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Two *Streptomyces* Species Producing Antibiotic, Antitumor, and Anti-Inflammatory Compounds Are Widespread Among Intertidal Macroalgae and Deep-Sea Coral Reef Invertebrates from the Central Cantabrian Sea

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Abstract Streptomycetes are widely distributed in the marine environment, although only a few studies on their associations to algae and coral ecosystems have been reported. Using a culture-dependent approach, we have isolated antibiotic-active *Streptomyces* species associated to diverse intertidal marine macroalgae (*Phyllum Heterokontophyta*, *Rhodophyta*, and *Chlorophyta)*, from the central Cantabrian Sea. Two strains, with diverse antibiotic and cytotoxic activities, were found to inhabit these coastal environments, being widespread and persistent over a 3-year observation time frame. Based on 16S rRNA sequence analysis, the strains were identified as *Streptomyces cyaneofuscatus* M-27 and *Streptomyces carnosus* M-40. Similar isolates to these two strains were also associated to corals and other invertebrates from deep-sea coral reef ecosystem (*Phyllum Cnidaria*, *Echinodermata*, *Arthropoda*, *Sipuncula*, and *Anelida*) living up to 4.700-m depth in the submarine Avilés Canyon, thus revealing their barotolerant feature. These two strains were also found to colonize terrestrial lichens and have been repeatedly isolated from precipitations from tropospheric clouds. Compounds with antibiotic and cytotoxic activities produced by these strains were identified by high-performance liquid chromatography (HPLC) and database comparison. Antitumor compounds with antibacterial activities and members of the anthracycline family (daunomycin, cosmomycin B, galtamycin B), antifungals (maltophilins), anti-inflamatory molecules also with antituberculosis properties (lobophorins) were identified in this work. Many other compounds produced by the studied strains still remain unidentified, suggesting that *Streptomyces* associated to algae and coral ecosystems might represent an underexplored promising source for pharmaceutical drug discovery.

Keywords Avilés Canyon · Antracyclines · Lobophorins · Maltophilins · *Streptomyces cyaneofuscatus* · *Streptomyces carnosus*

Accession numbers HG965212 · HG965214 · HG965215 · HG965216

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Introduction

In nature, bacteria of the order *Actinomycetales*, particularly of *Streptomyces* genus, are the main producers of secondary

metabolites of great medical and industrial relevance. *Streptomyces* are responsible for the production of most of the discovered secondary metabolites with activity as antibiotics, antitumor, or immunosuppressive agents, etc. *Streptomyces* species are widely distributed in nature, in both terrestrial and marine environments. Although most of the known species are of terrestrial origin and have been isolated from soils along the past few decades (since the 1950s), indigenous marine actinomycetes indeed exist in the oceans and are widely distributed in different marine organisms (animals and plants), besides seawater and sediments [12, 30, 45, 58]. An increasing number of novel potent bioactive metabolites have been isolated from marine actinomycetes during the last decade [7, 18, 30, 35, 42]. Since the greatest biodiversity and metabolic variety occur in the oceans, marine actinomycetes are emerging as a source of novel drugs [50]. The marine environment has become a prime resource in search and discovery for novel products, and marine actinomycetes turn out to be important contributors [53]. For future success on natural product discovery, it has been proposed to increase efforts on the isolation of microorganisms from the marine environment and also on those living in association with plants and animals [11].

Marine macroalgae (seaweeds) remains as a relatively underexplored source in the search of *Streptomyces* producing bioactive substances, expected to play an important role for its survival in this habitat. Only a few reports describe the isolation of actinomycetes on seaweeds on coastal ecosystems from temperate and cold waters of the North Atlantic Ocean, particularly from Iberian Peninsula coasts [19] and from the Kiel Fjord in the Baltic Sea [47, 55]. Phylogenetic analysis revealed a great diversity of antibiotic-producing actinobacteria, belonging most of the isolates to the *Streptomyces* genus [47, 55].

Coral reefs are among the most productive marine ecosystems. Considered as the "rain forests" of the oceans, coral reefs are the source of a large group of structurally unique biosynthetic products with biomedical potential [41]. The most prolific source of bioactive compounds consists in coral reef invertebrates, mainly sponges, ascidians, molluscs, and bryozoans, and it is becoming evident that some of the compounds are indeed produced by invertebrate-associated microorganisms [37]. Although little is known about the diversity of coral-associated actinobacteria, recent reports from the China Sea coral ecosystems show that diverse cultivable actinomycetes are associated with soft corals [51, 57] and also with stony corals [56]. These associated actinomycetes produce some antibacterial agents believed to protect their host against pathogens [57].

In this line of evidence, we initially isolated predominant *Streptomyces* strains associated to diverse intertidal seaweeds, collected in the temperate Cantabrian Sea (Southern Bay of Biscay in the North of Iberian Peninsula), and then explored their biosynthetic potential of medical relevance. We report here the isolation of two bioactive *Streptomyces* species, their identification by 16S RNA analysis and phylogenetic analysis, the corresponding determination of their metabolic profile, and finally the identification of some of the produced secondary metabolites with antibacterial, antifungal, antitumor, and anti-inflammatory activities. We have also investigated the distribution of these species in other marine environments, mainly focusing on their association to invertebrates from deep-sea coral ecosystems from the Avilés Canyon in the central Cantabrian Sea and also in other habitats. Knowledge of *Streptomyces* ecological distribution is important to obtain novel biologically active natural products, but also for the conservation of these unique marine ecosystems.

Methods

Sampling of Marine Macroalgae

Intertidal seaweed samples were collected at a coastal location nearby Gijón, in the central Cantabrian Sea (N Spain, Bay of Biscay; 43° 32′ 31″ N, 5° 39′ 6″ W), within an intertidal system with a tidal range of 5–6 m. A total of 35 samples were collected at different seasons during three consecutive years (2010–2012). Algae were transferred into sterile plastic bags and kept at 4 °C until immediate processing upon arrival in the laboratory, within 12–24 h after sampling.

Sampling of Deep-Sea Coral Reef Invertebrates

A total of 87 deep-sea invertebrates were collected at the submarine Avilés Canyon in April–May 2013, onboard RV Sarmiento de Gamboa during the BIOCANT3 expedition. Benthonic species were collected using a 5-m length Agassiz trawl with a beam width of 5 m and towed during 1 h at four stations located inside (C5, C8) and outside the Avilés Canyon (P3, TP, Fig. 1). One specimen of the coronate jellyfish *Periphylla periphylla* was captured within 1,200- and 2,000-m depth at station C5 (Fig. 1) using a pelagic MOCNESS with a square, 1-m^2 frame and a 200 black-tinted nitex mesh sieve. After collection, invertebrate samples were aseptically and individually transferred to sterile plastic bags, washed with sterile marine water, and immediately processed in the onboard laboratory.

Sampling of Lichens

A total of about 120 lichen samples were collected in the North of the Iberian Peninsula (Northern Spain and Northern

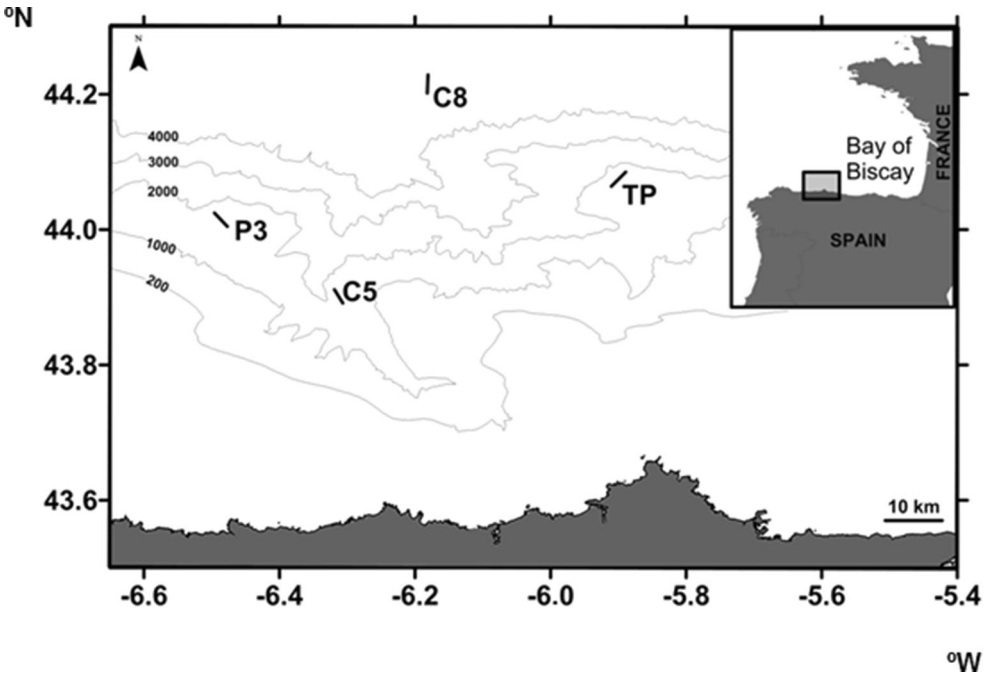

Fig. 1 Location of the Agassiz trawls where most of the deep invertebrate samples were obtained. *The lines* do not include the descending or ascending phases, only the periods where the trawl was in contact with the bottom

Portugal) and South of France since 2007. After collection, lichens were transferred into sterile plastic bags and kept at 4 °C until immediate processing upon arrival in the laboratory.

Sampling of Rain Water and Hailstone Precipitations

Different precipitations (rain water, hailstone, and snow) were taken within a year time at Gijón and Oviedo stations. Samples of 2–3 ml were collected in sterile recipients and immediately plated on selective media upon arrival in the laboratory, usually within 1–24 h.

Streptomyces Strain Isolation

All different samples were fragmented in empty Petri dishes, with the aid of a sterile scalpel or hammer in the case of stony corals, and transferred to tubes containing 1–2 ml of sterile marine water from the Cantabrian Sea. After vortex, 0.2 ml of each sample was plated on selective media containing the antifungal cycloheximide (80 μg/ml) and anti-Gram negative bacteria nalidixic acid (20 μg/ml), reported to be used previously for actinomycete isolation [21]. Different media were used, either prepared with distilled water or seawater from the same habitat: TSA (Merk) for seaweeds, TSA1/3 and Bleb 1/6 (Oxoid) for deep sea invertebrates and rain water samples, and TSA for lichens. Incubation was carried out for 2 weeks at 28 °C. Colonies growing on agar plates were selected based on different colony morphologies and pigment production. Isolates obtained in pure culture were frozen in 20 % glycerol at −20 and at −70 °C for long-term storage.

Biological Activities

Antimicrobial Bioassays

For antibiotic production, streptomycetes cultures were routinely grown on R5A medium [16]. Antibiotic production was determined by means of bioassays against the following microorganisms as indicators: the Gram-positive bacteria *Staphylococcus aureus* ATCC 6538P, the Gram-negative *Escherichia coli* ESS, and the yeast *Sacharomyces cerevisiae*. Bioassays against bacteria were carried out in TSA1/2 and for yeast in Sabouraud 1/2 (Pronadisa). These analyses were performed with agar plugs and also with ethyl acetate extracts from solid cultures.

Cytotoxic Assays

Determination of viable cells in cytotoxicity assays was carried out against two tumor lines: HeLa, from cervical carcinoma, and HCT116, from colorectal carcinoma, by using the Cell counting kit-8-(96992) from Sigma-Aldrich. Cytotoxic activities were determined with ethyl acetate extracts, obtained in both neutral and acidic conditions, for undiluted extracts and also for extracts diluted 1/10 and 1/100 times. Finally, 2 μl of each extract was added to each well containing 200 μl of cell suspension, and triplicate assays were carried out for every sample.

16S RNA Analysis Identification

The isolated strains were subjected to phylogenetic analysis based on 16S rRNA sequence analysis. DNA was extracted using a microbial DNA isolation kit (Ultra Clean, MoBio Laboratories, Inc.). The DNA was checked for purity, using standard methods [44]. The almost-complete 16S rRNA gene sequence of the bacterial strains was obtained by PCR amplification on a Genius thermocycler (Techne), followed by direct sequencing using primers 616V (forward) and 699R (reverse), as described [3], to target about 1,000 nt close to the 5′ end and primers P609D and P1525R to target positions 785–802 and 1525–1541, respectively (*Escherichia coli* numbering), as described [34]. The amplification mixture (50 μl) comprised 1 μl (50 pmol/μl) each of 616V and 699R primers, 0.5 μl (5U/μl) of DFS-Taq DNA Polymerase (Bioron), 5 μl of 10× incompleted reaction buffer (Bioron), 0.75 μl of $MgCl_2$ 100 mM, 1 μl dNTP mixture (containing 100 μM each of dATP, dGTP, dCTP, and dTTP, Roche), 35.75 μl of sterile filtered water (Milli-Q purification system, Millipore), and 5 μl of DNA template. The DNA templates were amplified by initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. Controls, devoid of DNA, were simultaneously included in the amplification process. The integrity of PCR products was assayed by development of single bands following electrophoresis for 25 min at 135 V in 1 % (*w*/*v*) agarose gels in Tris-borate EDTA buffer.

Sequences here obtained were compared to public sequences in databases using basically the BLAST program (Basic Local Alignment Search Tool) against the National Center for Biotechnology Information (NCBI) and EzTaxon.org server version 2 [10], submitted and deposited in the EMBL sequence database with Accession numbers HG965212, HG965214, HG965215, and HG965216.

Chromatographic Analysis

Routinely, compounds produced by *Streptomyces* strains were assessed in cultures on R5A solid medium. Agar plugs of about 7 ml that were taken from the plates were extracted with ethyl acetate in neutral and acidic (after addition of 1 % formic acid) conditions. The organic fraction was evaporated and the residue redissolved in 100 μl of a mixture of DMSO and methanol (50:50). These samples were analyzed by reversed phase chromatography in an Acquity UPLC equipment employing a BEH C18 column (1.7 μm, 2.1×100 mm, Waters), with acetonitrile and 0.1 % trifluoroacetic acid as solvents. Samples were eluted with 10 % acetonitrile during 1 min, followed by a linear gradient from 10 to 100 % during 7 min and an additional isocratic hold with 100 % acetonitrile during 2 min, at a flow rate of 0.5 ml/min and at a column temperature of 35 °C. Detection and spectral characterization of peaks were performed in both cases by photodiode array detection and Empower software (Waters).

Identification of Compounds by HPLC Analysis

The chromatographic system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detector and Kayak XM 600 Workstation (Agilent Technologies, Waldbronn, Germany). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435, and 500 nm, and UV-Vis spectra measured from 200 to 600 nm. Samples (10 ml) of the culture broths were centrifuged; the supernatants were adjusted to pH 5.0 and extracted with an equivalent volume of ethyl acetate. The mycelial pellet was extracted with 10-ml MeOH-acetone (1:1). The organic layers were concentrated, dried in vacuo, and resuspended in 1 ml MeOH. A total of 5-μl aliquots of the samples were injected onto an HPLC column (125×3 mm) fitted with a guard-column (20×3 mm) filled with 5-μm Nucleosil-100 C-18 (Maisch, Ammerbuch, Germany). The samples were analyzed by linear gradient elution using 0.1 % *ortho*-phosphoric acid as solvent A and acetonitrile as solvent B at a flow rate of 0.85 ml/min. The gradient was from 4.5 to 100 % for solvent A in 15 min with a 3-min hold at 100 % for solvent B. Evaluation was carried out by means of an in-house HPLC-UV-Vis database which contained nearly of 1,000 reference compounds, mostly antibiotics [17].

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Qualitative analysis was performed by GC-MS (Chromatograph Agilent 6890N coupled with a 5975B mass spectrometer) mainly as described for geosmin detection [24]. Volatiles released from *Streptomyces* strains during 2-week growth on R5A plates at 28 °C were absorbed onto 100 mg of activated charcoal (Norit GAC 1240) placed in the lid of each Petri dish. The charcoal was extracted with 0.5 ml of chloroform (Merck) for an hour, and then filtered through cotton wool. One microliter of each extract was analyzed by GC-MS as follows: capillary column, fused silica (30 m; 0.25-mm inside diameter; 0.25-mm film thickness); carrier gas, He (0.82 kPa on column injection); temperature program, isothermal for 1 min at 40 °C, change from 40 to 210 °C at a rate of 10 °C per min, and isothermal for 25 min at 210 °C; energy of ionization, 70 eV. The identity of these volatile compounds was determined by comparing their mass spectra with the Whiley and NIST (National Institute of Standards and Technology) libraries.

Results

Isolation of Bioactive *Streptomyces* Species Associated to Macroalgae from the Central Cantabrian Sea

Preliminary analysis of different intertidal marine macroalgae (brown, red, and green) collected in the central Cantabrian coast revealed the striking generalized presence of diverse cultivable *Streptomyces* populations. Morphologically different colonies of bacteria of this genus were isolated in a great number from many of the seaweed samples screened by using TSA selective media. All the isolates were able to grow in different media independently if they contain distilled water (TSA and R5A) or seawater from the same habitat and tolerate up to 7.5 % NaCl.

Among the predominant isolates from diverse seaweed species, two phenotypically different strains, M-27 and M-40 (Supplemental material 1), displaying strong antibiotic activities against Gram-positive, Gram-negative bacteria, and fungi (Supplemental material 2), were selected for further studies. When growing in R5A production media, the different isolates showed diverse phenotypic features and, according to mycelium, spore pigmentation and the color of pigments released to the medium, they were initially named as "Orange" and "Beige." The first strain showed an orange mycelium and pink pale spores, releasing an intense orange pigment to the medium, while displaying very strong antibacterial activities against both Gram-positive and Gram-negative bacteria. The strain initially named Beige showed both mycelium and spores of this peculiar color, not showing pigment production, and presented anti-Gram-positive activity.

In addition to antimicrobial activities, ethyl acetate extracts of the strains displayed diverse ranges of cytotoxic activities against two different tumor cell lines: HeLa and HCT116, being most active to the Orange strain against both cell lines even after 1/100 dilution (Supplemental material 3). Extracts of this strain were also active against DLD-1 and MC-F7 cell lines, from colorectal and breast adenocarcinomas, respectively (Santiago Cal, personal communication).

We focused on these widely distributed strains, which were further characterized by 16S RNA phylogenetic analysis. We studied their biogeographically distribution among different ecosystems and also, their metabolic profiling, with identification of some of the bioactive compounds produced.

Identification of Predominant Marine *Streptomyces* Strains by 16S RNA Phylogenetic Analysis

For taxonomic identification, an isolate of each type of strain was further analyzed for 16S RNA sequencing. The strain M-27 with Orange phenotype displayed 99.9 % similarity with the *S. cyaneofuscatus* (AY999770/Type strain JCM4364) belonging to the *S. griseus* clade [43], and we will refer to it as *S. cyaneofuscatus* M-27 strain (accession number HG965212). However, to the best of our knowledge, there is no precedent on the presence of this species in marine environments.

The strain M-40 with Beige phenotype showed 100 % similarity to *Streptomyces carnosus* (accession number KC522300), and we will refer to it as *S. carnosus* M-40 strain (accession number HG965214). Members of this species have been previously isolated from an intertidal sponge from the China Sea [54], and as far as we know, they have not been reported in terrestrial habitats.

Distribution of *Streptomyces* Species in Intertidal Seaweeds

The distribution of *Streptomyces* strains among the different seaweed species sampled is shown in Table 1. The presence of these bacteria in seawater obtained from the same habitat was of 2–4 orders of magnitude less than that in a similar volume of seaweed, depending on the season.

The strain *S. cyaneofuscatus* M-27 was first isolated in September 2010 from the perennial brown alga *Fucus spiralis*. This strain was isolated mainly from the inner part of the receptacles in the fucals *F. spiralis and Pelvetia canaliculata*, as observed after ethanol immersion of the alga during 3 min. While most of streptomycetes colonizing algal surfaces are eliminated with this treatment, colonies corresponding to this species were still isolated in great number from the receptacles inner part. Also, seasonal differences were found in this case, since the number of isolates is highly reduced in winter, when receptacles are inactive, thus suggesting a possible role in algal development. Similar strains were isolated from brown, red, and green algae during 2011 and 2012. *S. carnosus* M-40 strain was first isolated in October 2011, from *C. baccata*, and similar strains were found to be present in brown and red algae collected in 2012.

Distribution of *Streptomyces* Species Among Deep-Sea Coral Reef Invertebrates from the Avilés Canyon

Surprisingly, phenotype features, biological activities, and metabolic profiling (see below) suggest that similar isolates to *S. cyaneofuscatus* M-27 and *S. carnosus* M-40 strains were also found to inhabit deep ecosystems from the Avilés Canyon. Samples were collected in Spring 2013 during a cruise expedition aboard the Sarmiento de Gamboa oceanographic ship. Table 2 displays the different invertebrate hosts from deep-sea coral ecosystems from which similar strains were isolated.

As shown in Table 2, similar *S. cyaneofuscatus* M-27 isolates were found to be mainly associated to invertebrates of the *Phylum Cnidaria*, which includes colonial and solitary stony corals (*O. Scleractinia*), soft gorgonian corals (*O. Gorgonacea* and *O. Alcyonaea*), but also in some actinia,

Table 1 Intertidal seaweed-associated *Streptomyces* strains from the Central Cantabrian Sea collected at Gijón beaches (43° 32′ 31″ N, 5° 39′ 6″ W). Dates of strain isolation (a)

Host macroalgae	Similar isolates[a]/Phenotype	
	S. cyaneofuscatus M-27/ORANGE	*S. carnosus M-40*/BEIGE
Brown macroalgae *(P. Heterokontophyta)*		
Cystoseira baccata	M-52	M-40
	M-39	
	M-78	
Fucus spiralis	M-27	M-43
		M-73
Pelvetia canaliculata	M-75	
Bifurcaria bifurcata	M-47	M-104
Red macroalgae *(P. Rhodophyta)*		
Plocamium cartilagineum	M-92	
Halopteris scoparia		M-60
Mesophyllum lichenoides		M-115
Green macroalgae (*P. Chlorophyta)*		
(*P. Chlorophyta)Blidingia minima*	M-95	

[a] Date of isolation: M-52/Mar 2012, M-40/Oct 2011, M-39/Oct 2011, M-78/Mar 2012, M-27/Sep 2010, M-43/Mar 2012, M-73/Mar 2012, M-75 Mar 2012, M-47/Mar 2012, M-104/Sep 2012, M-92/May 2012, M-60/Apr 2012, M-115/Sep 2012, M-95/May 2012

Table 2 Similar *Streptomyces* isolates associated to coral reef invertebrates from the Avilés Canyon in the Cantabrian Sea

Sample/Number	Host invertebrate taxonomic group	Depth (m)/Station/Net	Similar *Streptomyces* isolates	
			S. cyaneofuscatus M-27	*S. carnosus* M-40
colonial coral/66A	*P. Cnidaria*, *Cl. Anthozoa*, *O. Scleractinia*, *Lophelia pertusa*	1800/TP/Agassiz		M-225
solitary coral/67	*P. Cnidaria*, *Cl. Anthozoa*, *O. Scleractinia*, *Desmophyllum sp.*	1800/TP/Agassiz	M-213	
solitary coral/14	*P. Cnidaria*, *Cl. Anthozoa*, *O. Scleractinia*	2000/C5/Agassiz	M-158	M-161
gorgonian coral/30	*P. Cnidaria*, *Cl. Anthozoa*, *O. Gorgonacea*	1500/P3/Agassiz	M-167	M-166
gorgonian coral/58A	*P. Cnidaria*, *Cl. Anthozoa*, *O. Gorgonacea*	1.800/TP/Agassiz	M-174	
gorgonian coral/59	*P. Cnidaria*, *Cl. Anthozoa*, *O. Gorgonacea*	1800/TP/Agassiz	M-189	M-187
gorgonian cora/ 61,62	*P. Cnidaria*, *Cl. Anthozoa*, *O. Gorgonacea*	1800/TP/Agassiz	M-226	M-196
alcyonarian coral/31	*P. Cnidaria*, *Cl. Anthozoa*, *O. Alcyonaea*	1500/P3/Agassiz	M-222	M-220
actinia/79	*P. Cnidaria*, *Cl. Anthozoa O. Actiniaria*	1800/TP/Agassiz		M-215
actinia/52	*P. Cnidaria*, *Cl. Anthozoa O. Actiniaria*	4700/C8/Agassiz	M-192	
jellyfish/8	*P. Cnidaria*,*Cl. Scyphozoa*, *O. Coronatae*, *Periphyla periphyla*	2000/C5/Mocness		M-152
jellyfish/51	*P. Cnidaria*,*Cl. Scyphozoa*, *O. Coronatae*, *P. periphyla*	4700/C8/Agassiz		M-171
ofiuroid/58B	*P. Echinodermata*, *Cl. Ofiuroidea*	1800/TP Agassiz	M-176	
ofiuroid/87	*P. Echinodermata*, *Cl. Ofiuroidea*	1800/TP Agassiz		M-208
ofiuroid/72	*P. Echinodermata*, *Cl. Ofiuroidea*	1800/TP Agassiz		M-183
asteroid/73	*P. Echinodermata*, *Cl. Asteroidea*	1800/TP Agassiz		M-212
holoturia/3	*P. Echinodermata*, *Cl Holothuroidea*, *O. Molpadia*, *Hedingia sp.*	2000/C5/Agassiz		M-156
decapod/20	*P. Arthropoda*, *Cl.Malacostraca*; *O. Decapoda*, *Stereomastis sp.*	2000/C5/Agassiz		M-165
decapod/56	*P. Arthropoda*; *Cl.Malacostraca*; *O. Decapoda*	4700/C8/Agassiz		M-173
sipunculid/12	*P. Sipuncula*, *Cl. Sipunculida*, *O. Sipunculiformes*, *Sipunculus sp.*	2000/C5/Agassiz		M-154
polychaete/70	*P. Anelida*, *Cl. Polychaeta*	1800/TP/Agassiz	M-180	M-210

ofiuroid, and polychaeta specimen. *S. carnosus* M-40 seems to be more widely distributed since in addition to the *P. Cnidaria*, it was isolated from different species of *P. Echinodermata*, *P. Arthropoda*, *P. Sipuncula*, and *P. Anelida*. In the Avilés Canyon, similar strains to these two species were also isolated from invertebrates collected at all depths tested up to 4,700 m (Table 2), thus indicating the barotolerant behavior of these streptomycetes, able to survive under 470 atm of hydrostatic pressure, but also at very low temperatures, up to 2–4 °C.

Presence of Similar *Streptomyces* Strains in Other Habitats

In the course of previous actinomycete isolation from terrestrial lichens in the North of Iberian Peninsula (Northern Spain and Northern Portugal), strains displaying similar phenotypic, antibiotic production, and metabolite profile features to *S. cyaneofuscatus* M-27 and *S. carnosus* M-40 were found. Identification by 16S RNA, bioactivity assays, and UPLC revealed that these isolates are similar to the respective marine strains. As shown in Table 3, T-178 terrestrial strain (HG965215) displays 100 % similarity to *S. cyaneofuscatus* M-27 (accession number HG965212), and T-145 terrestrial strain (HG965216) displays 100 % similarity to *S. carnosus* M-40 (accession number HG965214), and to our knowledge has not been reported in terrestrial habitats. Strikingly, these inland lichen isolates collected from forest and mountain habitats were able to grow in seawater prepared media, and under saline conditions up to 7.5 % NaCl, being phenotypically and metabolically indistinguishable from the marine-borne strains.

Recently, the species described here have been repeatedly isolated from different precipitations from tropospheric clouds (rain water and hailstone) collected at Gijón and Oviedo stations all along the last year (2013–2014).

Metabolite Profiling Analysis and Identification of Secondary Metabolites Produced

To uncover the biosynthetic abilities of the two identified species, ethyl acetate extracts of R5A solid cultures after 7 days of growth were analyzed by ultra-performance liquid chromatography (UPLC). This was followed by the screening for secondary metabolites by HPLC analysis and by means of UV-visible absorbance spectral libraries [17]. Although most of the compounds produced by these strains remain unidentified, some of them were identified as shown in Figs. 2 and 3. The chromatograms shown are maxplots, i.e., chromatogram at absorbance maximum for each analyte, obtained from spectrophotometric detection in the range from 210 to 500 nm.

Figure 2a shows the metabolite profile of ethyl acetate extracts of *S. cyaneofuscatus* M-27 as determined by UPLC chromatographic analysis, and Fig. 2b displays the chemical structures of the identified bioactive synthesized compounds. This strain has the ability to produce simultaneously several antitumor antibiotics of the anthracycline family (Table 4), three of them identified as daunomycin (**1**), cosmomycin B (**2**), and galtamycin B (**4**), respectively (Fig. 2a, b). Different members of the antracycline/angucycline family still remain unidentified (Fig. 2a). Galtamycin B, to our knowledge, has

Table 3 Similar *Streptomyces* strains isolated from other sources in the North of the Iberian Peninsula

Streptomyces strain	Source	Similar isolate/date of isolation	Localization (geographical coordinates)
S. cyaneofuscatus M-27			
	Terrestrial lichen	T-178/Nov2009	Muniellos (43°10′3″N, 6°30′44″W)
	Terrestrial lichen	T-35/Jun2008	La Vecilla (42°50′56″N, 5°24′23″W)
	Terrestrial lichen	T-140/May2009	Cuntis (42°38′2″N, 8°33′50″W)
	Terrestrial lichen	T-163/Mar2010	Guimaraes (41°26′15″N, 8°18′12″W)
	Rain water	A-62/18Jan2014	Gijón (43°32′31″N, 5°39′6″W)
	Rain water	A-65/27Jan2014	Gijón (43°32′31″N, 5°39′6″W)
	Hailstone	A-73/25Mar2014	Oviedo (43°21 23 N, 5° 52′14″W)
S. carnosus M-40			
	Terrestrial lichen	T-145/May2009	Tiatordos (43°11′44″N, 5°13′4″W)
	Terrestrial lichen	T-146/May2009	Sobrefoz (43°10′15″N, 5°10′35″W)
	Rain water	A-3/25Feb2013	Oviedo (43°21 23 N, 5° 52′14″W)
	Rain water	A-39/2Oct2013	Gijón (43°32′31″N, 5°39′6″W)
	Rain water	A-60/18Jan2014	Gijón (43°32′31″N, 5°39′6″W)
	Rain water	A-66/27Jan2014	Gijón (43°32′31″N, 5°39′6″W)

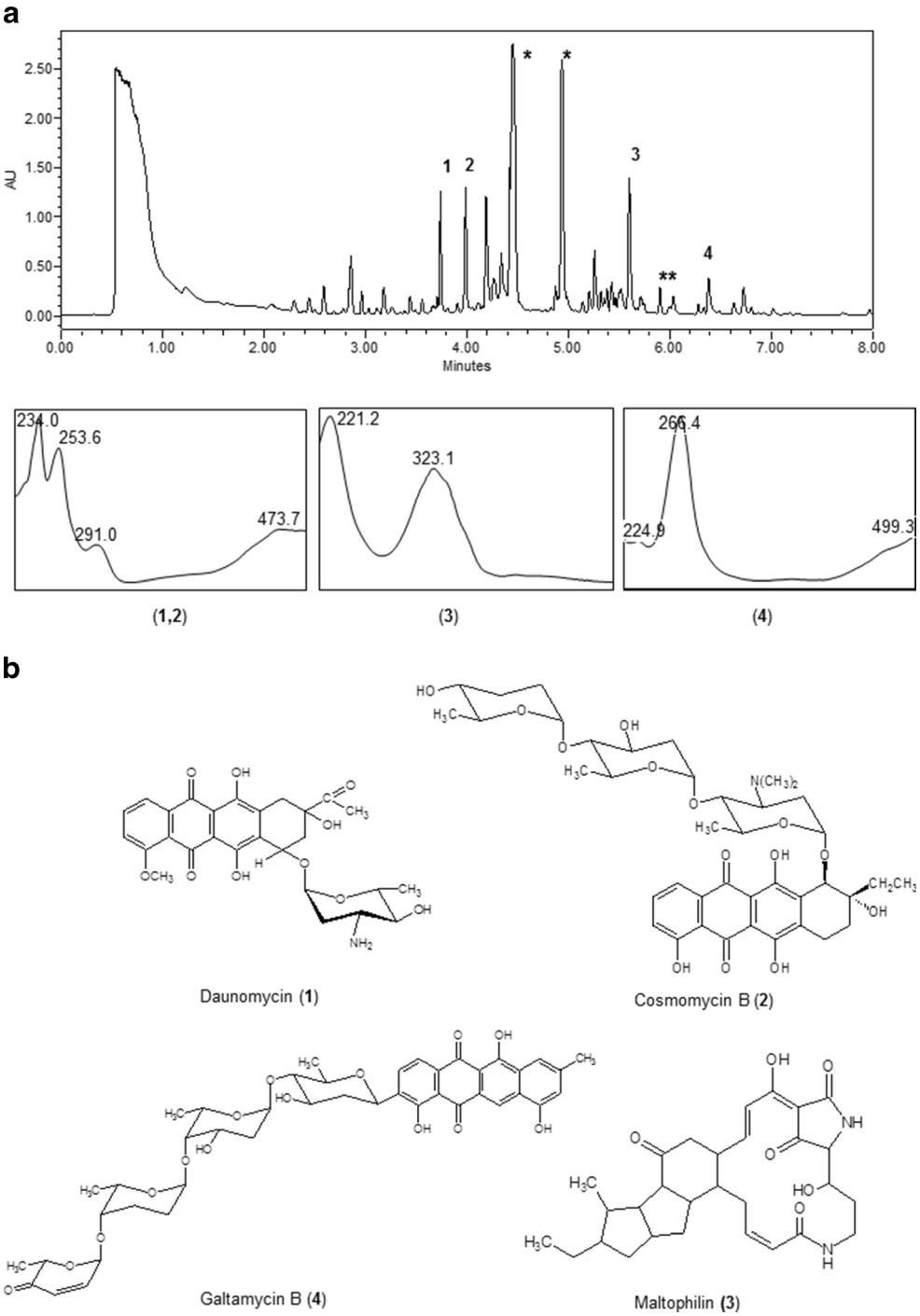

Fig. 2 Chromatogram of extract of *S. cyaneofuscatus* M-27. **2a** Peak numbers indicate the identified compounds and correspond to the following: daunomycin (**1**), cosmomycin B (**2**), maltophilin (**3**), and galtamycin (**4**). The peaks corresponding to unidentified anthracyclines (*) and unidentified maltophilin (**) are also shown. The *lower part of the figure* represents U.V. absorption spectra of the identified molecules. **2b** Chemical structures of bioactive secondary metabolites identified in extracts of *S. cyaneofuscatus* M-27

not been previously reported to be produced neither by *Streptomyces* nor in marine environments. In addition, *S. cyaneofuscatus* M-27 produces the antifungal macrolactam maltophilin (**3**) as well as an unidentified derivative.

Several compounds belonging to the lobophorines family have been detected in ethyl acetate extracts form *S. carnosus* M-40 (Fig. 3), among them only lobophorine B (**7**) has been identified. Lobophorines A and B are macrolides related to antibiotics of the Kijanimicin class with anti-inflammatory and antituberculosis properties (Table 4). It has been reported that an *S. carnosus* strain associated to a sponge from the China Sea produces lobophorines C and D with citotoxic activities [54].

Also produced by this strain are germicidin A (**5**) and B (**6**), both pyrone compounds (Fig. 3a, b) displaying a physiological role in spore germination and hypha elongation (Table 4).

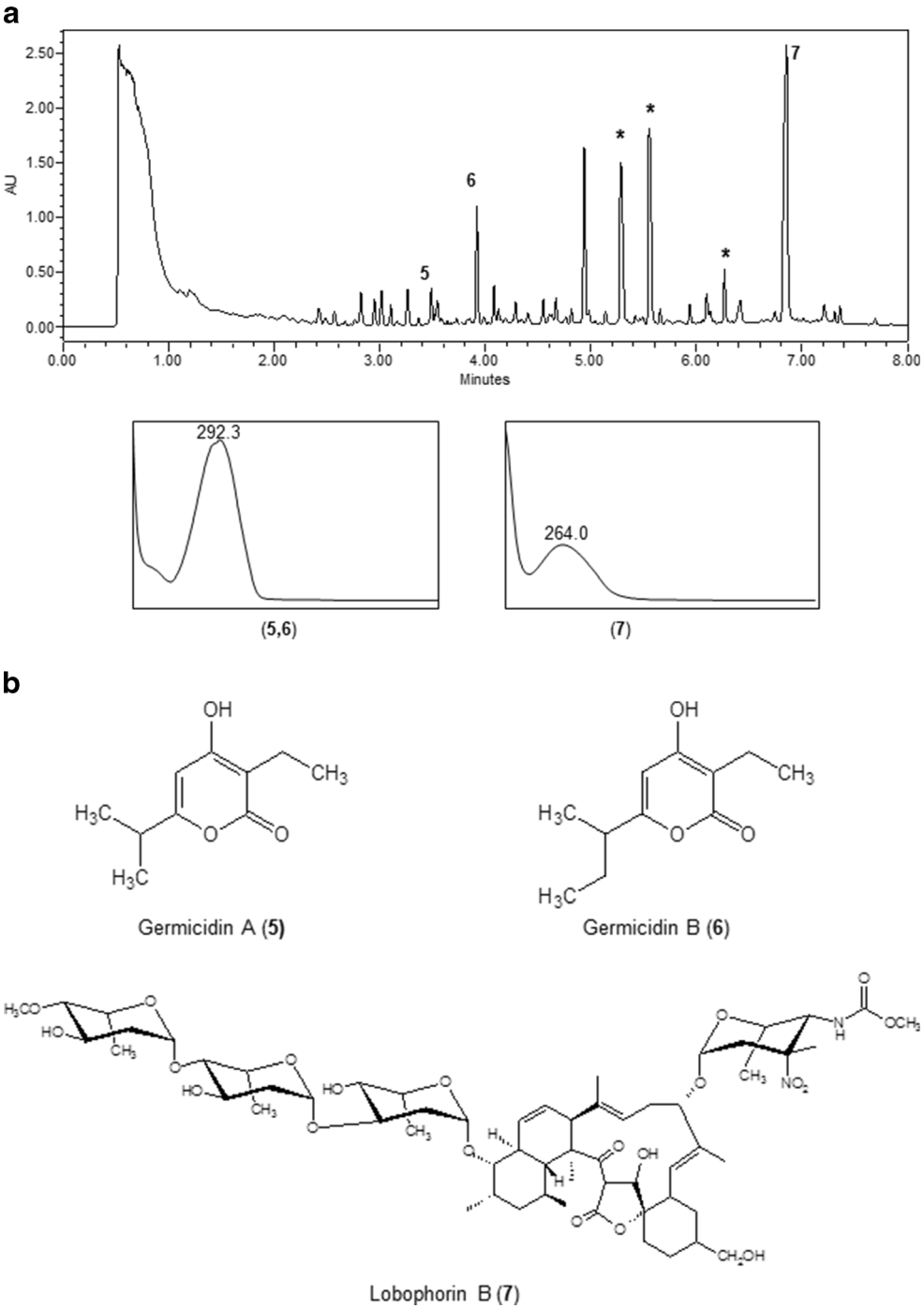

Fig. 3 Chromatogram of extract of *S. carnosus* M-40. **3a** *Peak numbers* indicate the identified compounds and correspond to the following: germicidin A (**5**), germicidin B (**6**), and lobophorin B (**7**). The peaks corresponding to unidentified lobophorines (*) are also shown. The *lower part of the figure* represents U.V. absorption spectra of the identified molecules. **3b** Chemical structures of secondary metabolites with biological activity identified in extracts of *S. carnosus* M-40

Volatile Metabolite Profiling Analyses by GC-MS and Comparison among the Different *Streptomyces* Species

Most major volatile compounds produced by the studied strains were identified by comparison with Wiley database as sesquiterpene aromatics (Supplemental material 4). Only one major peak is detected in M-27, while two major peaks were detected in M-40. The two major products in M-40 were identified as geosmin **(8)**, the compound responsible for the "earth smell" widespread among *Streptomyces* species [8, 26], and beta-patchoulene **(9)**, traditionally obtained from the plant *Pogostemon cablin* (patchouli) and used as fragrance agent in perfume industry, whose production by the *Streptomyces* genus has been recently reported [6]. The main product in M-27 corresponds to geosmin. Many other volatile compounds produced by these two strains still remain unidentified.

Table 4 Bioactive secondary metabolites produced by the isolated *Streptomyces* strains and their corresponding biological activities

Strain	Compound	Biological activities (reference)	Previously reported (reference)
S. cyaneofuscatus M-27			
	Daunomycin	Anticancer (acute lymphoblastic and myeloblastic leukaemias) [49]	*Streptomyces. peucetius* [22]
	Cosmomycin	Antitumor [32]; anti Gram-positive [32]	*Streptomyces violaceus* [32]
	Galtamycin	Antitumor [48]	*Micromonospora* sp. [48]
	Maltophilin	Antifungal [27]	*Stenotrophomonas maltophilia* [27]; *Streptomyces sp* [18]
	Geosmin	-	*Streptomyces* sp. [8, 26]
S. carnosus M-40			
	Lobophorin B	Anti-inflammatory [28]; anti-BCG and antituberculosis [9]	*Lobophora variegata* (brown alga) [28]
	Germicidins A, B	Spore germination and hypha elongation in *S. coelicolor* [2]	*Streptomyces. viridochromogenes* [39]; marine actinobacteria [45]
	Geosmin	-	*Streptomyces* sp. [8, 26]
	β-patchoulene	-	*Pogostemon cablin*; *Streptomyces albus* [6]

Discussion

Macroalgae and corals are complex hosts harboring a rich diversity of associated microorganisms with functions related to host health and defense. It has been recently suggested that marine macroalgae and epiphytic bacteria communities interact in a tight relationship as a unified functional entity or holobiont [14], term due to [36]. The seaweed holobiont concept is analogous to the coral holobiont, which describes a complex symbiosis between the coral animal and the associated microorganisms [5]. We report here that *Streptomyces* species producing bioactive secondary metabolites are widespread among marine macroalgae in intertidal ecosystems and, interestingly, also among different invertebrates from deep-sea bottoms, including cold coral reefs. Clearly, this finding has both ecological and pharmacological interest.

We have isolated diverse cultivable *Streptomyces* populations colonizing marine macroalgae. Our focus was on two particularly abundant and widespread antibiotic producing strains, identified as *S. cyaneofuscatus* M-27 and *S. carnosus* M-40 after 16S rRNA sequencing and phylogenetic analysis. The *Streptomyces*-seaweed associations reported here were specific and persistent within the 3-year timespan of our study, which also illustrated some seasonal variability. Although most of them seem to be epiphytic forms associated to the surface of the alga, S. *cyaneofuscatus* M-27 was clearly endophytic and associated to the receptacles of *Fucus spiralis* and *Pelvetia canaliculata*. In these hosts, S. *cyaneofuscatus* M-27 populations vary dramatically from winter, when the receptacles are inactive, to spring and summer, when these organs are mature. It has been shown that bacteria are the most common colonizers on macroalgae surfaces and can influence their growth at various developmental stages (e.g., reproduction, [4]), while secreting bioactive compounds that regulate seaweed morphogenesis and increase survival under contrasting environmental conditions [46]. Specific associations of bacterial populations with different parts of seaweeds, showing seasonal differences, have been reported [47], and also that epibacterial community patterns are host-specific but temporally variable [29].

S. cyaneofuscatus M-27 and *S. carnosus* M-40 were found both in intertidal seaweeds and in several deep-sea invertebrates at the Avilés submarine Canyon, especially in association with the reef-forming corals *Lophelia pertusa* and *Madrepora oculata*, very abundant in this Canyon [33]. Overall, it was very surprising to uncover that these two strains are able to colonize plants and animals living in very different habitats such as temperate intertidal ecosystems and cold water coral ecosystems up to 4,700-m depth. *Streptomyces* living on intertidal macroalgae are subject to a variable range of physiochemical environmental conditions in which the physical conditions, such as temperature, oxygen concentration, desiccation degree, exposure to sun light, and salinity, are constantly changing. In contrast, the deep-sea coral ecosystem, although extreme, is relatively constant from 1,800- to 4,700-m depth in terms of temperature (2–4 °C), oxygen concentration (7,7 mg/l aprox.), salinity (3.5 %), and darkness, whereas the hydrostatic pressure rises from 180 up to 470 atm. It follows that these *Streptomyces* species are barotolerant since they are able to grow at high hydrostatic pressure, in addition to atmospheric pressure.

These findings are also of interest in the *Streptomyces* genus biogeography. Similar strains to the marine-derived strains reported here were also isolated from terrestrial lichens (although restricted to specific geographical areas), which raises interesting questions concerning their origin and evolution. Comparative analysis of marine strains with similar terrestrial isolates from the inland lichens here described revealed that all of them grow and produce bioactive

compounds in culture media either prepared with marine or distilled water. It was really striking to find that all mentioned strains, independently of their marine or terrestrial origin, grow in media containing up to 7.5 % of NaCl. Although in general is assumed that marine streptomycetes are of terrestrial origin, other alternative scenarios could be also possible. As there was scientific literature on the fact that *Streptomyces* strains were found in tropospheric clouds [1], we also investigated their possible presence in rainwater and other precipitations taken over last year (2013–2014) in the same geographical region. With the recent finding of strains similar to *S. cyaneofuscatus* M-27 and *S. carnosus* M-40 isolated from different precipitations, rainwater, and hailstone, it is tempting to speculate that, in our planet, oceanic marine aerosols forming clouds might contribute to dissemination of streptomycetes spores from the sea to inland ecosystems.

Streptomyces-host interactions on marine ecosystems are also of pharmacological interest, since potential novel bioactive compounds might be produced in these interactions. Microbial communities associated with living organisms, such as corals, sponges, and algae, are influenced not only by physiochemical environmental factors but also by ecological interactions with their host organism [38]. New trends in marine research revealed that some of the natural products identified in marine organisms, initially thought to be invertebrate-derived, were later found to be produced by symbiotic or commensal microbes [20]. The "symbiont-product" hypothesis has emerged following this line of evidence [37, 40].

There is ample knowledge of retraction of the geographic range of several brown seaweed species in the Cantabrian Coast, some of them approaching regional extinction, a trend which has been explained at least partially by a global increase in sea surface temperature [13, 15, 31, 52]. The effect seems to be particularly evident in cold water algae of the order Fucales, to which *Fucus* and *Pelvetia canaliculata* belong. If there is some host specificity in the *Streptomyces*-alga association, such retraction may involve the loss of bacterial strains with potential medical interest. Likewise, climate change projections indicate that nearly 70 % of the deep corals in the world will experience corrosive effects by the end of this century due to acidification of the water, a direct consequence of the increasing levels of atmospheric CO_2 levels [23]. Comprehensive analysis of the deep-ocean communities is now starting (e.g., see [33] for an overview of deep biodiversity at the Avilés Canyon); thus, we may already be losing a startling, undocumented diversity of species and their interactions, including host-specific animal-*Streptomyces* associations. Clearly, these two aspects require a concerted research effort to document and preserve a crucial but potentially declining resource.

As a matter of fact, the streptomycetes reported here produce different bioactive secondary metabolites, with antibacterial, antifungal, cytotoxic, and anti-inflammatory activities. Comparative analysis of *Streptomyces* metabolites with natural product databases lead to the identification of several chemically diverse compounds, whereas many others remain unidentified, and some of them might be new. Some of the compounds here identified have not been previously found in the marine environment or reported in the genus *Streptomyces* [25, 35, 42, 50]. This finding mainly reinforces the idea that *Streptomyces* associated to intertidal marine macroalgae from coastal ecosystems, and deep-sea coral reefs invertebrates, might represent a promising resource for the discovery of novel natural products biologically active as pharmacological compounds for medical use. But also, the results of this work make us aware that knowledge of *Streptomyces* distribution is essential for the conservation of these unique and delicate marine ecosystems.

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