (Ninomiya et al., 2016). The 3’ flanking region consisted of: 12) a predicted multi-drug resistance (MDR) transporter belonging to the major facilitator superfamily (MFS) of membrane transport proteins (Kumar et al., 2016; Yan, 2015); 13) a predicted TetR/AcrR transcriptional regulator, which is a family of regulators reported to be involved in antibiotic resistance (Cuthbertson and Nodwell, 2013); 14) a hypothetical protein; and 15) another predicted MDR transporter belonging to the MFS superfamily. In contrast, the 3’ flanking region of the KJ40 strain \textit{sur} BGC, consisted of: 16) a group of four hypothetical proteins, which may represent pseudogene versions of the first MDR transporter identified in the other isolates (gene number 12 in Figure 6); 17) a predicted rearrangement hotspot (RHS) repeat protein, which is a family of proteins reported to be involved in mediating intercellular competition in bacteria (Koskiniemi et al., 2013); 18) a hypothetical protein; and 19) a MDR transporter belonging to the MFS superfamily, which, interestingly, is a homolog of protein number 15, which is present in all the other isolates.

The conserved gene synteny observed in the \textit{sur} BGC genomic region, particularly those positioned upstream of the main biosynthetic \textit{surABCD} genes, together with the observation that even the reading frames of the \textit{surE} and the \textit{surABCD} genes are conserved among all the genomes analysed, coupled with the previous phylogenetic and pan-genome analyses, suggest the following. Firstly, it
is very likely that these strains share a common ancestry and that the *sur* BGC genes had a common origin. Secondly, there is a strong evolutionary pressure ensuring the maintenance of not only gene synteny, but also of the reading frames of the main biosynthetic genes involved in the production of surugamides. The latter raises the question of which other genes in this region may be involved in the production of these compounds, or potentially conferring mechanisms of self-resistance to surugamides in the isolates, particularly since many of the genes have predicted functions that are compatible with the transport of small molecules and with multi-drug resistance. These observations are particularly interesting considering that these strains are derived from quite varied environments and geographic locations.

### 3.5. Growth, morphology, phenotype and metabolism assessment of *Streptomyces* sp. SM17 in complex media

In order to assess the metabolic potential of the SM17 strain (Manteca and Yagüe, 2019), particularly with respect to the production of surugamide A, the isolate was cultivated in a number of different growth media, within an OSMAC-based approach (Pan et al., 2019; Romano et al., 2018). While the SM17 strain was able to grow in SYP-NaCl, YD, SY, P1, P2, P3 and CH-F2 liquid media; the strain was unable to grow in Oatmeal and Sporulation media. The latter indicated an inability to metabolise oat and starch when nutrients other than yeast extract are not present. Morphologically, the SM17 strain formed cell aggregates or pellets in TSB, YD and SYP-NaCl, while this differentiation was not observed in the other media. Preliminary chemical analyses of these samples, employing Liquid Chromatography–Mass Spectrometry (UPLC-DAD-HRMS), indicated that secondary metabolism in SM17 was not very active when the strain was cultivated in SY, P1, P2, P3 and CH-F2 media. In contrast, significant production of surugamides was evidenced in the extracts from TSB, SYP-NaCl and YD media,
with characteristic ions at \( m/z \) 934.6106 (surugamide A) and 920.5949 (surugamide B) \([\text{M+Na}]^+\), which correlated with the formation of cell pellets and the production of natural products, as previously described in other *Streptomyces* strains (Manteca et al., 2008; Manteca and Yagüe, 2019).

### 3.6. Differential production of surugamide A by *Streptomyces* sp. SM17 and *S. albidoflavus* J1074

To confirm the production of surugamide A by the SM17 isolate, extracts from the TSB, SYP-NaCl and YD media were combined and purified using High Performance Liquid Chromatography (HPLC). The structures of the major compounds of the extract were subsequently analysed using Nuclear Magnetic Resonance (NMR) spectroscopy, which allowed for the identification of the chemical structure of the surugamide A molecule as major metabolite by comparison with reference NMR data (Figure 7) (Takada et al., 2013).
The isolates *Streptomyces* sp. SM17 and *S. albidoflavus* J1074 were subsequently cultivated in the aforementioned media in which the SM17 strain had been shown to be metabolically active, namely the TSB, SYP-NaCl, and YD media. This was performed in order to assess whether there were any significant differences in the production of surugamide A when different growth media are employed for the production of this compound, and to compare the levels of surugamide A produced by the SM17 and the J1074 isolates. The MeOH/DCM (1:1) extracts from the aforementioned cultures of SM17 and J1074 were subjected to Liquid Chromatography–Mass Spectrometry (UPLC-HRMS) to quantify the levels of surugamide A being produced under each condition (Table 1), using a surugamide A standard calibration curve (Figure S2).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Media</th>
<th>Percent (w/w) of Extract</th>
<th>Concentration of Surugamide A (mg/L) corrected in 5 mg/mL of extract</th>
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</thead>
<tbody>
<tr>
<td>SM17</td>
<td>TSB</td>
<td>2.44%</td>
<td>122.01</td>
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<tr>
<td>SM17</td>
<td>SYP-NaCl</td>
<td>10.60%</td>
<td>530.15</td>
</tr>
<tr>
<td>SM17</td>
<td>YD</td>
<td>1.13%</td>
<td>56.27</td>
</tr>
<tr>
<td>J1074</td>
<td>TSB</td>
<td>0.27%</td>
<td>13.32</td>
</tr>
<tr>
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<td>SYP-NaCl</td>
<td>3.55%</td>
<td>176.82</td>
</tr>
<tr>
<td>J1074</td>
<td>YD</td>
<td>0.09%</td>
<td>4.26</td>
</tr>
</tbody>
</table>

**Table 1:** Surugamide A production by SM17 and J1074 measured using different media.

The LC-MS quantification analysis (Table 1) indicated that both strains were capable of producing surugamide A in all the conditions tested. However, the SM17 strain appeared to produce considerably higher yields of the compound when compared to J1074, in all the conditions analysed. In addition, the *S. albidoflavus* J1074 isolate appeared to produce quite low levels of surugamide A when grown in TSB and YD media, accounting for less than 1% (w/w) of the extracts from these media. Interestingly, higher yields of surugamide A were produced in the SYP-NaCl medium in both strains, when compared with the levels of surugamide A produced by these strains when grown in TSB and the YD media (Table 1). In the SM17 culture in SYP-NaCl, surugamide A accounted for 10.60% (w/w) of the extract, compared to 2.44% and 1.13% from TSB and YD, respectively; while in J1074 it accounted for 3.55% (w/w) of the extract from the SYP-NaCl culture, compared to 0.27% and 0.09% from TSB and YD, respectively (Table 1). These results provide further insights into factors that are potentially involved in regulation the biosynthesis of surugamide A, in the *albidoflavus* phylogroup and in *Streptomyces* sp. SM17 in particular.
Firstly, it appears likely that surugamide A biosynthesis may be regulated, at least in part, by carbon catabolite repression (CCR). Carbon catabolite repression is a well described regulatory mechanism in bacteria that controls carbon metabolism (Brückner and Titgemeyer, 2002; Deutscher, 2008; Kremling et al., 2015; Stülke and Hillen, 1999), and which has also been reported to regulate the biosynthesis of secondary metabolites in a number of different bacterial species, including in *Streptomyces* isolates (Gallo and Katz, 1972; Inoue et al., 2007; Magnus et al., 2017; Romero-Rodríguez et al., 2016). While the TSB and the YD media contain glucose and dextrins as carbon sources, respectively; the complex polysaccharide starch is the carbon source in the SYP-NaCl medium. Therefore, it is reasonable to infer that glucose and dextrin may repress the production of surugamide A in *Streptomyces* sp. SM17 and in *Streptomyces albidoflavus* J1074, while starch does not. Further evidence for this can be found when considering the different production media previously employed in the production of surugamides by different *Streptomyces* isolates. For example, in the original research that led to the discovery of surugamides in *Streptomyces* sp. JAMM992 (Takada et al., 2013), the PC-1 medium (1% starch, 1% polypeptone, 1% meat extract, 1% molasses, pH 7.2) was employed for production of these compounds. Similarly to the SYP-NaCl medium employed in our study, the PC-1 medium also contains starch as the carbon source, together with another complex carbon source, namely molasses. Likewise, for the production of surugamides in *S. albidoflavus* strain LHW3101 (Zhou et al., 2019), the TSBY medium (3% tryptone soy broth, 10.3% sucrose, 0.5% yeast extract) was employed, which utilises sucrose as its main carbon source. In contrast, when elicitors were employed to induce the production of surugamides and their derivatives in the J1074 strain (Xu et al., 2017), by activating the *sur* BGC which appeared to be silent in this isolate; the R4 medium (0.5% glucose, 0.1% yeast extract, among other non-carbon related components) was employed, which utilises glucose as its main carbon supply, and, as shown in this study, it potentially...
represses the production of surugamide A. Thus, from these previous reports and from our observations, it appears likely that CCR plays an important role in regulating the biosynthesis of surugamides.

Secondly, it is important to note the presence of salts in the form of NaCl in the SYP-NaCl medium. As previously mentioned, genetic and phylogenetic analyses of the sur BGC indicated similarities between those BGCs belonging to aquatic saline-derived Streptomyces isolates (Figure 5); together with the likelihood that these sur BGCs might have had a common origin. Thus, it is plausible that this origin may have been marine, and hence the presence of salts in the growth medium may also have an influence on the biosynthesis of surugamide A. Different concentrations of salts in the form of NaCl in the culture medium have also previously been shown to impact on the chemical profile of metabolites produced in the marine-obligate bacteria Salinispora arenicola (Bose et al., 2015).

Nevertheless, it is interesting to observe that, despite the repression/induction of the biosynthesis of surugamide A observed when different media were employed, the SM17 isolate is clearly produces considerably higher amounts of surugamide A when compared to S. albidoflavus J1074 – reaching yields up to >13-fold higher in the YD medium, and around 3-fold higher when grown in the SYP-NaCl medium (Table 1).
4. **Conclusions**

Marine-derived bacteria, particularly those isolated in association with marine invertebrates, such as sponges, have been shown to be reservoirs of bioactive molecules, including those with antibacterial, antifungal, and anticancer activities. Among these newly identified bioactive compounds, the surugamides and their derivatives are of particular interest due to their clinically relevant bioactivities, i.e. anticancer and antifungal, and their original metabolic pathway.

Based on genome mining, this study identified the previously unreported capability of the marine sponge-derived isolate *Streptomyces* sp. SM17 to produce surugamide A and also sheds new light on factors such as the carbon catabolite repression (CCR) that may be involved in regulating production of this molecule. Phylogenomics analysis indicated that the *sur* BGC is commonly present in members of the proposed *albidoflavus* phylogroup, and that the *sur* BGCs present in different isolates derived from varied environmental niches may possess a common ancestry. Although high quality genomic data from this proposed *albidoflavus* phylogroup is still lacking, results presented here suggest that the *sur* BGCs derived from *Streptomyces* isolated from aquatic saline environment are more similar to each other, when compared to those isolated from terrestrial environments.

Chemical analysis was performed in order to assess differential production of surugamide A when comparing a marine *Streptomyces* isolate with a terrestrial *Streptomyces* isolate, namely SM17 and J1074 strains, respectively; following an OSMAC-based approach employing different culture media. This analysis showed that not only the marine-derived isolate SM17 was capable of producing more surugamide A when compared to J1074 under all the conditions tested, but also that the biosynthesis of surugamide A is likely to be influenced by the CCR, and potentially by the presence of salts in the growth medium. These results also
highlight the importance of employing an OSMAC-based approach even when
analysing the production of known compounds, since there is a clear difference in
the yields of surugamide A obtained when employing different culture media.
Thus, it is possible to gain further insights into the production of bacterial types of
compounds by 1) discovering strains that possess a higher capability to produce
these compounds; 2) establishing optimal conditions for the biosynthesis of their
production; and 3) providing a better understanding of the genetic and regulatory
mechanisms potentially underpinning the production of these compounds.
5. Materials and methods

5.1. Bacterial strains and nucleotide sequences

The *Streptomyces* sp. SM17 strain was isolated from the marine sponge *Haliclona simulans*, from the Kilkieran Bay, Galway, Ireland, as previously described (Kennedy et al., 2009). The *Streptomyces albidoflavus* J1074 strain was provided by Dr Andriy Luzhetskyy (Helmholtz Institute for Pharmaceutical Research Saarland, Germany). Their complete genome sequences are available from the GenBank database (Benson et al., 2018) under the accession numbers NZ_CP029338 and NC_020990, for *Streptomyces* sp. SM17 and *S. albidoflavus* J1074, respectively. The surugamides biosynthetic gene cluster (*sur BGC*) sequence used as a reference for this study was the one previously described in *Streptomyces albidoflavus* LHW3101 (GenBank accession number: MH070261) (Zhou et al., 2019). Other genomes used in this study’s analyses were obtained from the GenBank RefSeq database (Benson et al., 2018).

5.2. Phylogenetic analyses

The NCBI BLASTN tool (Camacho et al., 2009; Johnson et al., 2008) was used to determine the closest 30 *Streptomyces* strains with complete genome available in the GenBank RefSeq database (Benson et al., 2018) to the *Streptomyces* sp. SM17. Then phylogeny analysis was performed with the concatenated sequences of the 16S rRNA, and the housekeeping genes *atpD, gyrB, recA, rpoB*, and *trpB*. The sequences were aligned using the MAFFT program (Katoh and Standley, 2013), and the phylogeny analysis was performed using the MrBayes program (Ronquist et al., 2012). In MrBayes, the General Time Reversible (GTR) model of nucleotide substitution was used (Waddell and Steel, 1997), with gamma-distributed rates across sites with a proportion of invariable sites, with 1 million generations sampled every 100 generations. Final consensus phylogenetic tree generated by
MrBayes was processed using MEGA X (Kumar et al., 2018), with a posterior probability cut off of 95%.

Phylogeny analysis of the surugamides biosynthetic gene cluster (sur BGC) was performed by using the *S. albidoflavus* LHW3101 sur BGC nucleotide sequence as reference (Zhou et al., 2019), and searching for similar sequences on the GenBank RefSeq database using the NCBI BLASTN tool (Benson et al., 2018; Camacho et al., 2009; Johnson et al., 2008), only taking into account complete genomes. The genome regions with similarity to the *S. albidoflavus* LHW3101 sur BGC undergone phylogeny analysis using the same aforementioned tools and parameters.

5.3. Prediction of secondary metabolites biosynthetic gene clusters

In order to assess the similarities and differences between the *Streptomyces* isolates belonging to the *albidoflavus* phylogroup, in regard to their potential to produce secondary metabolites, BGCs were predicted in their genomes, using the antiSMASH (version 5) program (Blin et al., 2019). The predicted BGCs were then processed using the BiG-SCAPE program (Navarro-Muñoz et al., 2018), with the MIBiG database (version 1.4) as reference (Medema et al., 2015), and similarity clustering of gene cluster families (GCFs) was performed. The similarity network was processed using Cytoscape (Shannon et al., 2003).

5.4. Gene synteny analysis

The genome regions previously determined to share similarities with the *S. albidoflavus* LHW3101 sur BGC were manually annotated, for the known main biosynthetic genes (surABCD), the penicillin binding protein (PBP)-like peptide cyclase and hydrolase surE gene, and the gene with regulatory function surR (Matsuda et al., 2019a, 2019b; Ninomiya et al., 2016; Thankachan et al., 2019; Xu et al., 2017; Zhou et al., 2019). This was performed using the UniPro UGENE toolkit.
(Okonechnikov et al., 2012), the GenBank database and the NCBI BLASTN tool (Benson et al., 2018; Camacho et al., 2009; Johnson et al., 2008). The gene synteny and reading frame analysis was performed using the UniPro UGENE toolkit (Okonechnikov et al., 2012) and the Artemis genome browser (Rutherford et al., 2000).

5.5. Diagrams and figures

All the Venn diagrams presented in this study were generated using the venn package in R (Dusa, 2018; R Core Team, 2018), and RStudio (RStudio Team, 2015). All the images presented in this study were edited using the Inkscape program (available from https://inkscape.org/).

5.6. Strains culture, maintenance, and secondary metabolites production

The same culture media and protocols were employed for both isolates Streptomyces sp. SM17 and Streptomyces albidoflavus J1074. Glycerol stocks were prepared from spores collected from soya-mannitol (SM) medium after 8 days of cultivation at 28°C and preserved at -20°C. To verify the secondary metabolites production profile, spores were cultivated for 7 days on SM agar medium at 28°C, then pre-inoculated in 5 mL TSB medium, and cultivated at 28°C and 220 rpm for 2 days. Then 10% (v/v) of the pre-inoculum was transferred to 30 mL of the following media: TSB; SYP-NaCl (1% starch, 0.4% yeast extract, 0.2% peptone & 0.1% NaCl); YD (0.4% yeast extract, 1% malt extract & 4% dextrin pH 7); P1 (2% glucose, 1% soluble starch, 0.1% meat extract, 0.4% yeast extract, 2.5% soy flour, 0.2% NaCl & 0.005% K2HPO4 pH 7.3); P2 (1% glucose, 0.6% glycerol, 0.1% yeast extract, 0.2% malt extract, 0.6% MgCl2.6H2O, 0.03% CaCO3 & 10% sea water); P3 (2.5% soy flour, 0.75% starch, 2.25% glucose, 0.35% yeast extract, 0.05% ZnSO4×7H2O, 0.6% CaCO3 pH 6); CH-F2 (2% soy flour, 0.5% yeast extract, 0.2% CaCO3, 0.05% citric acid, 5% glucose, pH 7.0); SY (2.5% soluble starch, 1.5% soy flour, 0.2% yeast extract &
0.4% CaCO3 pH7); Sporulation medium (2% soluble starch & 0.4 yeast extract); and Oatmeal medium (2% oatmeal). These were cultivated at 28°C and 220 rpm for 4 days in TSB; and for 8 days in SYP-NaCl, YD, SY, P1, P2, P3, CH-F2, Sporulation, and Oatmeal media. Once the bioprocess was completed, the broth was frozen at -20°C for further chemical analysis.

5.7. Metabolic profiling, compound isolation and chemical structure analysis

The *Streptomyces* broth of TSB, SYP-NaCl and YD medium cultures (180 mL) was exhaustively extracted using a solvent mixture of 1:1 MeOH:DCM yielding a crude extract (3.89 g). This crude extract was first separated using SPE on C\textsubscript{18} bonded silica gel (Polygoprep C18, 12%, 60Å, 40-63 µm), eluting with varying solvent mixtures to produce five fractions: H\textsubscript{2}O (743.62 mg), 1:1 H\textsubscript{2}O:MeOH (368.6 mg), MeOH (15.4 mg), 1:1 MeOH:DCM (10.9 mg), DCM (8.2 mg). The final three fractions (MeOH, 1:1 MeOH:DCM, DCM, 34.5 mg) were then combined and subject to analytical reverse phase HPLC on a Waters Symmetry C18 5 µm, 4.6 x 250 mm column. The column was eluted with 10% MeCN (0.1% TFA)/90% H\textsubscript{2}O (0.1% TFA) for 5 min, then a linear gradient to 100% MeCN (0.1% TFA) over 21 min was performed. The column was further eluted with 100% MeCN (0.1% TFA) for 6 min. After the HPLC was complete a linear gradient back to 10% MeCN (0.1% TFA)/90% H\textsubscript{2}O (0.1% TFA) over 1 min and then further elution of 10% MeCN (0.1% FA)/90% H\textsubscript{2}O (0.1% FA) for 4 min was performed. This yielded pure surugamide A (0.8 mg). Surugamide A was characterised using MS and NMR data to confirm the structure for use as an analytical standard.

Surugamide A was quantified in the broth using LC-MS analysis on an Agilent UHR-qTOF 6540 mass spectrometer. The column used for separation was Waters equity UPLC BEH C18 1.7 µm 2.1 x 75 mm. The column was eluted with 10% MeCN (0.1% FA)/90% H\textsubscript{2}O (0.1% FA) for 2 min, then a linear gradient to 100%
MeCN (0.1% FA) over 6 min was performed. The column was further eluted with 100% MeCN (0.1% FA) for 4 min. After the UPLC was complete a linear gradient back to 10% MeCN (0.1% FA)/90% H2O (0.1% FA) over 1 min and then further elution of 10% MeCN (0.1% FA)/90% H2O (0.1% FA) for 3 min was performed before the next run. The MS detection method was positive ion. A calibration curve was produce using the LC-MS method above and injecting the pure surugamid A at seven concentrations (100, 25, 10, 2, 1, 0.2, 0.1 mg/L). 30 mL of each Streptomyces strain in broth were extracted using a solvent mixture of 1:1 MeOH:DCM three times to yield a crude extract. These extracts were resuspended in MeOH and filtered through PTFE 0.2 µm filters before being subject to the above LC-MS method.

The surugamide A calibration standards 1-7 and the six extracts were analysed using the Agilent MassHunter Quantification software package. This allowed the quantification of surugamide A in the extracts based on the intensity of peaks in the chromatogram with matching retention time and exact mass.
6. References


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7. **Supplementary material**

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<th>Isolate</th>
<th>Number of bases</th>
<th>Number of CDSs</th>
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<td>6,182</td>
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**Table S1:** Genome statistics determined using the Prokka program, of the *Streptomyces* isolates genomes obtained from GenBank and determined to belong to the *albidoflavus* phylogroup.

**Figure S1:** Venn diagram representing the presence/absence of groups of orthologous genes in the *albidoflavus* phylogroup genomes (namely strains FR-008, J1074, KJ40, SM17, and SM254), also including the *Streptomyces koyangensis* VK-A60T genome.
Figure S2: Calibration curve for surugamide A, determined using LC-MS and pure surugamide A at seven concentrations (0.1, 0.2, 1, 2, 10, 25, 100 mg/L).
Chapter 4

*In silico* screening and heterologous expression of a Polyethylene Terephthalate hydrolase (PETase)-like enzyme (SM14est) with Polycaprolactone (PCL)-degrading activity, from the marine sponge-derived strain *Streptomyces* sp. SM14

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1. Abstract

Plastics, such as the polyethylene terephthalate (PET), are widely used for various industrial applications, due to their physicochemical properties which are particularly useful in the packaging industry. However, due to improper plastic waste management and difficulties in recycling; post-consumer plastic waste has become a pressing issue for both the environment and for human health. Hence, novel technologies and methods of processing plastic waste are required to address these issues. Enzymatic-assisted hydrolysis of synthetic polymers has been proposed as a potentially more efficient and environment-friendly alternative to the currently employed methods. Recently, a number of PET hydrolases have been described, and in particular a PETase derived from the Ideonella sakaiensis 201-F6 (IsPETase), which appears to be the most efficient and substrate-specific bacterial PET hydrolase enzyme discovered to date. In order to further investigate the class of PETase-like enzymes, we employed an in silico-based screening approach on the biotechnologically relevant genus Streptomyces, including terrestrial and marine isolates; in a search for potential PETase homologs. From a total of 52 genomes analysed, we were able to identify 3 potential PETase-like enzymes, all of which were derived from marine-sponge associated Streptomyces isolates. A candidate PETase-like gene (SM14est) was identified in Streptomyces sp. SM14. Further in silico characterisation of the SM14est protein sequence and its potential three-dimensional structure were performed and compared to the well characterised IsPETase. Both the serine hydrolase motif Gly-x1-Ser-x2-Gly and the catalytic triad Ser, Asp, His are conserved in both sequences. Molecular docking experiments indicated that the SM14est enzyme possessed the capacity to bind plastics as substrates. Finally, polyesterase activity was confirmed using a polycaprolactone (PCL) plate clearing assay which is a model substrate for plastics degradation; following heterologous expression of SM14est in E. coli, with secretion being facilitated by the native Streptomyces signal peptide. These findings provide further insights into this important class of PETase-like enzymes.
2. Introduction

Plastics are materials that have been produced on a large scale from the 1950s onwards, and since then have been widely used for various applications, and have become almost indispensable in modern society (Geyer et al., 2017; Jambeck et al., 2015; Lebreton and Andrady, 2019). In the 1960s, plastics accounted for less than 1% of municipal solid waste in the United States, but steadily increased to around 10% by 2005 in countries with middle to high income (Geyer et al., 2017; Jambeck et al., 2015). This was largely due to their advantageous properties, such as their low production cost and bio-inertia, which are particularly useful for the packaging industry, when compared to other materials. This has resulted in the use of plastics in the packaging sector, which accounts for around 40% of the plastic converter demand in Europe (Lebreton and Andrady, 2019; PlasticsEurope, 2018). However, some of these aforementioned characteristics have resulted in plastics becoming a critical problem from an environmental perspective; as many synthetic plastics are highly recalcitrant to biodegradation and can persist for long periods of time in the environment (Wei and Zimmermann, 2017a).

In 2017, there was an estimated worldwide plastics production of 348 million tonnes – an increase from the 335 million tonnes estimated for the previous year, and this does not include polyethylene terephthalate (PET)-, polyamide (PA)- and polyacryl-fibres) (PlasticsEurope, 2018). At the current rate, this number is expected to double in the next 20 years (Lebreton and Andrady, 2019). In the past decade, plastic waste management policies have helped considerably in reducing post-consumer plastic waste being disposed in the environment. For example, in Europe more collected plastic waste (31.1% of 27.1 million tonnes of collected plastic waste in 2016) was submitted to recycling rather than to landfills, for the first time. However, landfills and incineration for energy recovery still account for
27.3% and 41.6% of the collected plastic post-consumer waste, respectively (PlasticsEurope, 2018).

Notwithstanding this, the aforementioned metrics do not take into account global mismanaged plastic waste which enters the natural environment, at locations others than landfills. A recent study has estimated that between 60 and 99 million metric tonnes of mismanaged plastic waste was produced globally in 2015, and that this number could triple by 2060 (Lebreton and Andrady, 2019).

Mismanaged plastic waste is particularly concerning when it effects the marine environment. It has been calculated that in 2010 between 4.8 and 12.7 million metric tons of plastic waste entered our oceans, and this data only accounted for coastal countries (Jambeck et al., 2015). Sunlight and other weathering effects cause the fragmentation of plastic debris into milli- and micro-metric particles (< 5mm), which are defined as micro-plastics (Geyer et al., 2017; Lebreton and Andrady, 2019). These micro-plastics are now believed to be ubiquitous in soil and aquatic environments, and are commonly ingested by animals (Lebreton and Andrady, 2019; Santillo et al., 2017). This is especially concerning, since micro-plastics can absorb and concentrate pollutants present in the ocean and transfer them along the food chain, particularly to seafood species that are consumed by humans (Santillo et al., 2017). Highlighting the issue of the ubiquitous presence of micro-plastics in the marine environment, a recent study detected the ingestion of micro-plastics by deep-sea amphipods, at depths ranging from 7,000 to 10,890 meters (Jamieson et al., 2019). Additionally, the deepest ever sub diving recorded to date has registered the presence of plastics on the ocean floor, at a depth of 10,927 meters (Street, 2019). It is alarming to find plastics, which are materials with a history of less than a century of large-scale production, already being so widespread in nature; with the potential for extensive negative impacts, many of which have yet to be fully realised. Hence, better plastic waste management and processing solutions are urgently required.
Currently, the majority of plastic waste recycling is based on mechanical recycling (collection, sorting, washing and grinding) (Ragaert et al., 2017). However, the presence of organic and inorganic impurities in post-consumer plastic waste presents a huge challenge for mechanical recycling (Drzyzga and Prieto, 2019). On the other hand, chemical recycling has been applied as an alternative for improved plastic waste management processes, in which the plastic polymers can be converted into raw materials that can be used for the synthesis of chemicals, fuels, or virgin plastics (Drzyzga and Prieto, 2019). Strictly chemical methods, however, require the use of toxic chemicals and high temperatures, and can also be quite costly (Awaja and Pavel, 2005; Wei and Zimmermann, 2017b). Therefore, enzymatic hydrolysis of synthetic polyesters has been proposed as a potentially more efficient and environment friendly method for the recycling of plastic waste (Drzyzga and Prieto, 2019; Wei and Zimmermann, 2017b, 2017b).

In the past decade, a number of bacterial enzymes capable of degrading synthetic polyesters, including the widely used polyethylene terephthalate (PET), have been identified (Kawai et al., 2019; Wei and Zimmermann, 2017b). These enzymes are commonly classified as members of the cutinase, lipase and esterase classes of enzymes, and to date have mainly been identified in thermophilic actinomycetes, particularly in the genus *Thermobifida* (Silva et al., 2011; Wei and Zimmermann, 2017b). More recently, in 2016, Yoshida and co-workers isolated a bacterium from a PET plastic bottle recycling plant in Sakai, Japan, that was capable of degrading and assimilating PET as its major energy and carbon source – namely *Ideonella sakaiensis* 201-F6 (Yoshida et al., 2016). The protein identified as being responsible for the hydrolysis of PET (ISF6_4831) was then defined as a PETase (or PET hydrolase) enzyme (EC 3.1.1.101) (Yoshida et al., 2016). The PETase from *Ideonella sakaiensis* 201-F6 has been shown to possess a relatively higher enzymatic activity and substrate specificity for PET than other previously
described PET hydrolases, in addition to the ability to degrade PET at moderate temperatures (around 30°C) (Joo et al., 2018; Yoshida et al., 2016). Since then, a number of studies have been undertaken in a concerted effort to characterise this enzyme and the underlying metabolic and biochemical processes involved in the degradation of PET (Chen et al., 2018; Han et al., 2017; Joo et al., 2018; Liu et al., 2018).

The *Streptomyces* genus, member of the Actinomycetales order, is well-known to produce compounds and enzymes of industrial and clinical interest, particularly antibiotics, for which it is considered the largest producer in the microbial world (Hwang et al., 2014; Ser et al., 2017; Spasic et al., 2018; Wavve et al., 2001). Recent efforts to exploit the biotechnological potential of *Streptomyces* species have largely focused on the identification of bioactive small molecules and secondary metabolites biosynthetic gene clusters (Chevrette et al., 2019; Manteca and Yagüe, 2019). In this respect, the focus has started to shift towards *Streptomyces* isolates derived from varied niche environments, such as those isolated from the marine environment, which are still not well characterised and majorly unexplored organisms, when compared to the previously more commonly studied soil-derived isolates (Dharmaraj, 2010; Hassan et al., 2017; Jin et al., 2018; Xu et al., 2018).

*Streptomyces* isolates from soil ecosystems have also been studied for their synthetic polyesters-degrading capabilities (Calabia and Tokiwa, 2004; Shivlata and Satyanarayana, 2015). These include *Streptomyces* sp. strain MG (Tokiwa and Calabia, 2004) and *Streptomyces thermoviolaceus* (Chua et al., 2013), which can degrade polycaprolactone (PCL); together with *Streptomyces bangladeshensis* 77T-4, which degrades poly(D-3-hydroxybutyrate) (PHB) (Hsu et al., 2012). Given that marine *Streptomyces* sp. SNG9 had previously been reported to degrade PHB (Mabrouk and Sabry, 2001), coupled with the fact that marine *Streptomyces* isolates...
are likely to have been exposed to plastics and/or microplastics in marine ecosystems – in particular those isolates which are associated with marine sponges (phylum *Porifera*), which filter large quantities of seawater (up to 24000 L of water per day/Kg sponge) on a daily basis to obtain nutrients (Food and Agriculture Organization of the United Nations, 2017; Godefroy et al., 2019; Taylor et al., 2007) – we reasoned that marine sponge-derived *Streptomyces* species may possess enzymes with an ability to degrade synthetic polymers. In this study, we screened a number of *Streptomyces* species, including both terrestrial and marine-derived isolates, using an *in silico*-based analysis to interrogate their genomes for potential PETase homologs. A candidate PETase-like gene was identified in *Streptomyces* sp. SM14 and enzyme activity was confirmed following heterologous expression of this gene in *Escherichia coli*. This is the first report of a PETase-like enzyme being identified in a marine sponge derived *Streptomyces* spp. isolates, and we believe that this study provides further insights into our current knowledge of this important class of enzymes.
3. Material and methods

3.1. Data sets

The reference data set was comprised of 15 amino acid sequences of enzymes with previously demonstrated synthetic polyesters-degrading capabilities (Table 1) (Danso et al., 2018; Joo et al., 2018; Kawai et al., 2019; Wei and Zimmermann, 2017b; Yoshida et al., 2016). A lipase from *Streptomyces exfoliatu*s (PDB ID: 1JFR), which is a cutinase-like enzyme (Kawai et al., 2019; Wei et al., 1998), was also included in the reference data set (Table 1), which although not possessing demonstrated polyester-degrading activity, served as an outgroup for the subsequent *in silico* analyses.

The *Streptomyces* genomes data set comprised of 52 *Streptomyces* genome sequences obtained from GenBank (Benson et al., 2018), including 23 genomes from terrestrial isolates, and 29 from marine isolates (Table S1). Open reading frames (ORFs) and their respective translated amino acid sequences were obtained using Prokka (Seemann, 2014).
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Source</th>
<th>UniProt accession</th>
<th>GenBank accession</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>ISF6_483 (IsPETase)</td>
<td><em>Ideonella sakaiensis</em> strain 201-F6</td>
<td>A0A0K8P6T7</td>
<td>GAP38373</td>
<td>Yoshida et al., 2016</td>
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<td>Cut190</td>
<td><em>Saccharomonospora viridis</em></td>
<td>W0TJ64</td>
<td>BAO42836</td>
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<td>Tcur_1278</td>
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<td>ACY96861</td>
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<td>Tha_Cut1</td>
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<td>E9LVH7</td>
<td>ADV92525</td>
<td>Wei et al., 2014</td>
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<td>Thh_Est</td>
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<td>H6WX58</td>
<td>AFA45122</td>
<td>Ribitsch et al., 2012a</td>
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<td>Chen et al., 2010</td>
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<td>Lipase (1JFR)</td>
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<td>Q56008</td>
<td>AAB51445</td>
<td>Wei et al., 1998</td>
</tr>
</tbody>
</table>

Table 1: Reference data set comprising of 15 PETase-like enzymes with demonstrated PET-degrading activity, including the ISF6_483 protein from *Ideonella sakaiensis* strain 201-F6 (IsPETase), and additionally the cutinase-like lipase from *Streptomyces exfoliatus* (PDB ID: 1JFR).
3.2. Bacterial strains

*Streptomyces* strain SM14 was isolated from the sponge *Haliclona simulans* (class *Demospongiae*, order *Haplosclerida*, family *Chalinidae*) which was sampled by SCUBA diving at a depth of 15 m in Kilkieran Bay, Galway, Ireland (N 53°18′56.6″, W 09°40′08.4″) as previously described (Kennedy et al., 2014). The NEB® 5-alpha and the BL21(DE3) competent *E. coli* cells were obtained from New England Biolabs Inc., USA.

3.3. Protein homology search and phylogeny analysis

Potential PETase-like proteins were identified in the *Streptomyces* genomes data set by performing an homology search using BLASTP (e-value threshold of 1e-30, maximum subject sequence length of 400 aa) (Altschul et al., 1990; Camacho et al., 2009). Protein alignments were performed using Muscle (Edgar, 2004), and phylogeny analysis was performed using MEGA X (maximum likelihood statistical method; 500 bootstrap replications; LG+G+F model) (Kumar et al., 2018).

3.4. PCL plate clearing assay

Polycaprolactone (PCL) plate clearing assays were performed based on previously described studies (Murphy et al., 1996; Nawaz et al., 2015; Nishida et al., 1998; Nishida and Tokiwa, 1993). PCL with an average molecular weight of 80,000 was used (Sigma-Aldrich®). PCL emulsion was prepared with 1% m/v of PCL in acetone, at 50°C with magnetic stirring. Water, agar (1.5% m/v) and LB medium (2% m/v) were added to the emulsion, at 50°C with magnetic stirring until the acetone evaporated. The medium was then autoclaved and poured into plates. Strains were inoculated onto the plates and incubated at 28°C for up to 12 days. For enzyme activity assessment using *E. coli* as the heterologous host, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium at a concentration of 0.5 mM and plates were incubated at 28°C for up to 4 days. As a negative control, *E.
coli BL21(DE3) containing the pET-20b(+) plasmid without the insert did not show any PCL-degrading activity (Figure S1). It has recently been reported that E. coli BL21(DE3) can be employed as a host system in screens for polyesterase activity, as it does not possess PCL-degrading capabilities (Molitor et al., 2019). Additionally, 12 other marine sponge-derived Streptomyces isolates were also assayed for polyesterase activity using the PCL plate clearing assay. SM1, SM3, SM4, SM7, SM8, SM9, SM11, SM13, SM17, and FMC008 which had previously been isolated from the marine sponge Haliclona simulans (Kennedy et al., 2009), together with B188M101 and B226SN101 isolated from the deep sea sponges Lissodendoryx diversichela and Inflataella pellicula respectively (Jackson et al., 2018); were grown in LB medium + 1% PCL emulsion at 28°C for 12 days (data not shown).

3.5. Protein structure analysis, modelling and molecular docking

Amino acid sequence analysis was performed and graphically represented using ESPript 3.0 (Robert and Gouet, 2014). In silico protein structure prediction was performed using the SWISS-DOCK webserver (Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Waterhouse et al., 2018), and the UCSF Chimera software was used for structure analysis and three-dimensional model rendering (Pettersen et al., 2004). Molecular docking experiments were performed using AutoDock Vina, MGLtools (http://mgltools.scripps.edu/), AutoDockTools (ADT) and UCSF Chimera (Pettersen et al., 2004; Sanner, 1999; Trott and Olson, 2009), with the model substrate BHET (Bis(2-hydroxyethyl) terephthalate, Zinc database ID: ZINC02040111) molecule as the ligand (Irwin et al., 2012; Irwin and Shoichet, 2005).

3.6. Heterologous expression

An Escherichia coli codon-optimised version of the PETase-like gene was designed, and was synthesised by Eurofins Genomics (Ebersberg, Germany). A 5’
NdeI restriction site, a C-terminal His	extsubscript{6} tag, and a stop codon followed by a 3\textquoteleft XhoI restriction site were added to the gene sequence. Alignment of the nucleotide sequences of the original SM14est gene and the codon-optimised version is shown in Figure S2. The synthetic gene was PCR amplified using Phusion Green High-Fidelity DNA Polymerase (Thermo Scientific\textsuperscript{TM}) (primers and conditions detailed in Table S2), and was subcloned into the pET-20b(+) plasmid (Novagen\textsuperscript{®}), resulting in the pET20b:SM14est vector construct (Figure 1), using the NEB\textsuperscript{®} 5-alpha competent E. coli (New England Biolabs Inc., USA) for vector construction and maintenance. The signal peptide of the native protein was predicted using SignalP 5.0 (Almagro Armenteros et al., 2019), and it was maintained in the final construct. The expression vector was then transformed into BL21(DE3) competent E. coli (New England Biolabs Inc., USA) for heterologous protein expression. Confirmation of the insert was performed via 1) restriction digestion of the plasmid DNA with the NdeI and XhoI restriction enzymes followed by gel electrophoresis analysis, and 2) via Sanger sequencing of the insert region of the plasmid, amplified using the T7 standard vector primers (Table S2).
Figure 1: Graphical representation of the pET20b:SM14est plasmid, constructed for the heterologous expression of the SM14est protein in *E. coli*. The insert (SM14est) and other important features of the plasmid are represented and labelled accordingly.
4. Results and Discussion

4.1. In silico screening of PETase-like proteins in *Streptomyces* genomes and enzyme activity assessment

Previous studies have identified enzymes with plastic-degrading capabilities which have been isolated from different organisms, amongst these were the *Ideonella sakaiensis* strain 201-F6, and isolates from the genera *Thermobifida*, *Thermomonospora*, and *Saccharomonospora* (Danso et al., 2018; Joo et al., 2018; Kawai et al., 2019; Wei and Zimmermann, 2017b). This class of enzymes is commonly referred to as PETase or PETase-like, due to their ability to hydrolyse PET, although at different levels of efficiency.

From the bacterial PETase-like class of enzymes discovered to date, the PETase from *Ideonella sakaiensis* strain 201-F6 (referred to from now on as IsPETase) is the one that has received most attention from the scientific community; as it is considered the enzyme which displays the best PET hydrolase activity and substrate specificity which has been discovered thus far (Joo et al., 2018; Kawai et al., 2019; Yoshida et al., 2016). A total of 15 of the most prominent PETase-like enzymes were selected to build the reference data set (Table 1), which was then used to search for potential homologous proteins in the *Streptomyces* genomes data set. As described previously, the *Streptomyces* genomes data set comprised of 52 genome sequences, including 23 terrestrial *Streptomyces* isolates, and 29 isolates derived from the marine environment – 20 of which had previously been isolated by our group and for which genome sequence was available (Table S2) (Almeida et al., 2018; Jackson et al., 2018; Kennedy et al., 2009).

By applying a sequence similarity search approach using BLASTP (Altschul et al., 1990; Camacho et al., 2009), 34 homologous proteins from 32 *Streptomyces* strains were identified, of which the majority were from marine isolates (22 in total). These were then selected for further phylogeny analysis using MEGA X (Kumar et al., 2018). The amino acid sequences of the reference data set, and the *Streptomyces* potential PETase-like
homologs were aligned using the Muscle program (Edgar, 2004), and a maximum
likelihood phylogenetic tree was generated, with 500 bootstrap replicates. The resulting
consensus phylogenetic tree, with a 50% bootstrap value cut-off, comprised of four main
clades (Figure 2). Clade number 1 appeared to include *Streptomyces* isolates that were
previously reported to share genetic similarity to the type strain *Streptomyces albus*
J1074, all of which shared >99.50% 16S rRNA gene sequence similarity amongst each
other (Ian et al., 2014; Zaburannyi et al., 2014). Clade number 2 showed less obvious
similarities between all the members of the clade. While it included the strains SM1, SM3
and SM4, that were isolated from the marine sponge *Haliclona simulans* and shared high
similarity in their 16S rRNA sequences (>99%) (Jackson et al., 2018; Kennedy et al., 2009),
it also included the sponge isolate *Streptomyces* sp. 13-12-16 that shared less 16s rRNA
similarity with the aforementioned strains (~98%), and the soil isolate *Streptomyces*
*glaucescens* strain GLA.O, which in addition to being isolated from a completely distinct
environment, also shares <99% 16S rRNA similarity with the other strains in the clade.
Clade number 3 appeared to be the most diverse, with *Streptomyces* isolated from varied
sources, including: soil, marine sediment, and those isolated from marine sponges,
lichens and insects (Bianchetti et al., 2013; Kennedy et al., 2009; Ohnishi et al., 2008; Shin
et al., 2013; Xu et al., 2018). Clade 3 also included the lipase from *Streptomyces exfoliatus*
(indicated with an asterisk in Figure 2), suggesting that these enzymes are likely to be
cutinase-like lipases (Kawai et al., 2019; Wei et al., 1998). Most interesting, however, was
clade number 4, which clearly included all of the PETase-like enzymes used in the
reference data set, indicating that they may share similar evolutionary processes and
history that differentiate them from the other proteins considered in this analysis, which
may possibly have led to their ability to degrade synthetic polyesters. It is noteworthy
that 3 proteins from marine *Streptomyces* isolates were also included in clade 4,
specifically protein sequences from the *Streptomyces* sp. SM12, *Streptomyces* sp. SM14,
and *Streptomyces xinghaiensis* S187 isolates, which is a strong indicator that these
enzymes may possess plastic-degrading capabilities.
Figure 2: Phylogenetic tree of potential PETase homologs identified in the *Streptomyces* genomes, including terrestrial and marine (highlighted in cyan) *Streptomyces* isolates. The enzymes with known PET-degrading activity are highlighted in yellow. The red star indicates the *Ideonella sakaiensis* IsPETase, the pink star a PETase-like protein from the *Streptomyces* sp. SM14, and the asterisk the lipase from *Streptomyces exfoliatus*. The numbers in the branches indicate the percentage of bootstrap replicates (with a 50% cut-off from 500 replicates) in which the associated taxa clustered together.
Subsequent amino acid sequence analysis showed that the SM12 and SM14 proteins are in fact identical, so additional analysis proceeded with the SM14 strain. The enzyme activity was confirmed with a PCL plate clearing assay, in which the SM14 strain was grown in LB medium + 1% PCL emulsion at 28°C for 12 days (Figure 3). PCL is a synthetic polyester that has previously been used as a model substrate to assess both PETase and cutinase enzymatic activities (Danso et al., 2018; Nyyssölä et al., 2013). The zone of clearing demonstrates the synthetic polyester-degrading capability of the Streptomyces sp. SM14 isolate (Figure 3), which is presumably due to the protein identified from the in silico screening (Figure 2). Therefore, for the purposes of this study, the SM14 protein will from now on be referred to as SM14est, as it is likely to be a potential polyesterase enzyme. The SM14est gene sequence was deposited in the GenBank database under the accession number BK010828.

Figure 3: PCL plate clearing assay with the Streptomyces sp. SM14 strain incubated at 28°C after 12 days.
4.2. Protein structure analysis

4.5.5. Amino acid sequence, conserved residues and domains

An amino acid sequence comparison between the SM14est and the IsPETase was performed, using previously described PETase enzyme sites as reference (Joo et al., 2018). The amino acid sequences were aligned using the Muscle algorithm in MEGA X (Edgar, 2004; Kumar et al., 2018), and the alignment and amino acid residues were analysed in ESPript 3.0 (Figure 4) (Robert and Gouet, 2014). The amino acid alignment showed that 41% of the amino acids in SM14est were identical to the IsPETase, and that an additional 19% of the SM14est amino acids shared similar biochemical properties to the IsPETase. The serine hydrolase motif Gly-x1-Ser-x2-Gly is conserved in both sequences (residues in IsPETase: Gly158-Trp159-Ser160-Met161-Gly162; residues in SM14est: Gly154-His155-Ser156-Met157-Gly158). The catalytic triad is also conserved in both sequences (residues in IsPETase: Ser160, Asp206, His237; and in SM14est: Ser156, Asp202, His234) (Figure 4 and Table S3), which is to be expected given that this catalytic triad has been shown to be crucial for enzymatic activity in this class of enzymes. In previous site-direct mutagenesis experiments performed with the IsPETase protein, substitution of any of the residues within the catalytic triad resulted in a complete disruption of the catalysis process (Joo et al., 2018; Liu et al., 2018). One major difference between the IsPETase and our SM14est is that the former possesses two disulphide bonds (the first between Cys273 and Cys289, and the second between Cys203 and Cys239), while the latter has none. Although disulphide bonds are generally related to higher protein thermostability, it has been proposed that the second disulphide bond of IsPETase is connected to its enzymatic activity, since it is positioned in close proximity to the enzyme’s active sites, and substitution of this disulphide bond via site-directed mutagenesis experiments resulted in a drastic decrease in PET hydrolysis (Joo et al., 2018; Liu et al., 2018). However, the requirement of this
extra disulphide bond may be exclusive to the IsPETase, since other PETase-like cutinases have PET hydrolase activity and high thermostability without possessing this disulphide bond (Kawai et al., 2019).

**Figure 4:** Amino acid sequence alignment of the IsPETase and the SM14est proteins, generated using MEGA X, Muscle, and ESPript 3.0. Identical residues are highlighted in red boxes, and the ones with similar biochemical properties are highlighted in yellow boxes. The serine hydrolase motif Gly-x1-Ser-x2-Gly and the IsPETase extended loop are highlighted in black boxes; the catalytic triad is indicated by a star; the sub-site I and sub-site II are indicated by a blue and a red triangle, respectively. The disulphide bond 1 and disulphide bond 2 are indicated by blue and red ellipses, respectively.

Previous molecular docking analysis of the IsPETase, using a four-MHET molecule that would mimic PET (2-hydroxyethyl-(mono-hydroxyethyl terephthalate))₄ or 2-HE(MHET)₄ as ligand; together with site-directed mutagenesis analysis, suggests that the enzyme possess two sub-sites (Joo et al., 2018). Sub-site I has been proposed to be responsible for the binding of the first
MHET moiety, and thus for stabilisation of the ligand. Meanwhile, sub-site II has been proposed to be responsible for accommodating the other three MHET moieties, partially leading to the superior PET degradation by the IsPETase in comparison to other PETase-like enzymes. The sub-site I, which consists of 4 residues, is conserved in both IsPETase and SM14est (residues in IsPETase: Tyr87, Met161, Trp185, Ile208; residues in SM14est: Tyr88, Met157, Trp181, Ile204) (Figure 4 and Table S3). This implies that both enzymes have a similar mode of binding to the substrate. However, major differences exist between the two sequences in the 12-residue sub-site II region (residues in IsPETase: Thr88, Ala89, Trp159, Ile232, Asn233, Ser236, Ser238, Asn241, Asn244, Ser245, Asn246, Arg280; residues in SM14est: Thr89, Ala90, His155, Leu229, Arg230, Gly233, Phe235, Asn238, Asn241, Thr242, GAP, Gly277) (Figure 4 and Table S3). These differences in the sub-site II region in both proteins could lead to different binding affinities to the moieties of the PET polymer. Another important difference between the two protein sequences is in the loop connecting $\beta8$ and $\alpha6$, in which IsPETase appears to possess 3 extra amino acids in comparison to SM14est (Asn246, Gln247, Ala248) (Figure 4). This extended loop has been proposed to be an important structural feature of the IsPETase. When compared to a cutinase from *Thermobifida fusca* KW3, the extended loop seems to provide a conformation that allowed the formation of a continuous cleft on sub-site II; and hence accommodation of the third and fourth MHET moieties; therefore once more potentially explaining the superior enzymatic activity of IsPETase (Joo et al., 2018).

It has been suggested that the aforementioned protein structural differences between the IsPETase and the cutinase from *Thermobifida fusca* KW3, namely the absence of two disulphide bonds; differences in the sub-site II residues and the lack of an extended loop; could result in a reduced efficiency in the degradation of PET when compared to the IsPETase, and it has been proposed that proteins with these
characteristics could be classified into the type I category of PETase-like enzymes, which also seems to be the case for the SM14est protein (Joo et al., 2018).

4.5.6. Protein three-dimensional structure prediction and molecular docking

The function and stability of proteins is closely linked to its conformation, or folded/native state (Balcão and Vila, 2015; England and Haran, 2011; Lumry and Eyring, 1954). To provide further insights into the potential functionality and conformation of the SM14est protein, a three-dimensional structure of the protein was in silico predicted using SWISS-MODEL (Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Waterhouse et al., 2018). The cutinase 1 (Thc_Cut1) from Thermobifida cellulosilytica (PDB ID: 5LUI) was used (Ribitsch et al., 2017) as a template for the model prediction, which generated a model with a GMQE score of 0.76 and a QMEAN Z-score of -1.76, indicating a reliable predicted model (Figure 5B). When compared to the structure of the IsPETase (Figure 5A), the predicted structure of the SM14est shows many similarities, with both belonging to the \( \alpha/\beta \) hydrolase superfamily (Hotelier et al., 2004; Nardini and Dijkstra, 1999; Ollis et al., 1992), displaying a similar arrangement of 9 \( \beta \)-sheets and 7 \( \alpha \)-helices (Figure 5).

The arrangement of the catalytic triad residues is also quite similar, as highlighted in Figure 5A and Figure 5B, which may partially explain the synthetic polyester-degrading activity of these enzymes. With respect to observed potential differences between the two protein structures, the most striking differences; as previously shown in the amino acid sequence comparison, were the lack of disulphide bonds in the SM14est, and the absence of an extended loop between \( \beta8 \) and \( \alpha6 \). The latter, as previously mentioned, has been proposed to be linked to proper accommodation of the MHET moieties constituting the PET polymer, and therefore the superior enzymatic activity of the IsPETase (Joo et al., 2018).
Figure 5: Three-dimensional protein structure comparison and molecular docking.

A) IsPETase three-dimensional structure (PDB ID: 5XJH), the catalytic triad (Ser160, Asp206, His237) is circled in red; the two disulphide bonds (DB1 and DB2) are indicated with arrows.

B) Predicted three-dimensional structure of the SM14est protein, generated using SWISS-MODEL, with the crystal structure of cutinase 1 from Thermobifida cellulosilytica as template (PDB ID: 5LUI). The catalytic triad (Ser156, Asp202, His234) is circled in red.

C) Molecular docking simulation performed using AutoDock Vina, with BHET as substrate, indicated with an arrow, detailing the binding pocket, which is circled in red. The catalytic triad residues are shown as sticks. Structures were analysed and rendered using the UCSF Chimera software.
Molecular docking experiments were then performed, to analyse the likelihood of the SM14est enzyme possessing the capacity to bind plastics as substrates using an in silico-based approach; as well as determining the most probable binding mode of the protein to the ligand. To this end, the AutoDock Vina program was used for the protein-ligand molecular docking simulations (Trott and Olson, 2009), using the previously predicted SM14est structure and the BHET molecule as the ligand. BHET has previously been used as a model substrate for PET degradation both in vitro and in silico through molecular docking studies (Hantani et al., 2018; Joo et al., 2018; Liu et al., 2018). The molecular docking experiment generated 6 binding modes with energy ≤ −5.0 kcal/mol, similar to the binding modes energy values that had previously been described in similar molecular docking experiment performed with IsPETase and BHET (Liu et al., 2018). The binding modes were analysed in more detail under the UCSF Chimera software (Pettersen et al., 2004), and the best mode with the lowest binding energy is represented in Figure 5C, which highlights the binding pocket of the enzyme, the catalytic triad, and the proposed binding mode of the BHET molecule. The protein structure and molecular docking analyses results further emphasise the potential of the SM14est to degrade plastics, and highlight the structural features that may facilitate this enzymatic activity.

4.3. Heterologous expression of SM14est

To determine whether the SM14est gene does possess polyesterase activity, the gene was firstly codon-optimized for E. coli to facilitate heterologous expression in this host. The gene was then cloned into the expression vector pET-20b(+), generating the construct pET20b:SM14est, in which the native signal peptide sequence was maintained (Figure 1). The construct was then transformed into E. coli BL21(DE3) and transformants were tested for activity by performing a PCL plate clearing assay (Figure 6). A halo of clearing, which is indicative of PCL
degradation, was observed following one day of incubation (Figure 6A), with the activity subsequently increasing after 2, 3 and 4 days (Figure 6B, C, and D, respectively). The negative control with E. coli BL21(DE3) containing the pET-20b(+) plasmid without the insert did not show any PCL-degrading activity (Figure S1).

![Figure 6](image.png)

**Figure 6:** PCL plate clearing assay with the E. coli BL21(DE3)(pET20b:SM14est) heterologous host, incubated at 28°C after A) 1 day; B) 2 days; C) 3 days; D) 4 days.

Another interesting observation was that the E. coli host successfully exported the heterologously expressed SM14est enzyme, when the native Streptomyces sp. SM14 signal peptide sequence was present in the expression construct (Figure 1, Figure 6). The signal peptide sequence was predicted to consist of the first 25 amino acids of the protein sequence, with a cleavage site probability of 0.9316, and to belong to the general secretory (Sec) pathway, with a likelihood value of 0.9608. In heterologous protein expression systems involving E. coli, successfully secretion and maintenance of the native protein confirmation can sometimes be challenging, and in this case potentially so, due to the fact the SM14est protein originates from such a distant host, *i.e.* a Streptomyces isolate (Freudl, 2018). Several Streptomyces genes encoding different enzymes have previously been heterologously expressed in E. coli (Spasic et al., 2018); including a xylanase from *S. mexicanus* HY-14 JQ943651 (Kim et al., 2014); a laccase from *S. coelicolor* (Sherif et al., 2013); a protease from *S. koyangensis* (Ben Elhoul et al., 2015); a glucose isomerase from Streptomyces sp. SK (Ben Hlima et al., 2013); and
an esterase from *S. lividans* (Wang et al., 2016). In these cases, however, unlike with the heterologous expression of the SM14est in *E. coli*, the native signal sequence was not employed in the expression constructs.

A number of different signal peptide sequences have previously been employed to ensure the secretion of PETase and PETase-like enzymes from *E. coli*. A PET carboxylesterase from *Thermobifida fusca* has been expressed and secreted from *E. coli* using a pelB leader sequence (Oeser et al., 2010), while a PET hydrolase has also previously been expressed and secreted from *Bacillus subtilis* using a native PETase signal peptide (SP_{PETase}) (Huang et al., 2018). In addition, a Sec-dependent signal sequence from *E. coli* has also recently been used to express the IsPETase, resulting in the production of the extracellular enzyme using *E. coli* (Seo et al., 2019). The successful secretion of heterologously expressed PETases is important not only for their subsequent purification, and biochemical characterisation (Han et al., 2017; Joo et al., 2018; Liu et al., 2018), but also for their potential development as recombinant PET-degrading microbes with utility in environmental remediation strategies. Therefore, it is interesting to note that we were able to efficiently heterologous express the PETase-like SM14est enzyme from *Streptomyces* sp. in *E. coli*, without the requirement to change its native signal peptide sequence, with extracellular synthetic polyester-degrading activity being observed in a PCL plate clearing assay. To our knowledge this is the first report of a PETase-like enzyme being identified in a marine sponge-derived *Streptomyces* spp. isolate, and we believe that the PETase-like SM14est enzyme will help provide further insights into our current knowledge of this important class of synthetic polyester-degrading enzymes.
5. Conclusion

Plastics such as the polyethylene terephthalate (PET) have been commonly used in storage materials and in synthetic fabrics, and their resistance to biodegradation has resulted in their accumulation in terrestrial and marine ecosystems at an alarming rate. In an attempt to alleviate this problem, much recent scientific interest has focused on the enzymatic hydrolysis of these types of synthetic polyesters, including PET. While a number of PETase and PETase-like enzymes have been identified and biochemically characterised, there is still much to be learned about this class of enzymes. In addition, more information on their structure, activity, and how widespread they are distributed in nature is required; and if they can ultimately be improved using genetic and protein engineering and applied in bioremediation strategies on an industrial scale.

Although the *Streptomyces* genus is well studied with respect to the production of bioactive compounds, less is known about their potential to produce enzymes with synthetic polyester-degradation activities. In this study, based on an *in silico* screening approach, we were able to identify a PETase-like enzyme, namely SM14est, with synthetic polyester-degrading activity, which was isolated from the marine sponge-derived strain *Streptomyces* sp. SM14, with enzyme activity being confirmed *in vitro* with the heterologous expression of the protein in *E. coli* using PCL plate clearing assays. Importantly, an active heterologously expressed SM14est protein was secreted from *E. coli* with the native *Streptomyces* SM14est signal peptide sequence. This will facilitate not only the future biochemical characterisation of the protein, but also its potential utility in other bioremediation-based applications targeting synthetic polyesters.
6. References


Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and


Trott, O., and Olson, A. J. (2009). AutoDock Vina: Improving the speed and


### Supplementary material

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**Table S1**: *Streptomyces* genome sequences employed in this study, obtained from the GenBank database when indicated. The isolation source is discriminated as marine and non-marine environment.
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**Table S2:** Primers and respective PCR amplification conditions employed in this study. The primer pair SM14est-fw and SM14est-rev was employed to subclone the synthetic SM14est gene sequence into the pET-20b(+) vector. The primer pair T7 and T7_term (T7 standard primers) was employed to confirm the insert in the construct pET20b:SM14est.
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<td>Arg280</td>
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**Table S3:** Comparison of residues constituting the catalytic triad, sub-site I, and sub-site II in IsPETase and SM14est. Residue substitutions in SM14est are indicated with an asterisk, and those substitutions involving amino acids with similar biochemical properties are indicated with two asterisks.
Figure S1: PCL plate clearing assay at 28°C. Left: *E. coli* BL21(DE3)(pET20b:SM14est); right: *E. coli* BL21(DE3) negative control.

Figure S2: Nucleotide sequences alignment of the native SM14est protein codon usage (SM14est-WT) and the *E. coli* codon optimised synthetic version of the gene (SM14est-OPT). Darker boxes indicate identical nucleotides, while the lighter boxes indicate differences. Features such as the signal peptide sequence, and the engineered His$_6$ tag and restriction sites (*Ndel* and *XhoI*) are indicated accordingly.
Chapter 5

General Discussion and Concluding Remarks
1. General discussion

1.1. Marine sponge-derived *Streptomyces* species isolates: the importance of morphological, phenotypical, and genetic characterisation, and their potential as sources of novel bio-active compounds and enzymes

Despite the fact that *Streptomyces* species isolates are well-known to have the capacity to produce numerous bio-active molecules of pharmacological, clinical, and biotechnological interest (Mohr, 2016; Watve et al., 2001), there is still a lack of information regarding *Streptomyces* isolates derived from the marine environment. Although recent research efforts have succeeded in identifying novel compounds with relevant bio-activities from *Streptomyces* found associated with marine sponges (Indraningrat et al., 2016), as shown in Chapter 1, very few studies have to date focused on the characterisation of these isolates at a genetic, phenotypical and morphological level (Ian et al., 2014; Tian et al., 2016).

In Chapter 2, we analysed two *Streptomyces* spp. isolates, namely strains SM17 and SM18, that were isolated from the marine sponge *Haliclona simulans*, and which had previously been shown to possess bio-activity against clinically relevant pathogens (Jackson et al., 2018; Kennedy et al., 2009). SM17 has been shown to possess inhibitory activity against *Escherichia coli* NCIMB 12210, MRSA, and *Candida* species; while SM18 inhibited the growth of MRSA and *Bacillus subtilis* 1A40 (Jackson et al., 2018; Kennedy et al., 2009). In the study performed in Chapter 2, we employed genomics and culture-based phenotypical characterisation aimed at providing novel insights with respect to genetic traits that may be related to environmental niche adaptations in SM17 and SM18, as well as their potential to produce secondary metabolites (Almeida et al., 2019). State-of-the-art sequencing technology was employed involving single molecule real-time sequencing (SMRT), using the PacBio RSII sequencing platform; which allowed for the assembly of
high-quality complete genome sequences for both SM17 and SM18 strains. This resulted in, to our knowledge; two of the first complete genome sequences of marine sponge-derived *Streptomyces* spp. isolates, which in itself represents an important milestone and a data resource on these interesting bacteria, which will be available to the wider scientific community. This also demonstrated that the PacBio RSII sequencing platform provides excellent outcomes with respect to sequencing the genomic DNA of uncharacterised environmental *Streptomyces* isolates.

A series of comparative analyses were performed in Chapter 2, between the marine sponge-derived isolates SM17 and SM18 and their closest terrestrial relatives, namely *Streptomyces albidoflavus* J1074 (referred to as *S. albus* J1074 in Chapter 2) and *Streptomyces pratensis* ATCC 33331, respectively, which were determined following phylogenomics-based analysis. Phenotypical differential growth assessment showed that the marine sponge-derived isolates SM17 and SM18 possessed an increased fitness to grow and differentiate in the presence of salts in the growth medium, when compared to their terrestrial counterparts. This was particularly true when comparing the SM17 and J1074 isolates, for which the terrestrial J1074 appeared to struggle to grow in the medium enriched with salts, while the marine SM17 thrived. Thus, it became clear that investigating genetic differences between the marine and terrestrial isolates might provide insights with respect to adaptations possessed by these marine-derived strains when compared to their terrestrial counterparts, at a genetic level.

Prediction of secondary metabolite biosynthetic gene clusters (BGCs) results presented in Chapter 2 showed that the marine sponge-derived isolates SM17 and SM18 possessed the potential to produce a range of diverse secondary metabolites, some of which share similarities with known compounds, and that are also unique in comparison to their terrestrial counterparts. This analysis further highlights the
importance of exploring marine microbiomes, particularly those associated with marine sponges, in the search for novel bioactive compounds.

Interestingly, the genomic analysis in the search for BGCs showed that while the SM18 isolate possessed siderophore-encoding BGCs, such as mirubactin and coelichelin, it did not possess a BGC encoding for the production of the desferrioxamines siderophores; which until our study were believed to be present in all *Streptomyces* species (Cruz-Morales et al., 2017; Tierrafría et al., 2011). This unprecedented characteristic could represent an adaptive and/or evolutionary trait, which would be interesting to further investigate. For example, it would be interesting to analyse the ability of the SM18 strain to grow in presence/absence/different concentrations of iron in the culture medium, and how it compares to other *Streptomyces* strains that possess desferrioxamines BGCs.

An in-depth comparative genomics between SM17, SM18, J1074 and ATCC 33331 allowed us to identify a subset of genes that are commonly present in the marine-derived isolates, and which are absent in their terrestrial counterparts. These genes were *in silico* functionally characterised; and some were proposed to represent environmental niche adaptations (ENA) that these marine isolates may possess, at a genetic level. These adaptations included resistance to osmotic stress; transcriptional regulation; symbiotic interactions; antimicrobial compounds production and resistance; ABC transporters; and horizontal gene transfer (HGT) and other potential defence-related features.

Further genomic analysis regarding the ENA gene pool, including *Streptomyces* isolates from other environments that were determined to be phylogenetically related to the SM17 and SM18 strains; indicated the presence of some of potential adaptation genes in other isolates. However, the majority of these isolates were derived from environments in which similar stresses, as those that the SM17 and SM18 are subjected to, could be experienced; since all of them were
isolated either from rhizosphere soil (which is known to present osmotic stress, for example), aquatic saline environments, or from symbiotic communities. Thus, this suggests that it is more likely that these adaptations may be related to specific stresses or environmental conditions, instead of general “marine” adaptations, in contrast to what has been suggested by previous studies (Ian et al., 2014; Penn and Jensen, 2012; Sun et al., 2018). A good example of this is the \textit{nuo} operon, which has been proposed to be a marine adaptation in \textit{Salinispora}, \textit{Kocuria}, and other marine \textit{Streptomyces} species (Ian et al., 2014; Penn and Jensen, 2012; Sun et al., 2018); however, this operon was also shown in Chapter 2 to be present in \textit{Streptomyces} derived from rhizosphere soil and other aquatic saline environments, which could instead point to a trait involving osmoadaptation. Thus, in future studies, it would be interesting to assess the capability of these other environmental \textit{Streptomyces} isolates to grow in the presence/different concentrations of salts in the culture medium.

Among the genes previously identified to be commonly present in the marine sponge-derived isolates SM17 and SM18, while absent in their terrestrial counterparts J1074 and ATCC 33331, a total of 7 of these genes were also absent in the other isolates analysed in the study. These consisted of: two genes encoding hypothetical proteins; a gene encoding a darcynin family of bacterial proteins with unknown function; a gene encoding an AAA family ATPase protein; the \textit{yihX} gene, which seems to encode a phosphatase; the previously mentioned \textit{tauB/ssuB} gene, which appears to encode an ABC transporter; and the \textit{aldH} gene, which seems to encode a NADP-dependent aldehyde dehydrogenase. Although most of these genes seem to encode proteins with unknown functions, the previously mentioned TauB/SsuB and the YihX proteins may be related to a broader ability of the SM17 and SM18 strains for nutrient acquisition when compared to the other strains, specifically with respect to nitrate and sulfonate, and to phosphorus, respectively,
which are important resources for marine-derived bacteria (Hoppe HG, 2003; Karimi et al., 2018; Sebastian and Ammerman, 2009). Finally, interestingly, two of these genes, namely aldH and tauB/ssuB, were determined to be homologs of genes belonging to the marine isolates Vibrio harveyi ATCC 33843 and Methanocaldococcus jannaschii DSM 2661; result that further indicates their potential as adaptive traits to the conditions these isolates may be exposed to in marine environments.

1.2. Genome mining and the OSMAC approach as powerful methods for the identification of natural products in Streptomyces isolates

Chapter 3 followed up on some of the results presented in Chapter 2, in which the surugamides BGC (referred to as sur BGC) was predicted to be present in the Streptomyces sp. SM17 strain. More detailed phylogenomics analysis of this isolate, together with its closest relatives was performed, namely Streptomyces albidoflavus strain J1074, S. albidoflavus strain SM254, S. sampsonii strain KJ40, and Streptomyces sp. FR-008. In addition to sharing high similarities with respect to marker genes, i.e. the 16S rRNA gene, as well as the atpD, gyrB, recA, rpoB, and trpB genes, these isolates were also shown to share a considerably high number of genes, for which 5,177 genes (around 68% of their pan-genome) were determined to be conserved in all the isolates (i.e. the core genome), and 1,055 genes (around 14% of their pan-genome) were determined to be present in at least two isolates (i.e. the accessory genome). These results prompted us to propose the presence of the albidoflavus phylogroup, which has also been suggested in other studies (Labeda et al., 2014, 2017), and that all these strains are likely to be members of the same species. Hence, it is likely that the SM17 and FR-008 isolates in fact belong to the S. albidoflavus species, and that Streptomyces sampsonii KJ40 might have potentially been misassigned, which is in fact a common recurring issue in the taxonomy assignment of Streptomyces isolates. Good examples of this issue are coincidently
two strains analysed in this study that belong to the *albidoflavus* phylogroup, namely *S. albidoflavus* J1074 and *S. albidoflavus* SM254, which have previously been assigned as *S. albus* isolates, but which have more recently been re-assigned as *S. albidoflavus* isolates (Hamm et al., 2017; Labeda et al., 2017).

Interestingly, all of the members of the *albidoflavus* phylogroup that were investigated in Chapter 3 do possess the BGC encoding for the production of surugamides (*sur* BGC) (Ninomiya et al., 2016). Further analyses employing phylogenetics and gene synteny showed that this BGC is highly conserved amongst these isolates, and are in particular phylogenetically more similar between the strains derived from aquatic-saline environments, namely Streptomyces sp. SM17, *S. albidoflavus* LHW3101, and *S. albidoflavus* SM254. These results provided evidence that there is a strong evolutionary pressure towards maintaining the gene synteny – and even the reading frames of the main biosynthetic genes – of the *sur* BGC, and also that this BGC potentially may in fact have originated in the marine environment. Furthermore, the downstream region to the main biosynthetic genes have been shown to be conserved in all the isolates, with exception to the KJ40 strain. Whereas in the other strains the downstream region to the *sur* BGC encompassed 1) a predicted multi-drug resistance (MDR) transporter; 2) a predicted TetR/AcrR transcriptional regulator; 3) a hypothetical protein; and 4) another predicted MDR transporter; while the downstream region to the KJ40 *sur* BGC comprised of 1) a group of four hypothetical proteins, which may represent pseudogene versions of the first MDR transporter identified in the other isolates; 2) a predicted rearrangement hotspot (RHS) repeat protein; 3) a hypothetical protein; and 4) a MDR transporter. Thus, it would be interesting to evaluate the ability of the KJ40 isolate to produce surugamides and determine whether these downstream genes do in fact play a role in their production.
These results prompted us to investigate differences in the expression level of surugamides when comparing a marine isolate (SM17) with a terrestrial isolate (J1074) when grown in different culture conditions, employing an “One Strain Many Compounds” (OSMAC)-based approach (Bode et al., 2002; Pan et al., 2019; Romano et al., 2018). These two strains were grown in different complex media (namely SYP-NaCl, YD, SY, P1, P2, P3, CH-F2, Oatmeal, and Sporulation media), and their metabolic profile with respect to surugamide A production was analysed, employing Liquid Chromatography–Mass Spectrometry (UPLC-DAD-HRMS and UPLC-HRMS), High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy.

Surugamide A production was identified in the extracts from TSB, SYP-NaCl and YD media, and interestingly SM17 was shown to be able to produce more surugamide A than J1074 in all the conditions tested. In fact, production of surugamide A by SM17 reached yields of up to >13-fold higher in YD medium, and around 3-fold higher when grown in the SYP-NaCl medium when compared to J1074. Identifying bacterial isolates that possess a higher capacity to produce compounds of pharmacological interest under standard laboratory conditions is certainly an important step towards the development of new drugs and industrial processes.

In both SM17 and J1074, the production of surugamide A was higher when they were grown in SYP-NaCl medium. Interesting inferences can be made based on these results. Since the media analysed use different carbon sources, it is possible that carbon catabolite repression may play a major influence in the production of surugamides, as it has also been reported to play for other secondary metabolites (Brückner and Titgemeyer, 2002; Deutscher, 2008; Kremling et al., 2015; Magnus et al., 2017; Romero-Rodríguez et al., 2017; Stülke and Hillen, 1999). The SYP-NaCl medium has starch, a complex polysaccharide, as its main carbon source,
while TSB and YD have glucose and dextrins, respectively. Additionally, the presence of salts in the SYP-NaCl medium might have an influence on the expression of the *sur* BGC. It has been reported that, for example, in the marine-obligate actinobacteria *Salinispora arenicola*, different concentrations of salts in the culture medium can result in different metabolic profiles; with different types and/or quantities of secondary metabolites produced by this isolate being influenced by the salts in the medium (Bose et al., 2015). Hence, it would be interesting to, in future studies; assess the influence of different concentrations of salts in the medium in the production of secondary metabolites by the SM17, J1074 and other *Streptomyces* isolates, particularly with respect to their potential influence on the production of surugamides.

All of these conclusions were only possible thanks to an initial genome mining-based assessment of the SM17 genome, and subsequent culture-dependent analyses coupled with analytical chemistry; which provided novel insights on the distribution of the *sur* BGC in *Streptomyces* species, how conserved they are genetically, and that carbon catabolite repression could be involved in the production of surugamides. Thus, the results discussed in Chapter 3 ultimately show that these approaches can also be very useful for the further characterisation of compounds which have previously been isolated, such as the surugamides, and to provide novel insights into regulatory mechanisms for the production of secondary metabolites that can be ultimately useful for the optimisation of their production in pharmacological and industrial settings.

1.3. **The potential of marine-derived *Streptomyces* isolates to harbour enzymes with synthetic polyesters-degrading activities**

Chapter 4 also clearly demonstrates how genome mining-based approaches, coupled with culture-dependent assays and state-of-the-art synthetic biology can
be applied when investigating the biotechnological potential of *Streptomyces* isolates, however in a slightly different case scenario than the previous chapters.

Instead of searching for BGCs encoding for the production of secondary metabolites, alternatively, we employed an initial *in silico* screening based on genome mining in a search for enzymes of potential biotechnological interest. Specifically, enzymes with potential polyesterase activity were targeted, based on the following rationale. Firstly, as it has been mentioned in Chapter 1, the issue of plastic pollution is a serious problem, particularly when it comes to marine ecosystems (Geyer et al., 2017; Jambeck et al., 2015). In fact, micro-plastics are now believed to be ubiquitous in the oceans, and are likely to be consumed by marine animals, and even possibly by marine sponges (Geyer et al., 2017; Lebreton and Andrady, 2019). Thus, it is reasonable to assume that *Streptomyces* isolates found in association with marine sponges are likely to have been in contact with these ubiquitous micro-plastics; particularly given that some sponge species can filter up to 20,000 litres of seawater kg\(^{-1}\) h\(^{-1}\) (dry wt) (Food and Agriculture Organization of the United Nations, 2017; Taylor et al., 2007). Therefore, these microorganisms could have developed – or inherited, e.g. via horizontal gene transfer – mechanisms to degrade and assimilate these micro-plastics, potentially even using them as energy source.

A set of 15 nucleotide/amino acid sequences of known enzymes with PET hydrolase activity was used as a reference (Kawai et al., 2019; Wei and Zimmermann, 2017); and a set of 52 *Streptomyces* genome sequences from terrestrial (23 genomes) and aquatic environments (29 genomes) was screened for potential homologs to these reference PET hydrolase enzymes (referred to as PETase-like enzymes). Phylogeny analysis of such potential PETase homologs indicated that three protein sequences belonging to three marine-derived *Streptomyces* spp. isolates, namely *Streptomyces* sp. SM14, *Streptomyces* sp. SM12,
and *Streptomyces xinghaiensis* S187, shared high sequence similarities together with close phylogenetic relatedness to the reference PETases. Due to two main reasons, further analyses were performed with the SM14 strain and its potential polyesterase enzyme. Firstly, further sequence analysis showed that the protein sequences derived from the SM12 and SM14 strains were in fact identical, which may not be surprising since these two isolates were determined to be very closely related. Secondly, the SM14 strain had originally been isolated by our group and genome sequence for the strain was available from previous works (Jackson et al., 2018; Kennedy et al., 2009), hence this strain was readily available in our culture collection, in contrast with the *S. xinghaiensis* S187, to which we had no ready access to.

The polyesterase activity of the *Streptomyces* sp. SM14 isolate was assessed via a polycaprolactone (PCL) plate-clearing assay (Murphy et al., 1996; Nawaz et al., 2015; Nishida et al., 1998; Nishida and Tokiwa, 1993), and confirmed when a pronounced halo of clearing was observed. This prompted us to further investigate the enzyme potentially responsible for the observed activity (namely SM14est) employing both *in silico* and *in vitro* approaches.

In Chapter 4, by employing *in silico* methods, the amino acid sequence and the predicted three-dimensional structure of the SM14est protein was determined, employing information from the currently best studied PETase, namely the *Ideonella sakaiensis* 201-F6 PETase (referred to as IsPETase) (Joo et al., 2018; Yoshida et al., 2016). This enabled us to provide some interesting insights with respect to the structural characteristics of the SM14est protein and how they could be related with its polyesterase activity, together with how differences that we observed between SM14est and the IsPETase could be related to differences in the enzyme’s activities and affinities to plastics substrates. These inferences were also based on previous studies that had experimentally determined the crystal structure
of the IsPETase (Joo et al., 2018; Liu et al., 2018), and thus had identified some of
the structural features possessed by this enzyme that could, at least partially,
explain its superior activity and affinity to a PET substrate.

In particular, it has been determined that the SM14est amino acid sequence
shared significant similarities to the IsPETase, with 41% amino acid identity in
addition to 19% of the amino acids sharing similar biochemical properties to those
of the IsPETase. The SM14est is like the IsPETase in that it displays a similar
arrangement of 9 β-sheets and 7 α-helixes, and thus may belong to the same α/β
hydrolase superfamily. The serine hydrolase motif (Gly-x1-Ser-x2-Gly) present in
the IsPETase was also determined to be conserved in the SM14est, i.e. Gly154-
His155-Ser156-Met157-Gly158, as well as the catalytic triad (Ser156, Asp202,
His234). The sub-site I of the IsPETase, which has been reported to be involved in
the stability of the substrate (Joo et al., 2018), also appears to be conserved in the
SM14est, encompassing the four residues Tyr88, Met157, Trp181, Ile204 in the
SM14est amino acid sequence.

However, key sequence and structural differences were also identified when
comparing SM14est with the IsPETase. Major differences were observed in the 12-
residue sub-site II region, which has been suggested to provide a superior
accommodation of the moieties of the PET substrate (Joo et al., 2018). These
residues, in IsPETase, are Thr88, Ala89, Trp159, Ile232, Asn233, Ser236, Ser238,
Asn241, Asn244, Ser245, Asn246, Arg280; whereas in SM14est they have been
determined to encompass Thr89, Ala90, His155, Leu229, Arg230, Gly233, Phe235,
Asn238, Asn241, Thr242, an amino acid alignment gap, and Gly277. Also, the
IsPETase possesses an extended loop connecting β8 and α6, which seems to be
absent in SM14est. This extended loop has been proposed to provide a higher
optimisation of the enzyme structure for an improved accommodation of the
substrate, and hence a superior enzymatic activity and affinity described in the
IsPETase (Joo et al., 2018). Finally, two disulfide bonds that are present in the
IsPETase are absent in the SM14est, which may result in the SM14est being less
thermostable or in being less active, since one of these disulfide bonds (between
Cys203 and Cys239) is located in the vicinity of the catalytic site in the IsPETase
(Joo et al., 2018).

Finally, in order to confirm that the previously observed polyesterase
activity by the SM14 strain was indeed being performed by the in silico-determined
SM14est enzyme we heterologously expressed a codon-optimised synthetic version
of the SM14est gene in E. coli. The activity was confirmed by employing a PCL
plate-clearing assay, in a similar fashion to the assay previously performed with the
wild type SM14 strain.

Thus, in Chapter 4 an efficient method to identify PETase-like enzymes
based on genomic approaches is demonstrated, for which we were able to identify
and in silico characterise for the first time, to our knowledge, a polyesterase with
PCL-degrading activity derived from a marine sponge-associated Streptomyces
isolate. Secondly, an efficient method to heterologously express a PETase-like
enzyme from a Streptomyces strain is reported. The latter also represents a
potential improved activity of the enzyme, since the halos of clearing in the PCL plate-clearing assays employing the heterologous host occurred in a significant
shorter period of time; with the activity observed in the heterologous E. coli host
system after 4 days being comparable to the activity observed after 12 days in the
native SM14 host. These activity levels would however need to be determined in
future studies to confirm this observation. Even more interestingly, however, is the
fact that the E. coli heterologous host was able to express and secrete a functional
polyesterase enzyme, even when the native Streptomyces signal peptide sequence
was employed in the synthetic gene construct used to express the E. coli codon-
optimised version of the SM14est gene. This novel finding was unexpected and to
our knowledge is likely to be unprecedented, as a native *Streptomyces* signal sequence being employed to export a functional *Streptomyces*-derived protein in an *E. coli* host system is highly unusual, mostly due to differences in the secretion pathways between the two genera. The impact of this finding could potentially go way beyond the scope of this study, and further investigations should be undertaken with this signal peptide, and how it could potentially be employed for the heterologous expression of other *Streptomyces*-derived enzymes in an *E. coli* host. Ideally, it would also have been interesting to analyse the effects of the SM14est enzyme in the degradation of PET and other recalcitrant plastics, but due to time and technical restrictions this was unfortunately not possible.

Nevertheless, the results presented in Chapter 4 represent new advances in our understanding of this extremely important PETase-like enzyme family. In particular, we report for the first time on an enzyme belonging to this family being present in a marine sponge-derived *Streptomyces* isolate. In addition by reporting on how genomic approaches coupled with simple *in vitro* assays and synthetic biology-based approaches can be used to identify PETase-like homologs; we open up the possibility of determining how potentially widespread in nature these enzymes might be; and hope that this may lead to the identification of other members of this enzyme family, with potential utility in various biotechnological applications and in particular in the degradation of synthetic polyesters.
2. Concluding remarks

Although recent efforts have shifted towards the exploration of marine-derived bacterial species aimed at the identification and isolation of novel compounds with pharmacological and biotechnological interest, marine sponge-associated *Streptomyces* species as a group are still largely underexplored. Throughout the work performed in this thesis, novel insights with respect to their genetics, biology and ecological niche lifestyle adaptations have been proposed, together with highlighting their outstanding potential as sources of potential bioactive compounds and enzymes of biotechnological and pharmacological interest.

Chapter 2 represents one of the very few studies published to date in regard to the characterisation of marine sponge-derived *Streptomyces* isolates, particularly from a genomics standpoint. Specifically, two marine sponge-derived isolates, namely *Streptomyces* sp. SM17 and *Streptomyces* sp. SM18, which had been subjected to state-of-the-art genome sequencing, represent two of the first complete genomes of marine sponge-derived *Streptomyces* available to date. These isolates were compared with their closest soil-derived counterparts, with a particular aim of identifying potential environmental niche adaptations possessed by these marine isolates. Additionally, the work performed here was crucial to proceeding with the analyses performed in the studies presented in the following chapters, with respect to assessing and setting the best approaches regarding genomics and bioinformatics-based pipelines for the analysis of our marine *Streptomyces* isolates.

In Chapter 3, using some of the genomics approaches established in Chapter 2, a new strain capable of producing surugamides was identified, namely *Streptomyces* sp. SM17. The surugamide family of compounds appears to be very promising due to their previously determined antitumor and antifungal properties,
however very few studies focusing on this family of compounds have been published to date (Kuranaga et al., 2018; Matsuda et al., 2019; Ninomiya et al., 2016; Takada et al., 2013; Thankachan et al., 2019; Xu et al., 2017). Therefore, the results presented in Chapter 3 are important in the context of gaining a better understanding of this family of compounds, from both a genetic and evolutionary standpoint, and to determine optimal conditions for their production. It also reports on the identification of a Streptomyces strain (SM17) that appears to be able of produce high levels of surugamide A in standard culture conditions, at least when compared to the well-known strain Streptomyces albideflavus J1074.

Finally, in Chapter 4, the exploitation of the biotechnological potential of marine-derived Streptomyces isolates employing genomics and bioinformatics-based approaches is described. In particular, these approaches were employed for the identification of novel enzymes potential with synthetic polyesters-degrading activities. In this study, we were able to identify, for the first time, a PETase-like polyesterase (SM14est) with PCL-degrading activity from a marine-sponge derived Streptomyces isolate, namely Streptomyces sp. SM14. Further work with this enzyme, such as an in-depth biochemical characterisation, together with further assessment of its plastics-degrading capabilities using other substrates such as PET, should be performed and would provide further insights into this family of enzymes. Nevertheless, this study is fundamental in shedding new light on how widespread in nature this family of enzymes might be, as well as reporting on an in silico workflow that coupled with culture-dependent and state-of-the-art synthetic biology methods can facilitate the identification of novel enzymes belonging to this family. These enzymes may ultimately be used in bioremediation-based strategies targeting plastic pollution, particularly in marine environments, or in novel processes for plastic waste management.
The work presented in this thesis ultimately helps to set new milestones with respect to appropriate genomics-based approaches that can be employed to exploit marine sponge-derived *Streptomyces* isolates, for biotechnological and pharmacological applications, which have been demonstrated to be useful in this work. In particular these approaches proved useful in the identification of a strain which produces secondary metabolites of pharmacological interest, namely the SM17 strain which produces surugamides, and in uncovering the polyester degrading capabilities of marine *Streptomyces* isolates, with the identification, *in silico* characterisation and heterologous expression of the SM14est polyesterase derived from the SM14 strain.
3. References


Appendix I

Other publications and contributions

Abstract: *Streptomyces* sp. strain SM8, isolated from *Haliclona simulans*, possesses antifungal and antibacterial activities and inhibits the calcineurin pathway in yeast. The draft genome sequence is 7,145,211 bp, containing 5,929 predicted coding sequences. Several secondary metabolite biosynthetic gene clusters are present, encoding known and novel metabolites, including antimycin.

Contribution: Eduardo L. Almeida processed the 454 sequencing data obtained using a GS FLX Titanium system (Roche), assembled and annotated the *Streptomyces* sp. SM8 genome, performed quality assessments, data analysis, and wrote the manuscript.

**Abstract:** *Pseudomonas putida* strain CA-3 is an industrial bioreactor isolate capable of synthesizing biodegradable polyhydroxyalkanoate polymers via the metabolism of styrene and other unrelated carbon sources. The pathways involved are subject to regulation by global cellular processes. The draft genome sequence is 6,177,154 bp long and contains 5,608 predicted coding sequences.

**Contribution:** Eduardo L. Almeida processed the Illumina’s MiSeq paired-end sequencing data, assembled and annotated the *Pseudomonas putida* strain CA-3 genome, performed quality assessments, data analysis, and wrote the manuscript.
Appendix I


**Authors list:** Beatriz Gil-Pulido, Emma Tarpey, Eduardo L. Almeida, William Finnegan, Xinmin Zhan, Alan D.W. Dobson, and Niall O’Leary.

**Abstract:** Dairy processing generates large volumes of wastewater that require extensive nutrient remediation prior to discharge. Significant commercial opportunities exist therefore for cost-effective biotechnologies capable of achieving this requirement. In this study the authors evaluated the use of intermittently aerated sequencing batch reactors, (IASBRs), as a single-tank biotreatment system for co-removal of COD, nitrogen and phosphorus from synthetic dairy processing wastewater. Variation of the IASBR aeration rates, (0.8, 0.6 and 0.4 L/min), had significant impacts on the respective nutrient removal efficiencies and underlying microbial diversity profiles. Aeration at 0.6 L/min was most effective and resulted in >90% co-removal of orthophosphate and ammonium. 16S rRNA based pyrosequencing of biomass DNA samples revealed the family *Comamonadaceae* was notably enriched (>80% relative abundance) under these conditions. *In silico* predictive metabolic modelling also identified *Comamonadaceae* as the major contributor of several known genes for nitrogen and phosphorus assimilation (*nirK, nosZ, norB, ppK, ppX and phbC*).

**Contribution:** Eduardo L. Almeida performed the *in silico* metabolic modelling analysis reported in this work, employing 16S amplicon analysis tools such as QIIME and PICRUSt, together with statistical analysis such as Principal Component Analysis.
Appendix I


Abstract: The genus *Streptomyces* produces secondary metabolic compounds that are rich in biological activity. Many of these compounds are genetically encoded by large secondary metabolism biosynthetic gene clusters (smBGCs) such as polyketide syntheses (PKS) and non-ribosomal peptide synthetases (NRPS) which are modular and can be highly repetitive. Due to the repeats, these gene clusters can be difficult to resolve using short read next generation datasets and are often quite poorly predicted using standard approaches. We have sequenced the genomes of 13 *Streptomyces* spp. strains isolated from shallow water and deep-sea sponges that display antimicrobial activities against a number of clinically relevant bacterial and yeast species. Draft genomes have been assembled and smBGCs have been identified using the antiSMASH (antibiotics and Secondary Metabolite Analysis Shell) web platform. We have compared the smBGCs amongst strains in the search for novel sequences conferring the potential to produce novel bioactive secondary metabolites. The strains in this study recruit to four distinct clades within the genus *Streptomyces*. The marine strains host abundant smBGCs which encode polyketides, NRPS, siderophores, bacteriocins and lantipeptides. The deep-sea strains appear to be enriched with gene clusters encoding NRPS. Marine adaptations are evident in the sponge-derived strains which are enriched for genes involved in the biosynthesis and transport of compatible solutes and for heat-shock proteins. *Streptomyces* spp. from marine environments are a promising source of novel bioactive secondary metabolites as the abundance and diversity of smBGCs show high degrees of novelty. Sponge derived *Streptomyces* spp. isolates appear to display genomic adaptations to marine living when compared to terrestrial strains.

Contribution: Eduardo L. Almeida contributed with the bioinformatics-based analyses present in this paper, particularly those related to the analysis of secondary metabolism protein domains of interest present in the marine *Streptomyces* spp. analysed in this work, employing the antiSMASH program together with custom Python and R scripts for data processing.