Microb Ecol (2017) 73:338–352
DOI 10.1007/s00248-016-0845-2

ENVIRONMENTAL MICROBIOLOGY

Pharmacological Potential of Phylogenetically Diverse Actinobacteria Isolated from Deep-Sea Coral Ecosystems of the Submarine Avilés Canyon in the Cantabrian Sea

Aida Sarmiento-Vizcaíno[1] · Verónica González[1] · Alfredo F. Braña[1] · Juan J. Palacios[2] · Luis Otero[3] · Jonathan Fernández[2] · Axayacatl Molina[4] · Andreas Kulik[5] · Fernando Vázquez[1,2] · José L. Acuña[4] · Luis A. García[6] · Gloria Blanco[1]

Received: 15 May 2016 / Accepted: 24 August 2016 / Published online: 10 September 2016

Abstract Marine Actinobacteria are emerging as an unexplored source for natural product discovery. Eighty-seven deep-sea coral reef invertebrates were collected during an oceanographic expedition at the submarine Avilés Canyon (Asturias, Spain) in a range of 1500 to 4700 m depth. From these, 18 cultivable bioactive Actinobacteria were isolated, mainly from corals, phylum *Cnidaria*, and some specimens of phyla *Echinodermata*, *Porifera*, *Annelida*, *Arthropoda*, *Mollusca* and *Sipuncula*. As determined by 16S rRNA sequencing and phylogenetic analyses, all isolates belong to the phylum *Actinobacteria*, mainly to the *Streptomyces* genus and also to *Micromonospora, Pseudonocardia* and *Myceligenerans*. Production of bioactive compounds of pharmacological interest was investigated by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC-MS) techniques and subsequent database comparison. Results reveal that deep-sea isolated Actinobacteria display a wide repertoire of secondary metabolite production with a high chemical diversity. Most identified products (both diffusible and volatiles) are known by their contrasted antibiotic or antitumor activities. Bioassays with ethyl acetate extracts from isolates displayed strong antibiotic activities against a panel of important resistant clinical pathogens, including Gram-positive and Gram-negative bacteria, as well as fungi, all of them isolated at two main hospitals (HUCA and Cabueñes) from the same geographical region. The identity of the active extracts components of these producing Actinobacteria is currently being investigated, given its potential for the discovery of pharmaceuticals and other products of biotechnological interest.

Keywords Actinomycetes · Volatiles · Antimicrobial agents · Actinobacteria ecology · Bioactive secondary metabolites

Electronic supplementary material The online version of this article (doi:10.1007/s00248-016-0845-2) contains supplementary material, which is available to authorized users.

✉ Gloria Blanco
gbb@uniovi.es

[1] Departamento de Biología Funcional, Área de Microbiología, e Instituto Universitario de Oncología del Principado de Asturias, Universidad de Oviedo, 33006 Oviedo, Spain

[2] Servicio de Microbiología, Hospital Universitario Central de Asturias (HUCA), Oviedo, Spain

[3] Servicio de Microbiología Hospital de Cabueñes, Gijón, Spain

[4] Departamento de Biología de Organismos y Sistemas. Área de Ecología, Universidad de Oviedo, Oviedo, Spain

[5] Microbial Biotechnology, Interfaculty Institute of Microbiology and Infection Medicine, Eberhard Karls University Tübingen, Tübingen, Germany

[6] Departamento de Ingeniería Química y Tecnología del Medio Ambiente. Área de Ingeniería Química, Universidad de Oviedo, Oviedo, Spain

Introduction

Natural products continue to be a primary resource in biomedicine and biotechnology. New trends in the search for novel pharmaceutical compounds, such antibiotics for combating human pathogens, is focused on the search from unexplored habitats [79]. Marine environments are considered an emerging source for natural product discovery. Oceans constitute more than 70 % of our planet's surface, of which 92–93 % is deep sea whereas the coastal region represents only 7–8 %. It has been estimated that 60 % of the deep-sea region is covered by water of more than 2000 m depth [48]. Deep sea is an extreme environment with high pressure, low

temperature, darkness and low-oxygen concentration which has been revealed to be a worthy source for the discovery of new antibiotics [11].

Coral reefs are among the most productive marine ecosystems, being estimated that the biological diversity in these ecosystems is higher than in the tropical rainforests [32]. Coral reefs are the source of a large group of structurally unique natural products with biomedical relevance [70], and it is becoming evident that some of the compounds are indeed produced by invertebrate-associated microorganisms [61]. Some of the natural products identified in marine organisms, initially thought to be invertebrate-derived, are in fact produced by symbiotic microbes [27]; the "symbiont-product" hypothesis has emerged following this line of evidence [52, 58]. Although little is known about the diversity of coral-associated bacteria, previous reports show that cultivable Actinobacteria populations are associated with soft corals [81, 96] and stony corals [93], and also that many coral-associated Actinobacteria could produce antibacterial agents that may protect their hosts against pathogens [96]. Most of these studies concern tropical corals, less is known of cold water corals, which live in darkness and are also known as azooxanthellate, since they lack endosymbiotic algae unlike their tropical relatives [50].

Deep-sea corals, known from all the Earth's oceans, are threatened ecosystems very vulnerable to human activities [6, 50]. There are few reports concerning deep-sea coral-associated Actinobacteria, and all of them are related to the Northeast Atlantic Ocean, were both culture dependent [25] and culture-independent approaches [51] have been carried out. Lately, in the Cantabrian Sea (Biscay Bay), Northeast Atlantic, bioactive *Streptomyces* species have been found to be associated to corals and other invertebrates living up to 4700 m depth in the submarine Avilés Canyon [6, 66]. A novel Actinobacterium, *Myceligenerans cantabricum*, has been recently isolated from a deep-sea scleractinian solitary coral (Fam. *Caryophillidae*) at 1500 m depth [67].

As it is well established, Actinobacteria, especially streptomycetes, are the main producers in nature of structurally diverse bioactive secondary metabolites of pharmaceutical interest, particularly antibiotics and antitumor compounds. Although most of the known species are of terrestrial origin, since the beginning of this century is becoming evident that Actinobacteria indeed exist in the oceans and are widely distributed in marine ecosystems in association with diverse marine organisms [44, 48, 85]. As a matter of fact, marine actinomycetes have been isolated from marine sediments even at the deepest part of the oceans, up to 10,898 m in the Marianas Trench [17, 56]. Terrestrial Actinobacteria are known to play an important ecological role in recycling of organic matter and also producing natural compounds, which are expected to protect the hosts against pathogens [85]. However, in the marine environment, their ecological role, biogeographic distribution and evolutionary history are not so well known [48]. Due to the constant need for novel drugs to combat pathogen resistance or with lower toxicity for antitumor chemotherapy, marine Actinobacteria are emerging as a major source for novel bioactive natural products.

We report here the exploration of the biosynthetic and phylogenetic diversity of cultivable marine Actinobacteria collected from cold water coral reef ecosystems of the Avilés Canyon. Deep-sea Actinobacteria isolates were assessed to produce highly diverse bioactive natural products, mainly antibiotics and antitumor compounds, by UPLC and GC-MS analyses followed by identification by comparison to natural products databases. Antibiotic and cytotoxic activities were detected in extracts of the strains, and their pharmacological potential against a variety of resistant clinical pathogens and two different tumour cell lines, was also investigated.

Materials and Methods

Sampling of Deep-Sea Coral Reef Ecosystems

Eighty-seven deep-sea invertebrates were collected at the submarine Avilés Canyon in April–May 2013, onboard RV Sarmiento de Gamboa during the BIOCANT3 expedition. Benthic species were collected using a 5-m-length Agassiz trawl with a beam width of 5 m and towed during 1 h at 4 stations located inside (C5, C8) and outside the Avilés Canyon (P3, TP, Fig. 1). The depths of the different stations are 1.500 m (P3), 1.800 m (TP), 2000 m (C5), and 4700 m (C8). Physico-chemical conditions, such as temperature, salinity and oxygen concentration at different depths are indicated in Suppl. 1. After collection, invertebrate samples (corals, sea stars, worms, crabs, shrimps, barnacles, snails, sea squirts and sponges) were aseptically and individually transferred to sterile plastic bags, washed with sterile marine water and immediately plated onto selective media in the onboard laboratory and later frozen at −20 °C. Seawater samples were collected at depths of 1250, 1500, 2000 and 3000 m using an oceanographic rosette sampler fitted with a Seabird 911-plus CTD (conductivity-temperature-depth) probe.

Actinobacteria Strains Isolation

All different deep-sea invertebrate samples were placed on empty Petri dishes and fragmented 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 surface 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^{-1}) and anti-Gram negative bacteria nalidixic acid (20 μg mL^{-1}), reported to be used previously for Actinobacteria isolation [29]. In the case of water samples,

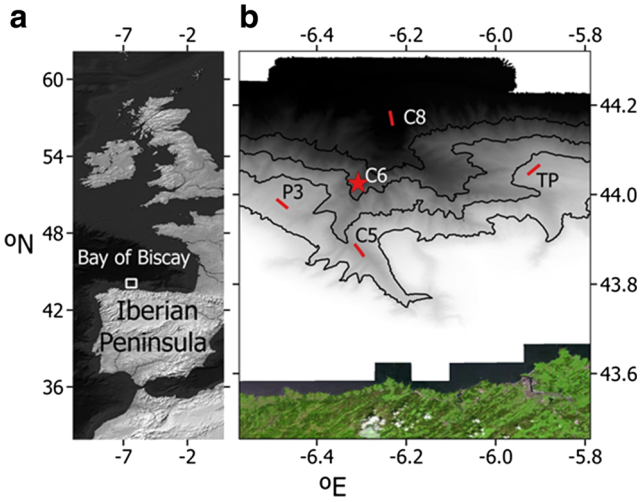

Fig. 1 Sampling locations. **a** General overview of the Western European Seas. *Inset box* indicates the location of the Avilés Canyon. Background is based on shaded relief and ocean bathymetry (courtesy of Natural Earth; http://www.naturalearthdata.com/). **b** Map of the Avilés Canyon. *Red lines* correspond to deep bottom trawls and *red star* indicates the position where water was sampled by means of Niskin bottles. *Grey background shading* corresponds to bottom depth according to Multibeam surveys (courtesy of Miquel Canals, University of Barcelona). *Black lines* correspond to the 1000, 2000, 3000 and 4000 m isobaths. Land is a composite of aerial, visible light ortophotos (courtesy of the Spanish National Plan of Aerial Ortophotography, PNOA)

1-mL aliquots were plated directly on selective agar plates. Different media were used, either prepared with distilled water or seawater from the same habitat: TSA1/3 and BLEB 1/6 (Oxoid). 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 °C and at −70 °C for long-term storage.

Bioactive Strains Selection

For antibiotic production, Actinobacteria cultures were routinely grown on R5A medium as previously described [6]. Antibiotic production was determined by means of bioassays against the following indicator microorganisms: the Gram-positive bacteria *Micrococcus luteus* ATCC 14452, the Gram-negative *Escherichia coli* ESS and the yeast *Saccharomyces cerevisiae*. Bioassays against bacteria were carried out in TSA 1/2 and for yeast in Sabouraud 1/2 (Pronadisa). These analyses were performed with agar plugs and also with ethyl acetate extracts obtained both in acid or neutral conditions (with 1 % formic acid) from solid cultures. Extracts were obtained from 7 mL of culture and resuspended in 50 μL of DMSO-methanol (1:1) from which 15 μL were loaded onto 6-mm-diameter AA Discs (Whatman), and the discs were allowed to fully dry before applying to culture media.

Antimicrobial Bioassays Against Clinic Pathogens

Extracts of the isolates in R5A medium were assayed against a panel of human pathogens (Table 1). Pathogens were isolated and identified in clinical microbiology laboratories from samples obtained in patients with clinical infections. Mueller-Hinton agar (Biomedics) was the culture media in bioassays against *E. coli, Acinetobacter baumannii, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Klebsiella pneumoniae, Morganella morganii*, *Staphylococcus aureus*, being supplemented according to the CLSI conditions [16] for *Streptococcus pneumoniae, Streptococcus pyogenes* and *Neisseria meningitidis*. Sabouraud (DIFCO) was used for *Candida* strains and Trypticasein soy agar *w*/5 % sheep blood (DIFCO) for *Corynebacterium urealyticum*. Brucella Broth (SIGMA) supplemented with hemin (5 μg/mL), vitamin K_1 (1 μg/mL) and lysed horse blood (5 % *v/v*) was used for *Bacteroides fragilis* and *Clostridium perfringens*.

For most Gram-positive and Gram-negative bacteria, the antimicrobial assays were performed by disk diffusion methodology [55] according to CLSI performance standards [16]. *Mycobacterium tuberculosis* susceptibility testing was done in Middlebrook 7H10 agar medium supplemented with 10 % OADC (Oleic acid, Albumin Fraction V Bovine, Dextrose, Catalase) and 0.5 % glycerol according to the agar proportion method for slowly growing mycobacteria [15].

Cytotoxic Assays

Determination of viable cells in cytotoxicity assays was carried out against two tumour 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 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 and Phylogenetic Analysis

The isolated strains were subjected to phylogenetic analysis based on 16S rRNA sequences analysis. DNA was extracted using a microbial DNA isolation kit (Ultra Clean, MoBio Laboratories, Inc.). The DNA was checked for purity, using standard methods [61, 64]. The almost-complete 16S rRNA gene sequence of the bacterial strains was obtained by PCR amplification as previously described [6]. Sequences here obtained were compared to public sequences in databases using basically the Basic Local Alignment Search Tool program (BLAST) against the National Center for Biotechnology

Table 1 Description of clinic microbial pathogens. The pathogens, with exception of *Mycobacterium tuberculosis* strains, are reported in this work for the first time

Clinic pathogen	Isolate	Hospital	Year	Antibiotic resistances
Gram-positives				
Mycobacterium tuberculosis H37Rv	ATCC 27294			–
Mycobacterium tuberculosis MDR-1	14595	SNRL-Spain	2013	Multiresistance[a]
Mycobacterium tuberculosis MDR-2	14615	SNRL-Spain	2013	Multiresistance[b]
Clostridium perfringens	103281	HUCA	2013	–
Corynebacterium urealyticum	1492	Cabueñes	2014	Multiresistance[c]
Enterococcus faecalis	8670	HUCA	2014	Ery, clin, tet
Enterococcus faecium	8043	HUCA	2014	Amp, quin, gen, str, ery
Listeria monocytogenes	72964	HUCA	2013	Cephalosporins
Streptococcus pneumoniae	64412	HUCA	2013	Ery
Streptococcus pyogenes	81293	HUCA	2013	–
Staphylococcus aureus	4312	Cabueñes	2014	Methicillin susceptible
Gram-negatives				
Acinetobacter baumanii	67169	Cabueñes	2013	Multiresistance[d]
Bacteroides fragilis	61592	HUCA	2013	Amo, tet
Escherichia coli	1336	Cabueñes	2014	Multiresistance[e]
Haemophilus influenzae	10155	HUCA		Cot
Klebsiella pneumoniae	67128	Cabueñes	2013	Multiresistance[f]
Morganella morganii	1179	Cabueñes	2014	Multiresistance[g]
Neisseria gonorrhoeae	6965	Cabueñes	2014	-
Neisseria meningitidis	71327	HUCA	2013	Clin
Pseudomonas aeruginosa	4192	Cabueñes	2014	Multiresistance[h]
Stenotrophomonas maltophilia	106446	HUCA	2013	Multiresistance[i]
Fungal				
Candida albicans	4579	Cabueñes	2014	–
Candida krusei	10528	Cabueñes	2014	Flu

Amk amikacin, *amo* amoxicillin, *amp* ampicillin, *cef* cefalotin, *cefa* cefazolin, *cefe* cefepime, *cefo* cefotaxime, *cefox* cefoxitin, *cefta* ceftazidime, *cefu* cefuroxime, *cip* ciprofloxacin, *clav* clavulanic acid, *clin* clindamycin, *cot* cotrimoxazole, *ert* ertapenem, *ery* erythromycin, *flu* fluconazole, *fos* fosfomycin, *gen* gentamicin, *imi* imipenem, *inh* isoniazid, *lev* levofloxacina, *nal* nalidixic acid, *nitro* nitrofurantoin, *nor* norfloxacin, *pip* piperacillin, *quin* quinolones, *rif* rifampicin, *str* streptomycin, *sulb* sulbactam, *sulf* sulfamethoxazole, *tazo* tazobactam, *tet* tetracycline, *tob* tobramycin, *trim* trimethoprim

[a] Inh, rif, emb

[b] Inh, rif, str, emb, amk, kan, cap

[c] Amp; amo/clav; ery; cot; cip; fos; nitro

[d] Amo/clav; amp; cef; cefa; cefe; cefo; cefox; cefta; cefu; cip; ert; fos; gen; nor; pip/tazo; tob; trim/sulf; amp/sulb

[e] Nal; amo/clav; amp; cef; cefa; cefe; cefo; cefu; cip; nor; tob; trim/sulf

[f] Nal; amo/clav; amp; cef; cefa; cefox; cefu; cip; nor; pip/tazo; tob

[g] Amo/clav; amp; cef; cefa; cefu; fos; nitro

[h] Amp/sulb; cip; fos; gen; lev; tob; trim/sulf

[i] Amo/clav; amp; cef; cefa; cefe; cefo; cefox; cefta; cefu; ert; imi; fos; gen; pip/tazo; tob; amk; amp/sulb

Information (NCBI) and EzTaxon.org server version 2 [14], submitted and deposited in the EMBL sequence database.

Phylogenetic analysis was performed using MEGA version 6.0 [82] after multiple alignment of data by CLUSTALO [74]. Distances (distance options according to the Kimura two-parameter model [39]) and clustering with the neighbour-joining [63] and maximum-likelihood [18] methods were determined using bootstrap values based on 1000 replications [19].

Identification of Compounds by HPLC Analysis

Routinely, compounds produced by *Streptomyces* strains were assessed in cultures on R5A solid medium. Agar plugs taken from the plates were extracted with ethyl acetate in neutral and acidic 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 analysed

by reversed phase chromatography. The chromatographic system consisted of an HP 1090 M 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 were analysed as previously reported [6], and evaluation was carried out by means of an in-house HPLC-UV–vis database which contained nearly of 1000 reference compounds, mostly antibiotics [21].

Gas Chromatography–Mass Spectrometry Analysis

Qualitative analysis was performed by gas chromatography–mass spectrometry (GC-MS) (Chromatograph Agilent 6890N coupled with a 5975B mass spectrometer) as described [7, 31]. The identity of these volatile compounds was determined by comparing their mass spectra with the Wiley and NIST (National Institute of Standards and Technology) libraries.

Results

Isolation of Bioactive Actinobacteria from Deep-Sea Ecosystems from the Avilés Canyon

Different Actinobacteria were isolated from deep-sea ecosystems in the Cantabrian Sea during oceanographic cruise BIOCANT3 aboard the Sarmiento de Gamboa in April–May 2013. Isolates originate from corals and other benthic organisms at depths ranging from 1500 to 4700 m and from seawater samples collected in the water column at depths of 1250, 1500, 2000 and 3000 m. Only Actinobacteria strains cultivable at atmospheric pressure and 28 °C were recovered on selective agar plates. Among a total of 78 actinobacterial isolates, 18 morphologically different microorganisms with antimicrobial activity, mainly streptomycetes, were selected for this study (Table 2). We observed that none of the isolates required seawater for growth; thus, R5A was selected as medium for antibiotic production. All isolated strains were initially tested for antimicrobial activity using agar diffusion assays. As determined by preliminary bioactivity assays with microbial indicator strains, isolates showed diverse antibiotic

Table 2 Bioactive Actinobacteria isolated from deep-sea coral reef ecosystems from the Avilés Canyon, measured as the diameters in mm of the zones of complete inhibition

Isolate	Sample/number	Host taxonomic group	Depth (m)/station/net	Antibiotic activity (mm)		
				M. lut	*E. col*	*S. cer*
M-157	Coral/14	*P. Cnidaria, O. Scleractinia*	2000/C5/Agassiz	30	22	–
M-169	Coral/30	*P. Cnidaria, O. Gorgonacea*	1500/P3/Agassiz	11	15	–
M-178	Sponge/76	*P. Porifera*	1800/TP/Agassiz	29	–	–
M-179	Polychaete/70	*P. Annelida*, *Cl. Polychaeta*	1800/TP/Agassiz	15	–	–
M-185	Coral/64	*P. Cnidaria, O. Gorgonacea*	1800/TP/Agassiz	15	–	–
M-186	Coral/59	*P. Cnidaria, O. Alcyonaea*	1800/TP/Agassiz	11	–	–
M-190	Coral/59	*P. Cnidaria*, *O. Alcyonaea*	1800/TP/Agassiz	15	–	–
M-192	Actinia/52	*P. Cnidaria, O. Actiniaria*	4700/C8/Agassiz	26	20	–
M-193	Starfish/37	*P. Echinodermata*, *Ceramaster sp.*	1500/P3/Agassiz	8	12	–
M-194	Coral/61	*P. Cnidaria, O. Gorgonacea*	1800/TP/Agassiz	18	17	12
M-204	Ofiuroid/63	*P. Echinodermata*, *Cl. Ofiuroidea*	1800/TP/Agassiz	13	–	12
M-207	Coral/66	*P. Cnidaria, Lophelia pertusa*	1800/TP/Agassiz	30	12	–
M-220	Polychaete/31β	*P. Annelida*, *Cl. Polychaeta*	1500/P3/Agassiz	27	–	–
M-227	Sea water	–	3000/CTD	26	16	–
M-228	Sea water	–	3000/CTD	15	–	–
M-231	Decapod/56	*P. Arthropoda, Colossendeis colossea*	4700/C8/Agassiz	13	–	–
M-235	Ofiuroid/88	*P. Echinodermata*, *Cl. Ofiuroidea*	1800/TP/Agassiz	14	–	–
M-237	Ofiuroid/89	*P. Echinodermata*, *Cl. Ofiuroidea*	1800/TP/Agassiz	19	–	–

O, order, *Cl* class

Table 3 Phylogenetic diversity of bioactive isolates

Strain	Species	EMBL accession number
M-157	*Streptomyces cyaneofuscatus*	LN824210
M-169	*Streptomyces cyaneofuscatus*	LN824211
M-178	*Streptomyces setonii*	LN824212
M-179	*Streptomyces albidoflavus*	Similar to LN626360 [67]
M-185	*Streptomyces cyaneofuscatus*	LN824213
M-186	*Streptomyces xiamenensis*	LN824214
M-190	*Streptomyces cyaneofuscatus*	LN824215
M-192	*Streptomyces cyaneofuscatus*	Similar to HG965212 [6]
M-193	*Myceligenerans cantabricum*	HG965211
M-194	*Micromonospora tulbaghiae*	LN824216
M-204	*Streptomyces halstedii*	LN824217
M-207	*Streptomyces carnosus*	LN824218
M-220	*Streptomyces carnosus*	Similar to HG965214 [6]
M-227	*Pseudonocardia carboxydivorans*	LN824219
M-228	*Pseudonocardia carboxydivorans*	LN824220
M-231	*Streptomyces sulfureus*	LN824221
M-235	*Micromonospora aurantiaca*	LN824222
M-237	*Micromonospora saelicesensis*	LN824223

activities, against *M. luteus*, not only as representative of Gram-positive bacteria but also against the Gram-negative *E. coli* and the yeast *S. cerevisiae*.

Taxonomic Identification, Phylogenetic Analyses and Distribution of Bioactive Isolates

For taxonomic identification of bioactive isolates, fragments of their 16S rDNA were sequenced. The nucleotide sequences were deposited in EMBL nucleotide sequence database, and the accession numbers are shown in Table 3. Phylogenetic analyses, based on 16S rRNA gene alignments, clearly demonstrated that all bioactive isolates belonged to the phylum *Actinobacteria*, since most of the isolates shared 99–100 % identity with known actinobacterial species. The relationship between the isolates and their nearest phylogenetic relatives is shown in the neighbour-joining phylogenetic tree presented in Fig. 2.

According to the results of phylogenetic analyses, all isolates belong to four different genera of four different taxonomical orders (order *Streptomycetales*, order *Micromonosporales*, order *Pseudonocardiales*, order *Streptomycetaceae*) among the phylum *Actinobacteria,* corresponding most of them to *Streptomyces* species. Some of the species were previously reported in marine environments, such as *Streptomyces cyaneofuscatus, Streptomyces carnosus, Streptomyces albidoflavus* and *Myceligenerans cantabricum*, previously isolated in the Cantabrian Sea [6, 66, 67], *Streptomyces xiamenensis* in deep sea sediments [94] and *Streptomyces sulfureus* in marine sediments [97]. The rest of the species have only been detected so far on terrestrial habitats, such as *Streptomyces setonii* [38] and *Streptomyces halstedii* [45]. *Pseudonocardia carboxydivorans* [54] and *Micromonospora aurantiaca* [34] were isolated from soils, *Micromonospora tulbaghiae* from leaves of the South African plant *Tulbaghia violacea* [40] and *Micromonospora saelicesensis* from nitrogen fixing leguminous plant root nodules [84].

Concerning the distribution of the actinobacterial strains in the Avilés Canyon, 8 out of 18 (M-157, M-169, M-190, M-194, M-204, M- 207, M-235, M-237) were isolated from a single invertebrate host and two from sea water (M-227,

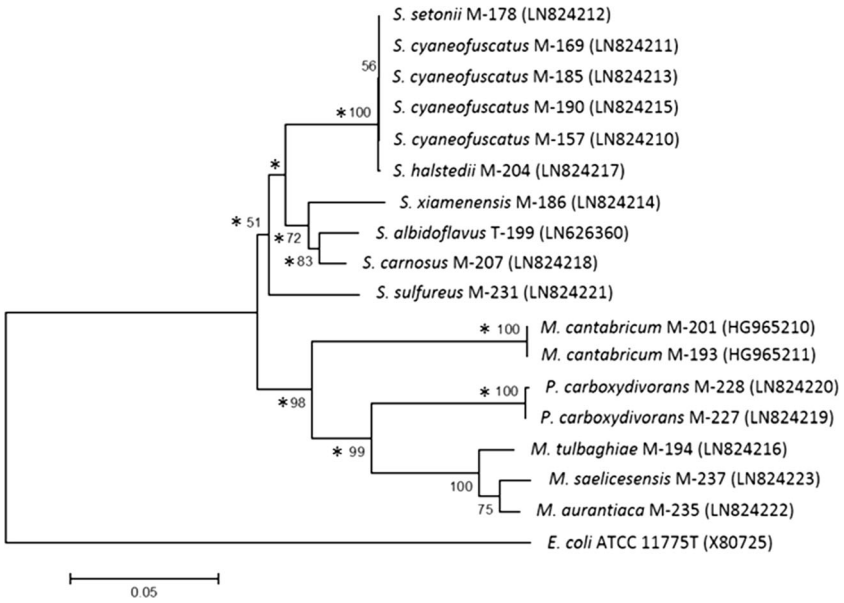

Fig. 2 Neighbour-joining phylogenetic tree obtained by distance matrix analysis of 16S rRNA gene sequences, showing their position and most closely related phylogenetic neighbours. *Numbers on branch nodes* are bootstrap values (1000 resamplings; only values >50 % are given). The sequence of *E. coli* ATCC 11775T was used as outgroup. *Asterisks* indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. *Bar*, 5 % sequence divergence

Table 4 Distribution of similar strains among corals and other invertebrates from the Avilés Canyon

Isolate	Similar isolates	Sample/number	Host taxonomic group	Depth (m)/station/net
S. setonii M-178	M-155	Sipunculid/12	*P. Sipuncula*, *Sipunculus sp.*	2000/C5/Agassiz
	M-347	Coral/61	*P. Cnidaria*, *O. Gorgonacea*	1800/TP/Agassiz
	M-446	Coral/28	*P. Cnidaria*, *O. Pennatulacea*	1500/P3/Agassiz
S. albidoflavus M-179	Marine, terrestrial and atmospheric hábitats [66]			
S. cyaneofuscatus M-185	M-191	Coral/59	*P. Cnidaria*, *O. Alcyonaea*	1800/TP/Agassiz
S. xiamenensis M-186	M-437	Decapod/11	*P. Arthropoda*, *Colossendeis colossea*	2000/C5/Agassiz
	M-515	Polychaete/70	*P. Annelida*, *Cl. Polychaeta*	1800/TP/Agassiz
	M-522	Scaphopod/71A	*P. Mollusca*, *O. Dentaliida*	1800/TP/Agassiz
S. cyaneofuscatus M-192	Marine algae and corals [6]			
M. cantabricum M-193	M-201 [67]	Coral/33	*P. Cnidaria*, *O. Scleractinia*	1500/P3/Agassiz
	M-199	Coral/30	*P. Cnidaria*, *O. Gorgonacea*	1500/P3/Agassiz
	M-201	Coral/33	*P. Cnidaria*, *O. Scleractinia*	1500/P3/Agassiz
	M-232	Coral/31	*P. Cnidaria*, *O. Alcyonaea*	1500/P3/Agassiz
	M-435	True whelk/39	*P. Mollusca*, *Colus sp.*	1500/P3/Agassiz
S. carnosus M-220	Marine algae and corals [6]			
S. sulfureus M-231	M-500	Coral/61	*P. Cnidaria*, *O. Gorgonacea*	1800/TP/Agassiz

M-228), whereas the remaining strains were found to be distributed among different deep-sea invertebrate hosts, mainly corals, from the same environment (Table 4). The distribution of *Micromonospora* strains has not been determined. *M. cantabricum* M-199 was found to be similar to the previously isolated *M. cantabricum* M-201, type strain for a novel species [67], which was selected for further analyses.

Metabolite Profiling Analysis and Identification of Secondary Metabolites Produced

To uncover the biosynthetic abilities of the studied species, ethyl acetate extracts of 7 days of growth R5A solid cultures were analysed and screened for secondary metabolites by HPLC analysis and by means of UV-visible absorbance spectral libraries [21]. Extracts of most of the strains showed complex metabolic profiles, suggesting their high potential as a source of secondary metabolites (data not shown). As an example, Fig. 3 displays a HPLC chromatogram of *S. halstedii* M-204 extract, with UV–vis spectra of the identified compounds. Based on retention times and absorption spectra, 15 products were identified (probability of more than 99.5 %) and 7 more were identified at family level of metabolic compounds level (Table 5). Suppl. 2 shows some the UV-visible absorbance spectra used and the criteria to make these assignments. The identified products are mainly antibiotics against bacteria and fungi, and antitumor, cytotoxic, antiparasitic, antiviral and anti-inflammatory compounds. However, the great majority of the secondary metabolites produced by the studied strains still remain unidentified.

Also, volatile metabolite profiling analyses by GC-MS was carried out for a representative isolate of each species. GC-MS chromatograms of M-157 and M-190 strains are provided as an example (Suppl. 3). Results shown in Table 6 allowed the identification of 22 volatile compounds by comparing their mass spectra with the Whiley and NIST natural products libraries. The compounds with more than 90 % identity to GC-MS Wiley and NIST databases were identified as in previous works [6, 7, 66]. Surprisingly, most of the identified volatile compounds display diverse anti-infective activities, mainly as antibacterial and antifungal and volatile molecules with antitumoral or cytotoxic activities were identified. It is remarkable that all strains produce dimethyldisulfide, a potent antifungal [69]. Among antitumor compounds, the multidrug resistance (MDR) reversal agent, β-elemene, of clinical use in breast cancer therapy [95] (not previously reported in bacteria), was produced by *S. xiamenensis* M-186 and *Streptomyces cyaneofuscatus* M-190. Other relevant compound of pharmaceutical interest is dihydro- β-agarofuran, a compound with antibacterial and antitumoral biological activities previously detected in plants and recently in *Streptomyces* species [7], which was found here to be produced by *M. aurantiaca* M-235 and *M. saelicesensis* M-237. Also, 1-limonene, with antibiofilm activity [80], has been identified in *M. aurantiaca* M-235.

Geosmin, characteristic volatile of *Streptomyces* species, was identified in all Actinobacteria studied, with exception of *S. cyaneofuscatus* M-157 (Suppl. 3). Also, β-patchoulene of application in perfume industry, recently found in *Streptomyces* [6], was here detected in *S. xiamenensis*

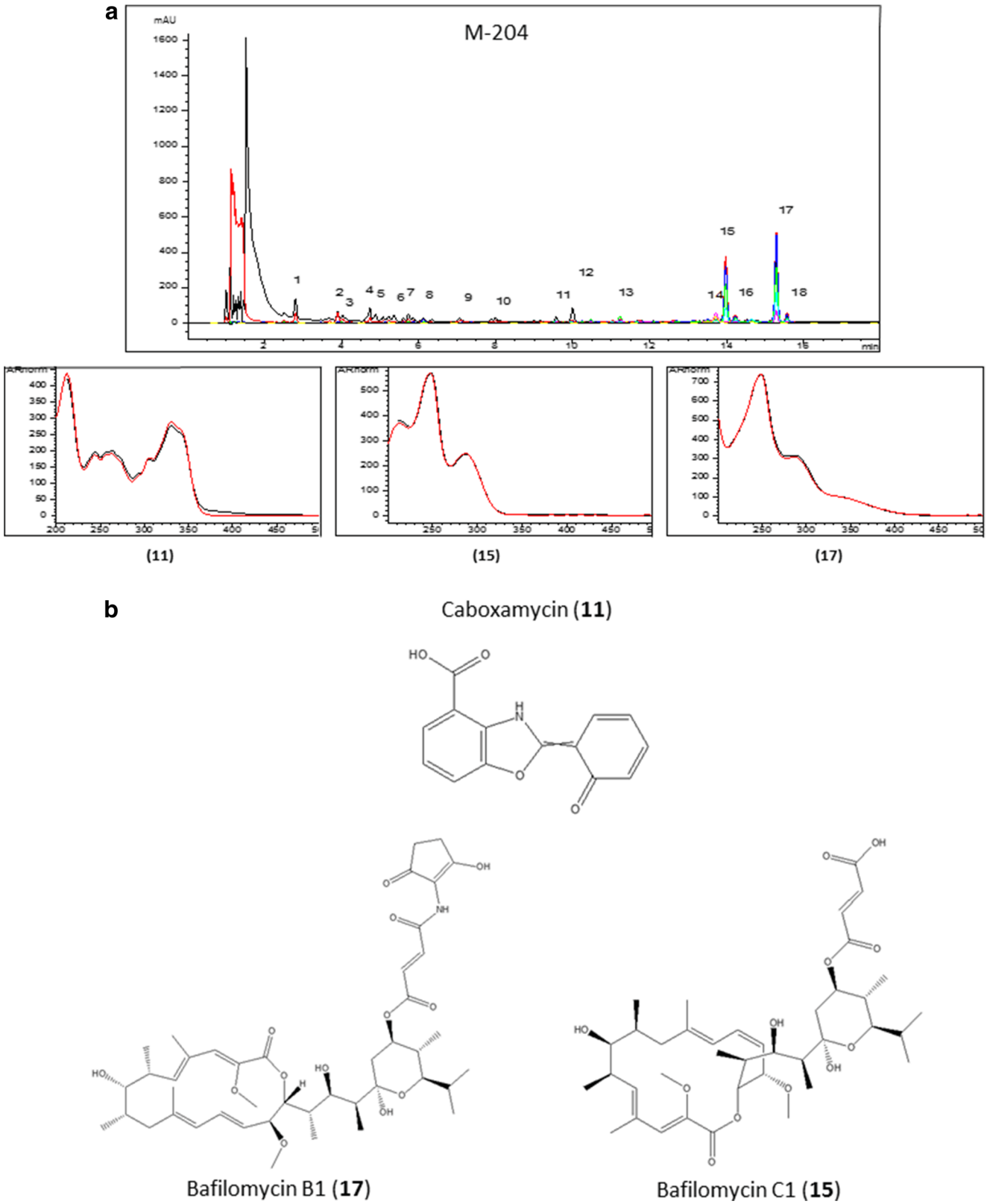

Fig. 3 Chromatogram of extract of *S. halstedii* M-204. **a** Peak numbers in the chromatograms indicate detected compounds, and some of them have been identified and correspond to the following: caboxamycin (11), bafilomycin C1 (2) and bafilomycin (B1). The lower part of the figure represents U.V. absorption spectra of the identified molecules. **b** Chemical structures of bioactive products identified in extracts of S. *halstedii* M-204

Table 5 Bioactive secondary metabolites produced by deep sea Actinobacteria strains and their corresponding biological activities

Compound	Strain	Biological activities
Aloesaponarin II	M-227	Anti-Gram-negative [23]
Anthranilic acid	M-186	Anti-Gram-positive [73]
Bafilomycin B1	M-204	Antifungal, anti-Gram-positive [24, 90]
Bafilomycin C1	M-204	Antifungal, anti-Gram-positive [24, 90]
Caboxamycin	M-204	Anti-Gram-positive, antitumor [35]
Cosmomycin	M-192	Antitumor, anti-Gram-positive [46]
Daunomycin	M-192	Antitumor [77]
Galtamycin	M-192	Antitumor [76]
Germicidin A, B	M-220	Spore germination, hypha elongation [2]
Lobophorin B	M-220	Anti-inflammatory, antituberculosis, anti-BCG [12, 36]
Lobophorin B-derivative	M-207	Unknown
Maltophilin	M-179, M-190,M-192	Antifungal [22]
Maltophilin-derivative	M-178, M-179, M-190	Unknown
NTK 250-A-derivative	M-231	Unknown
NTK 250-B-derivative	M-231	Unknown
Paulomycin A	M-179	Anti-Gram-positive, gonococcal and *Chlamydia* infections [4, 53]
Paulomycin B	M-179	Anti-Gram-positive, gonococcal and *Chlamydia* infections [4, 53]
Phenazine-derivative	M-178	Antimicrobial [30]
Phenelfamycin G	M-231	Anti-Gram-positive [9]
Phenelfamycin G-derivative	M-231	Unknown
Phenelfamycin H-derivative	M-231	Unknown
Valinomycin	M-185, M-190	Antibiotic, antiparasitary, antiviral [13, 57, 59]

M-186, *S. cyaneofuscatus* M-190 and *S. halstedii* M-204 extracts. Table 6 shows volatiles with interesting biological activities identified in the studied strains. These biological activities have been reported only for a few purified products (indicated with subpanel a in Table 6). The rest of the volatiles has been previously reported as constituents of essential oils of plant origin (in this case the biological activities are referred to the whole essential oil, not to the individual components) and, to our knowledge, have not been previously described in microorganisms.

Antibiotic Activity Assays Against Human Clinic Pathogens

Bioassays of ethyl acetate extracts of deep-sea Actinobacteria were performed against a panel of pathogenic clinic bacteria and fungi (Table 1) mainly isolated from human samples in the same geographical region (Asturias) at the Hospital Universitario Central de Asturias (HUCA, Oviedo) and Hospital de Cabueñes (Gijón). Disk diffusion assays were performed with discs prepared as described above ("Materials and Methods") in all cases, with exception of *M. tuberculosis* strains, for which a specific protocol was followed ("Materials and Methods").

Table 7 shows diverse antibiotic activities detected in 13 extracts (out of 18), measured as the halo diameters (mm) of the zones of complete inhibition, against a panel of 17 (out of 22) important clinic pathogens. In the case of *M. tuberculosis*, it is indicated as positive (+) or negative (−) antibiotic activities. No antibiotic activity was detected, however, against highly multiresistant Gram-negative clinic isolates (not included in the table), such as *P. aeruginosa*, *A. baumannii* and the enterobacteria *E. coli*, *K. pneumoniae* and *M. morganii.*

Strong antimicrobial activities were detected mainly against Gram-positive and Gram-negative pathogens, covering in some cases a wide spectrum of clinic bacteria in extracts obtained from producer Actinobacteria. Particularly active were the extracts of strains from the deepest stations, such as *S. sulfureus* M-231, isolated at 4700 m in association to the decapod *Colossendeis colossea*, and *P. carboxydivorans* M-227 isolated at 3000 m deep in the water column; and also all different *S. cyaneofuscatus* strains.

Table 6 Volatile compounds identified in deep sea Actinobacteria strains

Compound	Strain	Biological activities
1-Hexadecene	M-194, M-201	Cytotoxic [43]
1-Limonene	M-235	Anti-Gram positive, anti-Gram-negative [71]
		Cytotoxic [42]
		Antibiofilm activity[a] [80]
		Antifungal [72]
1 s, *cis*-Calamenene	M-157, M-169, M-190, M-204	Cytotoxic [62]
		Antidermatophytic [41]
Cadina-1,4-diene	M-157, M-169, M-190, M-204	Anti-Gram-negative anti-Gram-positive, antifungal [88];
		Antimalarial [1]
Calarene	M-153, M-169, M-178, M-190, M-204	Anti-Gram-negative anti-Gram-positive, antifungal [68, 89]
Decamethylcyclopentasiloxane	All strains	–
Dihydro-β-agarofuran	M-186, M-190, M-204	Antitumor, anti-VIH, immunosuppressant,
		MDR reversal[a], insecticidal [7, 26]
		Antituberculosis[a] [83]
Dimethyldisulfide	All strains	Antifungal[a] [69]
Epi-bicyclosesquiphellandrene	M-157	Antidermatophytic [41]
Geosmin	All strains, except M-157	–
Germacrene-D	M-186, M-190, M-204	Hepatoprotective[a] [87];
		anti-Gram positive, anti-Gram negative, antifungal;
		antitumor [10, 20, 75]
trans-β-Caryophyllene	M-157, M-169, M-178	Hepatoprotective [87];
		anti-Gram-positive, antifungal [75];
		antioxidant [5]
trans-β-Farnesene	M-207	Hepatoprotective [87];
		Cytotoxic, anti-Gram-positive [3]
α-Agarofuran	M-204	–
α-Cedrene	M-207	Hepatoprotective [87]
α-Copaene	M-157, M-169, M-190, M-204, M-235, M-237	Hepatoprotective [87];
		Anti-Gram-negative [49];
		anti-Gram-positive, antifungal [75]
α-Humulene	M-157	Cytotoxic [78]
α-Muurolene	M-157, M-204	Anti-Gram-positive, antifungal [28]
β-Elemene	M-186, M-190	MDR reversa l[a] [95];
		Anti-Gram-positive [33];
		Antimycobacterial [37]
β-Eudesmol	M-190	Antifungal [78];
		antitumor, antioxidant [8]
β-Patchoulene	M-220, M-235, M-237	–
β-Pinene	M-231	Anti-Gram-positive, antifungal [75];
		cytotoxic, antioxidant [47, 91]

[a] Bioassays carried out with pure compounds

Table 7 Antibiotic activities of extracts against a panel of diverse clinic microbial pathogens, measured as the diameters in mm of the zones of complete inhibition, except for *M. tuberculosis* strains for which a specific method was used

Clinic pathogen		Producer strain												
		S. cya M-157	*S. cya* M-169	*S. set* M-178	*S. alb* M-179	*S. cya* M-185	*S. cya* M-190	*S. cya* M-192	*S. hal* M-204	*S. car* M-207	*S. car* M-220	*P. car* M-227	*S. sul* M-231	*M. aur* M-235
Gram-positives														
Mycobacterium tuberculosis H37Rv	N/A	+	+	−	−	+	+	+	−	−	−	−	−	−
Mycobacterium tuberculosis MDR-1	N/A	+	+	−	−	+	+	+	−	−	−	−	−	−
Mycobacterium tuberculosis MDR-2	N/A	+	+	−	−	+	+	+	−	−	−	−	−	−
Streptococcus pyogenes	N	17	8	−	14	10	8	17	−	8	8	14	26	−
Streptococcus pneumoniae	N	15	8	−	10	8	8	16	−	13	13	15	26	8
Enterococcus faecalis	A	14	8	−	−	11	10	14	−	−	−	14	10	−
Enterococcus faecium	N	20	18	−	−	11	12	18	−	−	−	21	22	−
Listeria monocytogenes	N	12	8	−	−	−	−	10	−	14	10	−	21	−
Clostridium perfringens	A	13	8	−	−	8	8	12	10	−	−	18	16	−
Staphylococcus aureus	A	13	7	−	−	9	10	14	−	−	−	14	11	−
Corynebacterium urealyticum	A	17	7	−	7	7	7	18	−	17	15	20	20	−
Gram-negatives														
Neisseria meningitidis	A	15	8	−	−	10	8	16	−	16	10	18	24	−
Neisseria gonorrhoeae	A	10	−	10	−	12	11	10	−	−	−	21	16	−
Bacteroides fragilis	A	10	−	−	−	−	−	12	−	−	−	12	8	−
Haemophilus influenzae	N	−	−	−	−	−	−	−	−	−	−	10	8	−
Stenotrophomonas maltophilia	N	8	−	−	−	−	−	−	−	−	−	−	−	−
Fungal														
Candida albicans	N	−	7	−	15	−	8	−	−	−	−	−	−	−
Candida krusei	N	−	−	−	7	−	−	−	12	−	−	−	−	−

N neutral conditions for extraction, *A* acidic conditions for extraction

Cytotoxic Activity Assays Against Different Tumour Cell Lines

In addition to antibiotic activities, ethyl acetate extracts of most of the strains also displayed moderate cytotoxic activities against two different tumour cell lines: Hela and HCT116, with undiluted and 1:10 diluted extracts (Fig. 4). The fact that the 1/10 diluted extracts appear more active than the undiluted ones against both cell lines could be explained by assay interferences due to the high complexity of the sample, which might contain other compounds with antagonist activity only observed at high concentrations. This fact has been previously observed [6, 66]. Most of the extracts were found to be moderately active against both cell lines. In a few cases, such as *S. cyaneofuscatus* M-157 and M-192, the extracts were even active for highly diluted, 1:100 extracts (data not shown). *S. cyaneofuscatus* M-157 was associated to a stony coral (2000 m. depth) and *S. cyaneofuscatus* M-192 to an actinia from 4700 m depth.

Discussion

As a result of a multidisciplinary approach, we provide here direct evidence that phylogenetically diverse cultivable Actinobacteria populations colonize unexplored deep-sea ecosystems in the Avilés Canyon. The studied species have been identified as members of four genera: *Streptomyces*, *Micromonospora*, *Pseudonocardia* and *Myceligenerans*, which belong to four different taxonomical orders within the phylum *Actinobacteria*. These strains were isolated from deep waters of Arctic origin in a range of 1500–4700 m with temperatures of 2–4 °C, hydrostatic pressure of 150–470 atm, saline concentration of 34.8–35.2 psu (practical salinity unit) and oxygen dissolved of 7.5–7.7 mg/L. Since they can also be grown at atmospheric pressure, 28 °C and show no salt requirements, the strains result to be barotolerant, psychrotolerant and halotolerant. Most species belong to the *Streptomyces* genus and have been isolated from all depths tested in association with very diverse marine organisms, also in the water column. Concerning

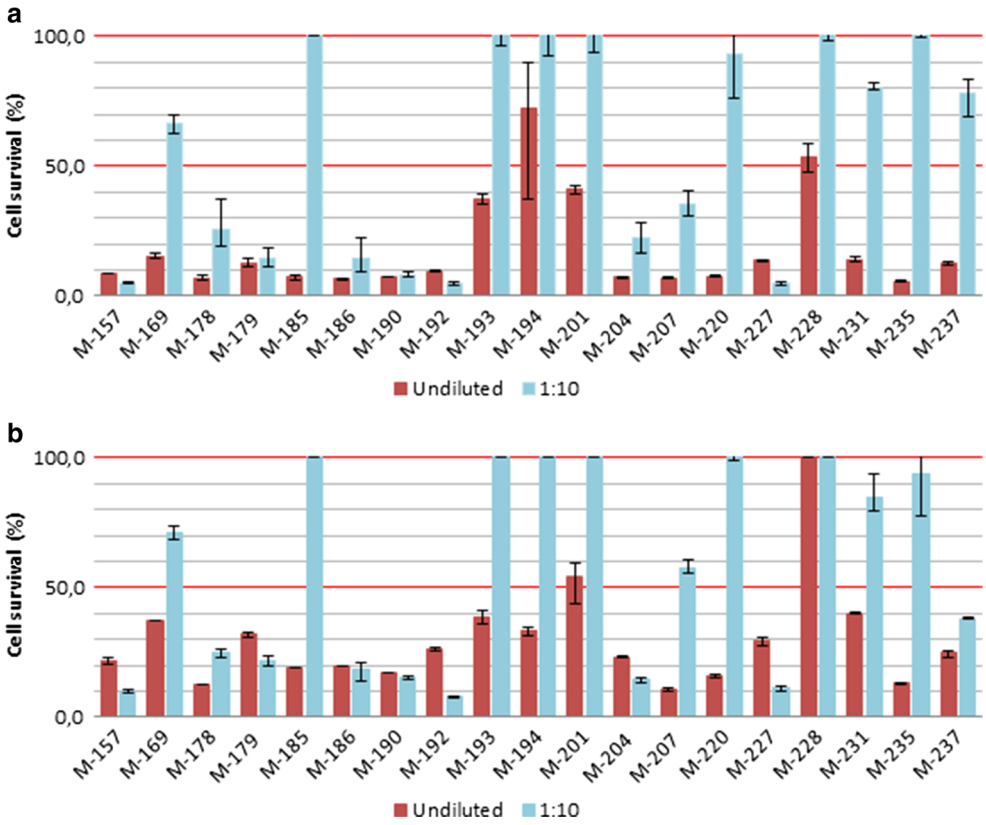

Fig. 4 Cell survival percentage in cytotoxicity assays with ethyl acetate extracts in acidic conditions from Actinobacteria isolates carried out against two different tumour cell lines: HeLa, from cervical carcinoma, and HCT116, from colorectal carcinoma

Micromonospora, although only three species were studied here, ongoing research reveals that this genus colonizes most studied depths (data not shown). In contrast, strains of novel species *M. cantabricum* were isolated only from corals and other invertebrates collected at 1500 m depth (temperature: 6.5 °C; salinity: 35.39 psu; density: 1027.78 Kg m^{-3}). This is slightly below the depth range from 700 to 1200 m, and above the density range between 1027.35 and 1027.65 kg m^3 typically found for cold water corals in this area, corresponding to a water mass of Mediterranean origin [65]. *P. carboxydivorans* strains were sampled in the water column at a depth of 3000 m (temperature: 2.8 °C; salinity: 34.94 psu; density: 1027.86 Kg m^{-3}), characterized by water masses of Arctic origin [86].

These deep-sea Actinobacteria here studied produce a wide number of secondary metabolites with diverse biological activities, mainly antibiotics. Interestingly, some of them are active against noteworthy antibiotic-resistant human pathogens, isolated at the most important hospitals from the same geographical area where Actinobacteria were collected. Surprisingly, the strains exhibiting highest activities, both antibiotic and cytotoxic, were picked up at the deepest stations in this submarine Canyon. Although several compounds were already identified, it is interesting that the great majority of molecules still remain unknown and some of them might be new. It must be highlighted that only the strains of *S. cyaneofuscatus* produce compounds with activity against antibiotic-resistant *M. tuberculosis*. As revealed by comparative analysis between bioactivity existing in identified compounds and in the extracts, there is evidence of highly bioactive extracts, mainly from *S. cyaneofuscatus* M-157 and *P. carboxydivorans* M-227. Compounds produced by these strains are likely to be novel, since no identification was achieved, thus providing a potential source for new natural products. In any case, the number of produced secondary metabolites is estimated to be even higher than the one shown here, since only apolar compounds were extracted and analysed so far, whereas possible polar products have not been studied at all. Besides the presence of diffusible secondary metabolites, particularly striking is the great reservoir of volatile molecules released by most of the Actinobacterial cultures here studied. Although volatile production in microorganisms has been overlooked for a long time, in the last decade, there is increasing evidence that microbial volatiles can act as communication signals or "infochemicals", in interactions among microbes and between microbes and their eukaryotic hosts; some of them displaying antibiotic activities against bacteria and fungi [69].

Furthermore, much of the metabolic potential of Actinobacteria is essentially hidden, not expressed under standard laboratory conditions, representing this silent or cryptic potential the great majority of the metabolome. While significant advances have been made in the field of marine biodiscovery, leading to the introduction of new classes of

therapeutics for clinical medicine, cosmetics and industrial products, most of what this natural ecosystem can really offer is essentially hidden from our screening methods [60]. The most successful approaches to activate silent biosynthetic gene clusters from marine microorganisms have been recently reviewed [60], as also have been metabolomic techniques of great relevance in natural product discovery [92]. Our findings so far, a total of 50 bioactive natural compounds (21 volatiles and 29 diffusible) show that deep-sea marine Actinobacteria from the Avilés Canyon represent an important unexplored source for natural products discovery, particularly antibiotics and other pharmacologically active metabolites of biotechnological interest. Ongoing research is not only focused in the study of potential novelty of some of these bioactive natural molecules, but also in the activation of the silent biosynthetic potential of these marine Actinobacteria.

Acknowledgments This study was financially supported by the Gobierno del Principado de Asturias (SV-PA-13-ECOEMP-62), Ministerio de Economía y Competitividad, Proyecto DOSMARES/BIOCANT (MICINN-10-CTM2010-21810-C03-02) and Consejería de Economía y Empleo del Principado de Asturias (TBR group). A.K. was supported by grants from the DFG (SFB766). The authors want to thank Ricardo Anadón and all other participants in the BIOCANT3 campaign and Santiago Cal for his help with cytotoxicity assays. We are also grateful to José L. Caso and José A. Guijarro for continuous support. We finally thank Miguel Campoamor, Marcos García and Cristina Sariego for their excellent technical assistance and M. Carmen Macián (CECT) for her help in the identification of the strains. This is a contribution of the Asturias Marine Observatory.

References

1. Afoulous S, Ferhout H, Raoelison EG, Valentin A, Moukarzel B, Couderc F, Bouajila J (2013) Chemical composition and anticancer, antiinflammatory, antioxidant and antimalarial activities of leaves essential oil of *Cedrelopsis grevei*. Food Chem Toxicol 56:352–362
2. Aoki Y, Matsumoto D, Kawaide H, Natsume M (2011) Physiological role of germicidins in spore germination and hyphal elongation in *Streptomyces coelicolor* A3(2). J Antibiot (Tokyo) 64:607–611
3. Araujo L, Moujir LM, Rojas J, Rojas L, Carmona J, Rondón M (2013) Chemical composition and biological activity of *Conyza bonariensis* essential oil collected in Mérida, Venezuela. Nat Prod Commun 8:1175–1178
4. Argoudelis AD, Brinkley TA, Brodasky TF, Buege JA, Meyer HF, Mizsak SA (1982) Paulomycins A and B. Isolation and characterization. J Antibiot (Tokyo) 35:285–294
5. Bagheri H, Abdul Manap MY, Solati Z (2014) Antioxidant activity of *Piper nigrum* L. essential oil extracted by supercritical CO_2 extraction and hydro-distillation. Talanta 121:220–228
6. Braña AF, Fiedler H-P, Nava H, González V, Sarmiento-Vizcaíno A, Molina A, Acuña JL, García LA, Blanco G (2015) 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. Microb Ecol 69:512–524
7. Braña AF, Rodríguez M, Pahari P, Rohr J, García LA, Blanco G (2014) Activation and silencing of secondary metabolites in *Streptomyces albus* and *Streptomyces lividans* after transformation with cosmids containing the thienamycin gene cluster from *Streptomyces cattleya*. Arch Microbiol 196:345–355
8. Britto AC, de Oliveira AC, Henriques RM, Cardoso GM, Bomfim DS, Carvalho AA, Moraes MO, Pessoa C, Pinheiro ML, Costa EV, Bezerra DP (2012) *In vitro* and *in vivo* antitumor effects of the essential oil from the leaves of *Guatteria friesiana*. Planta Med 78:409–414
9. Brötz E, Kulik A, Vikineswary S, Lim CT, Tan GY, Zinecker H, Imhoff JF, Paululat T, Fiedler HP (2011) Phenelfamycins G and H, new elfamycin-type antibiotics produced by *Streptomyces albospinus* Acta 3619. J Antibiot (Tokyo) 64:257–266
10. Buitrago A, Rojas J, Rojas L, Velasco J, Morales A, Peñaloza Y, Díaz C (2015) Essential oil composition and antimicrobial activity of *Vismia macrophylla* leaves and fruits collected in Táchira-Venezuela. Nat Prod Commun 10:375–377
11. Bull AT, Ward AC, Goodfellow M (2000) Search and discovery strategies for biotechnology: the paradigm shift. Microbiol Mol Biol Rev 64:573–606
12. Chen C, Wang J, Guo H, Hou W, Yang N, Ren B, Liu M, Dai H, Liu X, Song F, Zhang L (2013) Three antimycobacterial metabolites identified from a marine-derived *Streptomyces* sp. MS100061. Appl Microbiol Biotechnol 97:3885–3892
13. Cheng YQ (2006) Deciphering the biosynthetic codes for the potent anti-SARS-CoV cyclodepsipeptide valinomycin in *Streptomyces tsusimaensis* ATCC 15141. Chembiochem 7:471–477
14. Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 57:2259–2261
15. CLSI Susceptibility testing of *Mycobacteria*, *Nocardiae*, and other aerobic Actinomycetes, Approved Standard-Second Edition. CLSI document M-24-A2, Wayne, PA: clinical and Laboratory Standards Institute, 2011
16. CLSI (2014) Performance Standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. CLSI document M 100-S24. Clinical and Laboratory Standards Institute, Wayne
17. Colquhoum JA, Heald SC, Li L, Tamaoka J, Kato C, Horikoshi K, Bull AT (1998) Taxonomy and biotransformation activities of some deep-sea actinomycetes. Extremophiles 2:269–277
18. Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376
19. Felsenstein J (1985) Conference limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
20. Ferraz RP, Cardoso GM, da Silva TB, Fontes JE, Prata AP, Carvalho AA, Moraes MO, Pessoa C, Costa EV, Bezerra DP (2013) Antitumour properties of the leaf essential oil of *Xylopia frutescens* Aubl. (*Annonaceae*). Food Chem 141:196–200
21. Fiedler HP (1993) Biosynthetic capacities of actinomycetes. 1. Screening for secondary metabolites by HPLC and UV-visible absorbance spectral libraries. Nat Prod Lett 2:119–128
22. Fiedler HP, Bruntner C, Bull AT, Ward AC, Goodfellow M, Potterat O, Puder C, Mihm G (2005) Marine actinomycetes as a source of novel secondary metabolites. Antonie Van Leeuwenhoek 87:37–42
23. Fotso S, Maskey RP, Grün-Wollny I, Schulz KP, Munk M, Laatsch H (2003) Bhimamycin A to approximately E and bhimanone: isolation, structure elucidation and biological activity of novel quinone antibiotics from a terrestrial Streptomycete. J Antibiot (Tokyo) 56:931–941
24. Frändberg E, Petersson C, Lundgren LN, Schnürer J (2000) *Streptomyces halstedii* K122 produces the antifungal compounds bafilomycin B1 and C1. Can J Microbiol 46:753–758
25. Galkiewicz JP, Pratte ZA, Gray MA, Kellogg CA (2011) Characterization of culturable bacteria isolated from the cold-water coral *Lophelia pertusa*. FEMS Microbiol Ecol 77:333–346

26. Gao JM, Wu WJ, Zhang JW, Konishi Y (2007) The dihydro-beta-agarofuran sesquiterpenoids. Nat Prod Rep 24:1153–1189
27. Giddings LA, Newman DJ (2013) Microbial natural products: molecular blueprints for antitumor drugs. J Ind Microbiol Biotechnol 40:1181–1210
28. González AM, Tracanna MI, Amani SM, Schuff C, Poch MJ, Bach H, Catalán CA (2012) Chemical composition, antimicrobial and antioxidant properties of the volatile oil and methanol extract of *Xenophyllum poposum*. Nat Prod Commun 7:1663–1666
29. González I, Ayuso-Sacido A, Anderson A, Genilloud O (2005) Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences. FEMS Microbiol Ecol 54:401–415
30. Gorantla JN, Nishanth Kumar S, Nisha GV, Sumandu AS, Dileep C, Sudaresan A, Sree Kumar MM, Lankalapalli RS, Dileep Kumar BS (2014) Purification and characterization of antifungal phenazines from a fluorescent *Pseudomonas* strain FPO4 against medically important fungi. J Mycol Med 24:185–192
31. Gust B, Challis GL, Fowler K, Kieser T, Chater K (2003) PCR targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci U S A 100:1541–1546
32. Haefner B (2003) Drugs from the deep: marine natural products as drug candidates. Drug Discov Today 8:536–544, Review
33. Hashim SE, Sirat HM, Yen KH (2014) Chemical compositions and antimicrobial activity of the essential oils of *Hornstedtia havilandii* (*Zingiberaceae*). Nat Prod Commun 9:119–120
34. Hirsch AM, Valdés M (2009) *Micromonospora*: An important microbe for biomedicine and potentially for biocontrol and biofuels. Soil Biol Biochem 42:536–542
35. Hohmann C, Schneider K, Bruntner C, Irran E, Nicholson G, Bull AT, Jones AL, Brown R, Stach JE, Goodfellow M, Beil W, Krämer M, Imhoff JF, Süssmuth RD, Fiedler HP (2009) Caboxamycin, a new antibiotic of the benzoxazole family produced by the deep-sea strain *Streptomyces* sp. NTK 937. J Antibiot (Tokyo) 62:99–104
36. Jiang ZD, Jensen PR, Fenical W (1999) Lobophorins A and B, new antiinflammatory macrolides produced by a tropical marine bacterium. Bioorg Med Chem Lett 9:2003–2006
37. Julião Lde S, Bizzo HR, Souza AM, Lourenço MC, Silva PE, Tavares ES, Rastrelli L, Leitão SG (2009) Essential oils from two *Lantana* species with antimycobacterial activity. Nat Prod Commun 4:1733–1736
38. Kim KO, Shin KS, Kim MN, Shin KS, Labeda DP, Han JH, Kim SB (2012) Reassessment of the status of *Streptomyces setonii* and reclassification of *Streptomyces fimicarius* as a later synonym of *Streptomyces setonii* and *Streptomyces albovinaceus* as a later synonym of *Streptomyces globisporus* based on combined 16S rRNA/gyrB gene sequence analysis. Int J Syst Evol Microbiol 62:2978–2985
39. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
40. Kirby BM, Meyers PR (2010) *Micromonospora tulbaghiae* sp. nov., isolated from the leaves of wild garlic, *Tulbaghia violacea*. Int J Syst Evol Microbiol 60:1328–1333
41. Kuiate JR, Bessière JM, Zollo PH, Kuate SP (2006) Chemical composition and antidermatophytic properties of volatile fractions of hexanic extract from leaves of *Cupressus lusitanica* Mill. from Cameroon. J Ethnopharmacol 103:160–165
42. Kumar D, Sukapaka M, Babu GD, Padwad Y (2015) Chemical composition and in vitro cytotoxicity of essential oils from leaves and flowers of *Callistemon citrinus* from Western Himalayas. PLoS One 1, e0133823
43. Lai CS, Mas RH, Nair NK, Majid MI, Mansor SM, Navaratnam V (2008) *Typhonium flagelliforme* inhibits cancer cell growth in vitro and induces apoptosis: an evaluation by the bioactivity guided approach. J Ethnopharmacol 118:14–20
44. Lam KS (2006) Discovery of novel metabolites from marine actinomycetes. Curr Opin Microbiol 9:245–251
45. Levadoux W, Trani M, Lortie R, Kerr D, Groleau D (2002) Microbial resolution of baclofen by a new isolate of *Streptomyces halstedii*. J Biosci Bioeng 93:557–562
46. Li M, Chen YL (1986) Structural studies on rhodilunancins A and B. J Antibiot 39:430–436
47. Lone SH, Bhat KA, Bhat HM, Majeed R, Anand R, Hamid A, Khuroo MA (2014) Essential oil composition of *Senecio graciliflorus* DC: Comparative analysis of different parts and evaluation of antioxidant and cytotoxic activities. Phytomedicine 21:919–925
48. Manivasagan P, Venkatesan J, Sivakumar K, Kim S (2014) Pharmaceutically active secondary metabolites of marine actinobacteria. Microbiol Res 169:262–278
49. Martins Cde M, Do Nascimento EA, de Morais SA, de Oliveira A, Chang R, Cunha LC, Martins MM, Martins CH, Moraes Tda S, Rodrigues PV, da Silva CV, de Aquino FJ (2015) Chemical constituents and evaluation of antimicrobial and cytotoxic activities of *Kielmeyera coriacea* Mart. & Zucc. essential oils. Evid Based Complement Alternat Med 2015:842047
50. Morgan LE (2005) What are deep-sea corals? Curr: J Marine Educ 21:1–4
51. Neulinger SC, Järnegren J, Ludvigsen M, Lochte K, Dullo WC (2008) Phenotype-specific bacterial communities in the cold-water coral *Lophelia pertusa* (*Scleractinia*) and their implications for the coral's nutrition, health, and distribution. Appl Environ Microbiol 74:7272–7285
52. Noro JC, Kalaitzis JA, Neilan BA (2012) Bioactive natural products from Papua New Guinea marine sponges. Chem Biodivers 9:2077–2095
53. Novak E (1988) Treating *Chlamydia* infections with paulomycin. The Upjohn Company. Patent PCT/US1987/002420
54. Park SW, Park ST, Lee JE, Kim YM (2008) *Pseudonocardia carboxydivorans* sp. nov., a carbon monoxide-oxidizing actinomycete, and an emended description of the genus *Pseudonocardia*. Int J Syst Evol Microbiol 58:2475–2478
55. Patel JB, Tenover FC, Turnidge JD, Jorgensen JH (2011) Susceptibility test methods: dilution and disk diffusion methods. In: Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW (eds) Manual of Clinical Microbiology, 10th edn. American Society for Microbiology press, Washington, DC, pp 1122–1143
56. Pathom-Aree W, Stach JE, Ward AC, Horikoshi K, Bull AT, Goodfellow M (2006) Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. Extremophiles 10:181–189
57. Perkins JB, Guterman SK, Howitt CL, Williams VE 2nd, Pero J (1990) *Streptomyces* genes involved in biosynthesis of the peptide antibiotic valinomycin. J Bacteriol 172:3108–3116
58. Piel J (2009) Metabolites from symbiotic bacteria. Nat Prod Rep 26:338–362
59. Pimentel-Elardo SM, Kozytska S, Bugni TS, Ireland CM, Moll H, Hentschel U (2010) Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. Mar Drugs 8:373–380
60. Reen FJ, Romano S, Dobson AD, O'Gara F (2015) The sound of silence: activating silent biosynthetic gene clusters in marine microorganisms. Mar Drugs 13:4754–4783
61. Ryan RP, Dow JM (2008) Diffusible signals and interspecies communication in bacteria. Microbiology 154:1845–1858
62. Saab AM, Guerrini A, Sacchetti G, Maietti S, Zeino M, Arend J, Gambari R, Bernardi F, Efferth T (2012) Phytochemical analysis and cytotoxicity towards multidrug-resistant leukemia cells of

essential oils derived from Lebanese medicinal plants. Planta Med 78:1927–1931
63. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
64. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
65. Sánchez F, González-Pola C, Druet M, García-Alegre A, Acosta J, Cristobo J, Parra S, Ríos P, Altuna A, Gómez-Ballesteros M, Muñoz-Recio A, Rivera J, Díaz del Río G (2014) Habitat characterization of deep-water coral reefs in La Gaviera canyon (Avilés Canyon System, Cantabrian Sea). Deep-Sea Res II 106:118–140
66. Sarmiento-Vizcaíno A, Braña AF, González V, Nava H, Molina A, Llera E, Fiedler HP, Rico JM, García-Flórez L, Acuña JL, García LA, Blanco G (2016) Atmospheric dispersal of bioactive *Streptomyces albidoflavus* strains among terrestrial and marine environments. Microb Ecol 71:375–386
67. Sarmiento-Vizcaíno A, González V, Braña AF, Molina A, Acuña JL, García LA, Blanco G (2015) *Myceligenerans cantabricum* sp. nov., a barotolerant actinobacterium isolated from a deep cold water coral. Int J Syst Evol Microbiol 65:1328–1334
68. Satyal P, Chhetri BK, Dosoky NS, Poudel A, Setzer WN (2015) Chemical composition of *Nardostachys grandiflora* rhizome oil from Nepal—a contribution to the chemotaxonomy and bioactivity of *Nardostachys*. Nat Prod Commun 10:1067–1070
69. Schmidt R, Cordoves V, de Boer W, Raaijmakers J, Garbeva P (2015) Volatile affairs in microbial interactions. ISME J. doi:10.1038/ismej.2015.42
70. Seifried JS, Wichels A, Gerdts G (2015) Spatial distribution of marine airborne bacterial communities. Microbiol Open 4:475–490
71. Shahbazi Y (2015) Chemical composition and in vitro antibacterial activity of *Mentha spicata* essential oil against common food-borne pathogenic bacteria. J Pathog 2015:916305
72. Sharifi-Rad J, Hoseini-Alfatemi SM, Sharifi-Rad M, Sharifi-Rad M, Iriti M, Sharifi-Rad M, Sharifi-Rad R, Raeisi S (2015) Phytochemical compositions and biological activities of essential oil from *Xanthium strumarium* L. Molecules 20:7034–7047
73. Shou Q, Banbury LK, Maccarone AT, Renshaw DE, Mon H, Griesser S, Griesser HJ, Blanksby SJ, Smith JE, Wohlmuth H (2014) Antibacterial anthranilic acid derivatives from *Geijera parviflora*. Fitoterapia 93:62–66
74. Sievers F, Wilm A, Dineen DG, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M et al (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol 7:539
75. Silvério MS, Del-Vechio-Vieira G, Pinto MA, Alves MS, Sousa OV (2013) Chemical composition and biological activities of essential oils of *Eremanthus erythropappus* (DC) McLeisch (*Asteraceae*). Molecules 18:9785–9796
76. Ströch K, Zeeck A, Antal N, Fiedler HP (2005) Retymicin, galtamycin B, saquayamycin Z and ribofuranosyllumichrome, novel secondary metabolites from *Micromonospora* sp. Tü 6368. II. Structure elucidation. J Antibiot (Tokyo) 58:103–110
77. Stutzman-Engwall KJ, Hutchinson CR (1989) Multigene families for anthracycline antibiotic production in *Streptomyces peucetis*. Proc Natl Acad Sci U S A 86:3135–3139
78. Su YC, Hsu KP, Wang EI, Ho CL (2015) Chemical composition and anti-mildew activities of essential oils from different parts of *Michelia compressa* var.*formosana*. Nat Prod Commun 10: 665–668
79. Subramani R, Aalbersberg W (2013) Culturable rare Actinomycetes: diversity, isolation and marine natural product discovery. Appl Microbiol Biotechnol 97:9291–9321
80. Subramenium GA, Vijayakumar K, Pandian SK (2015) Limonene inhibits streptococcal biofilm formation by targeting surface-associated virulence factors. J Med Microbiol 64:879–890
81. Sun W, Peng C, Zhao Y, Li Z (2012) Functional gene-guided discovery of type II polyketides from culturable actinomycetes associated with soft coral *Scleronephthya* sp. PLoS One 7, e42847
82. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729
83. Torres-Romero D, Jiménez IA, Rojas R, Gilman RH, López M, Bazzocchi IL (2011) Dihydro-β-agarofuran sesquiterpenes isolated from *Celastrus vulcanicola* as potential anti-*Mycobacterium tuberculosis* multidrug-resistant agents. Bioorg Med Chem 19: 2182–2189
84. Trujillo ME, Kroppenstedt RM, Fernández-Molinero C, Schumann P, Martínez-Molina E (2007) *Micromonospora lupini* sp. nov. and *Micromonospora saelicesensis* sp. nov., isolated from root nodules of *Lupinus angustifolius*. Int J Syst Evol Microbiol 57:2799–2804
85. Valliappan K, Sun W, Li Z (2014) Marine actinobacteria associated with marine organisms and their potentials in producing pharmaceutical natural products. Appl Microbiol Biotechnol. doi:10.1007/s00253-014-5954-6
86. Van Aken HM (2000) The hydrography of the mid-latitude Northeast Atlantic Ocean. I: the deep water masses. Deep-Sea Res I 47:757–788
87. Vinholes J, Rudnitskaya A, Gonçalves P, Martel F, Coimbra MA, Rocha SM (2014) Hepatoprotection of sesquiterpenoids: a quantitative structure-activity relationship (QSAR) approach. Food Chem 146:78–84
88. Vukovic N, Milosevic T, Sukdolak S, Solujic S (2007) Antimicrobial activities of essential oil and methanol extract of *Teucrium montanum*. Evid Based Complement Alternat Med 4:17–20
89. Wang J, Zhao J, Liu H, Zhou L, Liu Z, Wang J, Han J, Yu Z, Yang F (2010) Chemical analysis and biological activity of the essential oils of two valerianaceous species from China: *Nardostachys chinensis* and *Valeriana officinalis*. Molecules 15:6411–6422
90. Werner G, Hagenmaier H, Drautz H, Baumgartner A, Zähner H (1984) Metabolic products of microorganisms. 224. Bafilomycins, a new group of macrolide antibiotics. Production, isolation, chemical structure and biological activity. J Antibiot (Tokyo) 37:110–117
91. Woguem V, Maggi F, Fogang HP, Tapondjoua LA, Womeni HM, Luana Q, Bramuccic M, Vitali LA, Petrelli D, Lupidi G, Papa F, Vittori S, Barboni L (2013) Antioxidant, antiproliferative and antimicrobial activities of the volatile oil from the wild pepper *Piper capense* used in cameroon as a culinary spice. Nat Prod Commun 8: 1791–1796
92. Wu C, Choi YH, van Wezel GP (2015) Metabolic profiling as a tool for prioritizing antimicrobial compounds. J Ind Microbiol Biotechnol. doi:10.1007/s10295-015-1666-x
93. Yang S, Sun W, Tang C, Jin L, Zhang F, Li Z (2013) Phylogenetic diversity of actinobacteria associated with soft coral *Alcyonium gracllimum* and stony coral *Tubastraea coccinea* in the East China Sea. Microb Ecol 66:189–199
94. You ZY, Wang YH, Zhang ZG, Xu MJ, Xie SJ, Han TS, Feng L, Li XG, Xu J (2013) Identification of two novel anti-fibrotic benzopyran compounds produced by engineered strains derived from *Streptomyces xiamenensis* M1-94P that originated from deep-sea sediments. Mar Drugs 11:4035–4049
95. Zhang GN, Ashby CR Jr, Zhang YK, Chen ZS, Guo H (2015) The reversal of antineoplastic drug resistance in cancer cells by β-elemene. Chin J Cancer 34:45
96. Zhang XY, He F, Wang GH, Bao J, Xu XY, Qi SH (2013) Diversity and antibacterial activity of culturable actinobacteria isolated from five species of the South China Sea gorgonian corals. World J Microbiol Biotechnol 29:1107–1116
97. Zhao X, Geng X, Chen C, Chen L, Jiao W, Yang C (2012) Draft genome sequence of the marine actinomycete *Streptomyces sulphureus* L180. J Bacteriol 194:4482