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ENVIRONMENTAL BIOTECHNOLOGY

Biotechnological potential of Actinobacteria from Canadian and Azorean volcanic caves

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Abstract Caves are regarded as extreme habitats with appropriate conditions for the development of Actinobacteria. In comparison with other habitats, caves have not yet been the target of intensive screening for bioactive secondary metabolites produced by actinomycetes. As a primary screening strategy, we conducted a metagenomic analysis of the diversity and richness of a key gene required for non-ribosomal peptide (NRP) biosynthesis, focusing on cave-derived sediments from two Canadian caves (a lava tube and a limestone cave) to help us predict whether different types of caves may harbor drug-producing actinobacteria. Using degenerate PCR primers targeting adenylation domains (AD), a conserved domain in the core gene in NRP biosynthesis, a number of amplicons were obtained that mapped back to biomedically relevant NRP gene cluster families. This result guided our culture-dependent sampling strategy of actinomycete isolation from the volcanic caves of Canada (British Columbia) and Portugal (Azores) and subsequent characterization of their antibacterial and enzymatic activities. Multiple enzymatic and antimicrobial activities were identified from bacterial of the *Arthrobacter* and *Streptomyces* genera demonstrating that actinomycetes from volcanic caves are promising sources of antibacterial, antibiofilm compounds and industrially relevant enzymes.

Keywords Caves · Actinobacteria · Metagenomics · Antimicrobial activity · Enzymatic activity

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Introduction

Primary and secondary metabolites from *Actinomycetales* (aka actinomycetes) are important sources of industrially relevant compounds (Lam 2006; Donadio et al. 2010; Miao and Davies 2010; Manivasagan et al. 2014). The isolation of compounds from these bacteria has increasingly shown to yield more unknown compounds suggesting the need for exploring alternative sources of biodiversity within such prolific bacteria (Katz et al. 2015). Goodfellow and Fiedler (2010) have suggested mining underexploited habitats for rare Actinobacteria and their metabolites as a strategy to circumvent this problem. Rare Actinobacteria from different types of soils were described as a potential source of novel metabolites (Tiwari and Gupta 2013; Guo et al. 2015). Caves, particularly those of volcanic origin, remain an underexploited reservoir of bacterial metabolites of potential industrial relevance (Cheeptham et al. 2013; Montano and Henderson 2013).

Both culture-independent and culture-dependent strategies are important tools in natural product research (Milshteyn et al. 2014). Culture-independent techniques may provide crucial information on biodiversity and thus help avoiding the most common pitfall in natural product research, i.e., rediscovery (Katz et al. 2015). Scanning electron microscopy

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(SEM) has also been used to provide a first, morphology-based, view on the bacterial diversity in cave habitats (Northup et al. 2011; Riquelme et al. 2015a).

To assess the bacterial diversity of cave environments, we chose to investigate non-ribosomal peptides (NRPs), a large and diverse family of bacterial metabolites. NRPs have diverse pharmacological properties including antibiosis, antifungal, and antitumor activities (Newman and Cragg 2012), and they are synthesized by large modular mega-enzymes called non-ribosomal peptide synthetases (NRPSs). NRPS enzymes are composed of discrete modules consisting of highly conserved protein domains that catalyze the incorporation of single amino acids into a growing peptide chain (Walsh 2004). With the exception of rare cases of convergent evolution, biosynthetic gene clusters that encode closely related structures appear to have arisen from vertical evolutionary changes (Fischbach et al. 2008). It is therefore possible to use the phylogenetic differences observed between individual NRPS domain sequences as a surrogate for predicting relationships among entire NRP gene clusters (Charlop-Powers et al. 2014; Chang and Brady 2014; Owen et al. 2015). Using this general approach, investigation of the diversity and richness of NRP biosynthesis in cave-derived sediments can be achieved.

Cave conditions may enhance the production of hydrolytic enzymes and antimicrobial compounds (Davies 2006; Nakaew et al. 2009; Hibbing et al. 2010; Cheeptham et al. 2013). Bacterial hydrolytic enzymes are of interest for a range of different industrial applications and are an expanding market area (Adrio and Demain 2014). Gelatinase, chitinases, cellulases, xylanases, pectinases, inulinases, xylanases, phytases, and DNases find applications in waste upcycling, bioethanol production, biotechnology, household care, cosmetic, pharma, textile, paper, and pulp industries, as well as in human and animal nutrition (Mazotto et al. 2011; Sanchez and Demain 2011; Prakash et al. 2013; Swartjes et al. 2013). In particular, actinomycetes are a promising source of biocatalysts (Miao and Davies 2010; Prakash et al. 2013). However, cave actinomycetes have rarely been screened for their enzymatic activities (Tomova et al. 2013).

Natural product screening is regarded as the most promising line for novel antibiotic discovery, which is needed to counteract the loss of effectiveness of the presently available chemotherapeutic choices (Kirst 2013; Silver 2015). In comparison with other habitats, cave actinomycetes have not yet been the target of intensive screening efforts regarding their antibacterial activity against pathogenic bacteria, with only a few reports published in mainstream journals in the last few years (Duangmal et al. 2012; Cheeptham et al. 2013; Montano and Henderson 2013; Rule and Cheeptham 2013). Their findings demonstrate a promisingly high probability of discovering a novel compound with different modes of action. However, in some cases, such as when assessing antibacterial activity against *Pseudomonas aeruginosa*, screening protocols may need to be shifted to include testing against biofilms. Biofilms are communities of bacteria encased in a self-synthesized polymeric matrix that may adhere to biotic or abiotic surfaces (Hall-Stoodley et al. 2004). This mode of growth protects bacteria from eradication by desiccation, nutrient deprivation, and antibiotic treatment (Gaddy and Actis 2009). Bacteria associated with biofilms may be up to 1000 times more resistant to antibiotics compared to planktonic cells, enabling cells to persist despite intensive antibiotic therapy (Mah and O'Toole 2001; Gaddy and Actis 2009). Additionally, there have been many studies on the activation of natural compounds with either full spectrum sunlight or ultraviolet light. These studies include the activation of biocidal compounds such as plant extracts, cow urine, and even some antibiotics (Cheeptham and Towers 2002; Upadhyay et al. 2010; Yuan et al. 2011).

The main goal of this research was the screening of actinobacterial isolates collected in volcanic caves from Azores (Portugal) and British Columbia (Canada) for their antibacterial and enzymatic activities, thus tapping into a still unexploited potential source of useful bacterial metabolites. To be able to meet such a goal, a preliminary investigation was conducted on the functional differences observed between individual non-ribosomal peptide biosynthesis (NRPS) domain sequences via an assignment of adenylation domains (AD) and known antimicrobial producing gene clusters to determine whether potential antimicrobial agent producers can be found in deeper and more pristine cave areas. Metagenomics and SEM data were used to guide the sampling effort in obtaining actinomycete isolates, and isolation targeted different activities, i.e., enzymatic profile, broad-spectrum antibacterial activity, antibiosis against planktonic cells, and biofilms of *P. aeruginosa* (with or without UV treatment).

Materials and methods

Sampling sites

Samples of sediments and colored microbial mats developing on cave walls and ceilings were collected from two Canadian caves and 12 Portuguese volcanic caves: (i) Helmcken Falls Cave, which is a volcanic cave in Wells Gray Provincial Park in Clearwater, British Columbia, Canada (Cheeptham et al. 2013); (ii) Raspberry Rising Cave, which is a limestone cave in Mount Tupper system in Glacier National Park, Revelstoke, British Columbia (Canada); (iii) Furna do Lemos (GL), Gruta dos Montanheiros (GM), Gruta da Ribeira do Fundo (GRF), and Gruta das Torres (GT) in Pico Island (Azores, Portugal); and (iv) Gruta das Agulhas (GA), Gruta dos Buracos (GB), Gruta dos Balcões (GBL), Gruta da Branca Opala (GBO), Gruta do Natal (GN), Gruta da Terra Mole (GTM), Gruta

dos Principiantes (GP), and Galeria da Queimada (GQ) in Terceira Island (Azores, Portugal).

Scanning electron microscopy (SEM)

Sediment samples from Helmcken Falls Cave were freeze-dried, fixed with osmium fumes as described in Cheeptham et al. (2013), and observed at the University of British Columbia (UBC) BioImaging Facility, using a Hitachi S4700 Field Emission SEM (Hitachi, Tokyo, Japan). Samples of small cave wall chips covered with microbial mats from Azorean volcanic caves were mounted directly on SEM sample stubs, sputter coated with Au-Pd film, and examined on a JEOL 5800 SEM, as described in Hathaway et al. (2014).

NRPS biosynthetic conserved domain pyrosequencing and assessment of NRP biodiversity of sediment samples from Canadian caves

Experimental setup Five sediment samples from two different Canadian caves and one forest soil sample (as a control) were collected and subjected to DNA extraction; the DNA extracted directly from each sample was column purified, and the crude environmental DNA extracts were used as templates in PCR reactions with barcoded degenerate primers designed to target NRPS AD sequences. The resulting AD amplicons, containing site-specific barcodes, were pooled and sequenced using the Illumina Miseq platform. Raw sequence reads were filtered for quality, de-barcoded to assign reads to a source environment, and then clustered at a distance of 5 % to generate A-domain operational taxonomic units (OTUs) for each cave site. These OTU tables were then used to assess AD richness, predict the presence of known gene cluster families and compare gene cluster content between samples. This experimental setup is detailed below.

Cave sediment collection Four sediment samples (designated Cave07-10) were collected from various locations in the main cavern of the Helmcken Falls Cave (Fig. 1).

As a baseline comparison, a forest soil sample collected at the mouth of Helmcken Cave was also included (sample 11). One sample (designated Cave06) was taken from the Raspberry Rising Cave.

Sediment DNA extraction DNA was extracted from samples using a simplified version of our previously published DNA isolation protocols (Brady 2007; Charlop-Powers et al. 2015). The modified protocol is as follows: 25 g of each sample was incubated in a 50-ml conical tube at 70 °C for 2 h in 25-ml of lysis buffer (2 % sodium dodecyl sulfate [*w*/*v*], 100 mM Tris-HCl, 100 mM EDTA, 1.5 M NaCl, 1 % cetyl trimethyl-ammonium bromide [*w*/*v*]). Large particulates were then removed by centrifugation (×4000*g*, 30 min), and crude DNA was precipitated from the resulting supernatant with the addition of 0.6 volumes of isopropyl alcohol. This precipitated DNA, known as environmental DNA or "eDNA," was collected by centrifugation (×4000*g*, 30 min), washed with 70 % ethanol and resuspended in a minimum volume of TE (10 mM Tris, 1 mM EDTA [pH 8]). Crude eDNA was passed through two rounds of column purification using the PowerClean system (MoBio, Carlsbad, CA). Purified eDNA was then diluted to 30 ng/μl and archived for use in PCR reactions.

First round PCR amplification Degenerate primers targeting conserved regions of the AD [A3F (5′-GCSTACSYSATSTACACSTCSGG) and A7R (5′-SASGTCVCCSGTSCGGTA) (Ayuso-Sacido and Genilloud 2005)] were used to amplify gene fragments from crude eDNA. Forward primers contained a 454 sequencing primer (CGTATCGCCTCCCTCGCGCCATCAG) followed by a unique 8 bp barcode, and the reverse primer contained the 454 adaptor sequence followed by the degenerate A7R primer sequence (5′-CTATGCGCCTTGCCAGCCCG CTCAGSASGTCVCCSGTSCGGTA-3′). The PCR reaction consisted of 25 μl of FailSafe PCR Buffer G (Epicenter, Madison, Wisconsin), 1 μl recombinant Taq Polymerase (Bulldog Bio, Portsmouth, NH), 1.25 μl of each primer (100 mM), 14.5 μl of water, and 6.5 μl of purified eDNA. PCR conditions for the first round of PCR were as follows: 95 °C for 4 min followed by 40 cycles of 94 °C for 0.5 min, 63.5 °C for 0.5 min, 72 °C for 1 min, and finally 72 °C for 5 min.

Second round PCR amplification The first round of PCR primers creates an amplicon that is ready for 454 sequencing. However, we sought to adapt our work to the Illumina platform by using a second round of PCR to attach adaptor sequences (Cimermancic et al. 2014). Following the example of a recent paper (Fadrosh et al. 2014), we designed four forward and four reverse primers that contained the MiSeq P5 and P7 sequences, respectively, a 10 bp barcode, a 1–4 bp spacer sequence, and 18 basepairs of homology to the 454 adaptors on the first primer (Supplementary Table S1). AD amplicons from the first round of PCR were pooled and cleaned with one round of Agencourt Ampure XP beads (Beckman Coulter, Brea, CA) according to the manufacturer's protocol at a DNA/bead ratio of 0.6. Four pools of amplicons were amplified with four sets of 454-to-Illumina exchange primers, using the following cycle protocol: 95 °C for 5 min followed by 8 cycles of 95 °C for 03 s, 55 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 5 min. DNA from four reactions were cleaned using another round of Ampure beads at a DNA/bead ratio of 0.6, verified for size-homogeneity using the TapeStation (Agilent), pooled at equimolar concentrations, and run on the MiSeq sequencing machine using v3 chemistry and 2 × 300 cycles.

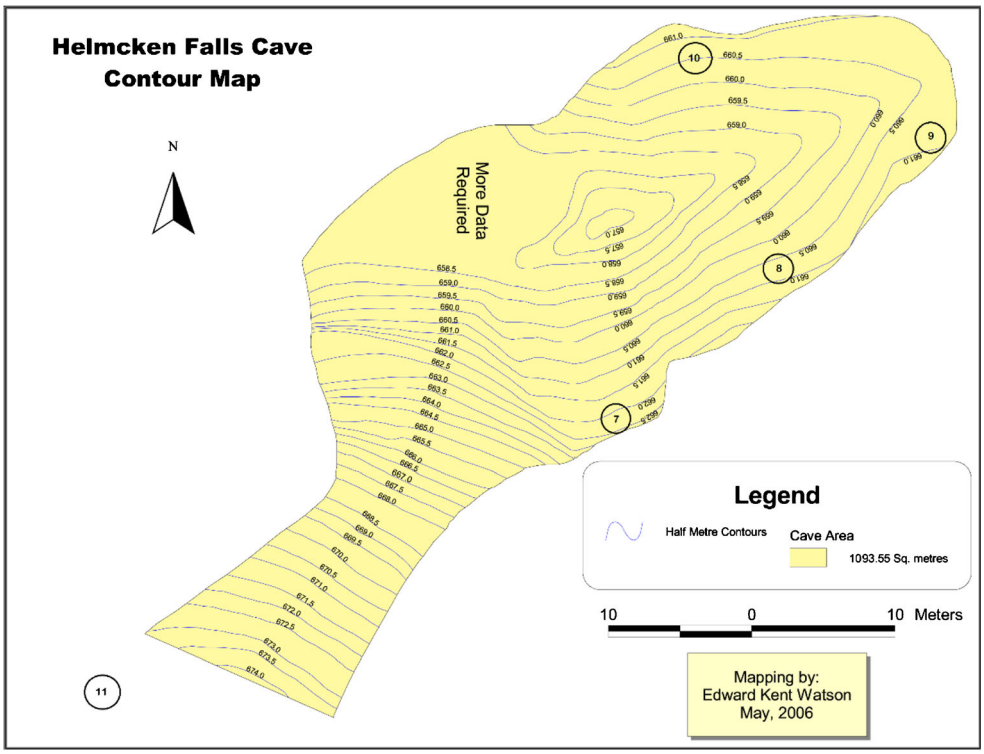

Fig. 1 Map of the Helmcken Falls volcanic cave (Canada) with the positions of the sampling sites. Samples designated Cave07 to 10 were collected from within the cave and were used for NRPS DNA sequencing while sample 11 was of forest origin and collected outside the cave. Sample 11 was used as a baseline control

A two-step PCR approach was employed so that we could continue to use the barcoded 454 PCR primers we have used in the analysis of other metagenomes (Fadrosh et al. 2014). The second round of PCR added an invariant sequence that is required for Illumina sequencing. As this second PCR step only runs for 8 cycles and is consistent with other protocols for attaching an invariant primer for unbiased sequencing, we expect that it introduced very limited if any additional amplification bias.

Raw reads for all samples are deposited online at the National Center for Biotechnology Information. Sequence read accessions and sample information can be found associated with the BioProject PRJNA324563 entitled Cave Soil Adenylation Domains. The accession numbers for the samples in this study are summarized in Supplementary Table S2.

Processing Illumina Miseq data Raw reads from the forward-facing primer were filtered for quality and assigned to samples using a multistage process. Low-quality base calls were trimmed, excised from the reads using the phred algorithm in seqtk, after which samples were de-barcoded using Qiime (version 1.9) (Caporaso et al., 2010). All reads were trimmed to 250 bp or discarded if they were shorter. These reads were then clustered at 97 % identity using USEARCH (version 7) (Edgar 2013), and chimeric sequences were removed using the default clustering tool. The consensus sequences at 97 % were used to re-cluster at 95 %, and the resulting "5 %" AD OTU tables were used for all subsequent rarefaction and diversity analyses.

Similarity analyses Ecological distances between samples were calculated using the Jaccard distance metric, a metric that assess the percentage of OTUs shared between two samples as a fraction of the OTUs in both samples (OTUA&B)/(OTUA + OTUB-OTUA&B] (Oksanen et al. 2015). Network plots were generated in Phyloseq (McMurdie and Holmes 2013), and the distances were also displayed in matrix format. In an effort to identify biomedically relevant NRP gene cluster families present in bacteria from cave sediments, we used the eSNaPD algorithm to analyze the sequence data obtained from each sample (Reddy et al. 2014). eSNaPD uses a basic BLAST search algorithm and a curated set of gene clusters to identify OTUs that are uniquely related, at high sequence identity, to known gene cluster families (Owen et al. 2015). Using 454 sequencing reads, eSNaPD was empirically validated to have an approximately 80 % success rate for correctly assigning amplicons to known gene cluster families. The Illumina data generated in this study were subjected to a similar eSNaPD threshold for analyzing 454 sequencing data.

Rarefaction analysis To assess the total AD diversity of amplicons obtained from the samples, we performed a rarefaction analysis where the OTU table was subsampled at a depth ranging from 1 to 700,000. At each sampling depth, the OTUs for each sample were randomly, independently subsampled ten times. For each sampling event, the Chao1 estimate of diversity was calculated (Chao 1984; Chao and Shen 2003), and the mean of which is displayed. Samples were also sampled evenly at a single fixed depth (346,870 reads; the

smallest number of reads per sample), and the Shannon and Simpson Diversity measurements were calculated for each sample (Shannon 1948; Simpson 1949).

Assignment of AD to known gene clusters AD amplicon reads were assigned to known biosynthetic gene clusters using a modification of the environmental Survey of Natural Product Diversity (eSNaPD, http://esnapd2.rockefeller.edu/) bioinformatic algorithm (Reddy et al. 2014). For Miseq forward reads, we used 250 bp of the 300 bp amplicon and a conservative e-value BLAST threshold of e-80 to assign domains to a gene cluster family.

Screening for enzymatic and antimicrobial activity from Azorean volcanic caves

Actinobacteria isolation. One hundred and forty-eight actinobacterial isolates were obtained from the walls and ceilings of volcanic caves from the Terceira and Pico Islands (Azores, Portugal). Sampling was carried out by touching speleothems, oozes, bacterial mats, and apparently non-colonized surfaces with the cotton tip of a sterile swab. These swabs were used to inoculate the surface of half-strength R2A (½ R2A) agar. The inoculated plates were incubated aerobically at temperatures close to those in the caves (15 °C for all caves, except the plates from Montanheiros cave, which were incubated at 11 °C), until visible growth was observed (3–5 days). Actinobacteria were selected from the observed growth in each plate on the basis of colony (typical of actinomycetes in shape, structure, and odor) and cell morphological characteristics (Gram-positive filaments) and purified by repeatedly streaking out onto ½ R2A. *Arthrobacter* isolates that had been sequenced in a previous work (unpublished results) were also included. Stock cultures were prepared on ½ R2A agar slants and kept at −4 °C for further testing.

Screening for antimicrobial activity The 148 actinobacterial isolates obtained were screened for their antimicrobial activity against *Proteus* sp. (collection of the Microbiology Laboratory–CITA-A), *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* (ATCC 9144 and 29,523), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 2785, *Listeria monocytogenes* ATCC 7466, and *Listeria innocua* ATCC 33090, by an agar diffusion assay, using the cross-streak technique (Guo et al. 2010). In short, the actinobacterial isolates were grown on a layer of ½ R2A at 11–15°, for 3–5 days, as a single streak across the diameter of the agar surface. An overlay of plate count agar (PCA) was carefully poured over the basis ½ R2A. After solidifying, the target bacteria were perpendicularly streaked. Plates were subsequently incubated at 37 °C for 24 h. Lack of growth on the target strains streaks in, at least, their central portion where they crossed the actinobacteria streak was considered as a positive result (inhibition of the target strain by the actinobacterial culture).

Screening for enzymatic activity Eighteen actinobacterial isolates from Gruta dos Buracos (1), Gruta dos Balcões (4), Gruta dos Montanheiros (8), and Gruta das Torres (5) were screened for their enzymatic activities. Amylase, pectinase, inulinase, cellulase, xylanase, chitinase, and DNase were tested using the culture media proposed by Babavalian et al. (2013), but omitting NaCl. Phytase activity was assessed using the culture medium proposed by Quan et al. (2001), whereas the screening for gelatinase activity was performed on the culture medium proposed by noticed Terzic-Vidojevic et al. (2009). Incubation was carried out at 15 °C for 7 days.

Identification of the active isolates Selected isolates displaying enzymatic and/or antimicrobial activity were identified by sequencing of the 16S rRNA gene. Genomic DNA was extracted from cultures grown in nitrate broth with the UltraClean Microbial DNA isolation kit (MoBio, Carlsbad, California) using manufacturer's protocol. The 16S rRNA gene was amplified by PCR with universal primers, 8 forward (5′-AGAGTTTGATCCTTGGCTCAG-3′) and 1492 reverse (5′-GCYTACCTTGTTACGACTT-3′) using Amplitaq. Reactions and amplification were carried out in a 50-μl volume, as described in Hathaway et al. (2014). Amplicons were cleaned and purified using the Qiagen PCR cleanup kit (Qiagen, Germantown, Maryland). Isolates were sequenced using the Big Dye Terminator Kit (Applied Biosystems) with primers 46 forward (5′-GCYTAAYACATGCAAGTCG-3′) and 1409 reverse (5′-GTGACGGGCRGTGTGTRCAA-3′) and precipitated with sodium acetate and ethanol. Sequences were read by the ABI 3130 Sequencer (Applied Biosystems). Searching for the closest relatives of the sequences was performed using the nucleotide BLAST algorithm (Altschul et al. 1997). Sequences were aligned by SINA aligner (1.2.11) (Pruesse et al. 2012). Sequence similarity matrices were built for each isolate and its BLAST best hit's sequence using BioEdit sequence editor (version 7.2.5) (Hall 1999). A list of GenBank accession numbers is provided in Supplementary Table S3.

Screening for antibiofilm activity in a Canadian volcanic cave

Three bacterial isolates (CM_A1A3, CM_PM58B, and CM_RA003) were originally isolated from sediment samples collected from the sample site 08 (Fig. 1) from the Helmcken Falls volcanic Cave (British Columbia, Canada) and used in this study due to their ability to inhibit biofilms of

Pseudomonas aeruginosa (Mason 2015). The three cave bacteria were used to inoculate test tubes containing each of the three different broth media: V-8 juice, International *Streptomyces* Project #2 (ISP2), R2A fermentation media (Cheeptham et al. 2013). These broth media were autoclaved at 121 °C for 15 min prior to inoculation. The inoculated test tubes were then cultured at 25 °C for 10 days with 100 rpm of agitation in an orbital shaker (New Brunswick Scientific Innova 42). Then, 800-μl samples of the cell-free extracts from each tube were taken at days 2, 4, 6, 8, and 10 and stored at −20 °C until used.

Screening of cell-free extracts against *P. aeruginosa* biofilms Culturing, exposure, and recovery of *P. aeruginosa* biofilms were done using the MBEC P&G assay device (Innovotech, Canada) according to "Standard Test Method for Testing Disinfectant Efficacy against *P. aeruginosa* Biofilm using the MBEC Assay" (ASTM E2799-12, 2012). The cell-free extracts were split into two groups: UV and non-UV-treated samples. The UV treatment consisted of exposing the 96-well microplate with the supernatant to UV light (254 nm) in a Labconco Class II biological safety cabinet, at room temperature. The negative controls were sterile TSB, V8, R2A, ISP2, and deionized water. The positive controls were 2 % Virkon solution and the antibiotics tetracycline HCl and ciprofloxacin HCl. The measurement of cell-free extract antibiofilm/antimicrobial activity was done with dilutions and viable cell counts. A preliminary surviving cell count was done using spot plate technique on large square plates (Thermo Scientific™ Nunc™ Square BioAssay dishes with 250 ml of TSA) to assess the antibiofilm/antimicrobial activity. The plate was divided into sections, and 10 μl of each sample was spot plated on the agar. The TSA plates were then incubated at 35 °C for 18 h. After incubation, each spot on the TSA plates was inspected to determine if any samples had no growth or reduced growth. Such samples were to be subjected to further measurement. Once identified, the samples were diluted from 10^{-1} to 10^{-4}. Then, 10 ml of each dilution was spot plated onto TSA plates and incubated at 35 °C for 18 h. After 18 h, the CFU/ml of each sample was calculated and then subjected to a 2 sample *t* test with pooled variances (after testing data for normality and equal variance) to determine if the difference was significant. A P value of <0.05 was considered significant.

Kirby-Bauer disk diffusion assay to determine planktonic antimicrobial activity The standard Kirby-Bauer (KB) disk diffusion assay (Bauer et al. 1966; Wilkins et al. 1972) was used to determine the antimicrobial activity of the cave isolates against planktonic *P. aeruginosa*. The sterile disks used were 8 mm Advantec paper disks (Tokyo, Japan). Eighty microliters of each cell-free extracts was impregnated on the disks in replicates of three. The negative controls were sterile TSB, V8, R2A, ISP2, and deionized water. The positive controls were 2 % Virkon solution and the antibiotics tetracycline HCl and ciprofloxacin HCl. A 1 % *v*/*v* inoculum of *P. aeruginosa* at 1.0 McFarland was transferred to 200 ml of sterile molten TSA, and then poured into the sterile Thermo Scientific™ Nunc™ Square BioAssay dishes and allowed to solidify. The impregnated disks were then placed onto the bioassay agar plates. The plates were incubated at 35 °C for 18 h; after incubation, the inhibitory zones were measured.

Results

Morphological observations

SEM revealed microbial structures with morphological characteristics typical of Actinobacteria in the sediment samples and colored microbial mats from the Canadian and Portuguese volcanic caves (Fig. 2). Most of the Actinobacteria-like mats comprised masses of spores with hairy surface (Fig. 2a, b) or spiny surface (Fig. 2c, d). They were arranged in clusters of single spores (Fig. 2b, d) or spore chains (Fig. 2c, e, f). Generally, the spores were of globose shape (Fig. 2d), but ovoid and rod-shaped spores were also frequently found (Fig. 2c, e). Spores with spiny surface were observed with variable spine sizes (Fig. 2d, e).

NRPS biosynthetic conserved domains and NRP biodiversity of Canadian cave sediment samples

Sample similarity To assess the overall relationship of NRPS systems in the Helmcken Falls and Raspberry Rising Caves, we isolated eDNA from six sediments and amplified the adenylation domains from those samples. After sequencing the amplicons, we assessed the similarity between samples by clustering the sequences into OTUs at 95 % similarity and comparing them using the Jaccard distance metric (Fig. 3a). The Jaccard distance is a ratio of the OTUs shared by two samples as a fraction of the total OTUs that both samples contain, which provides a simple way of assessing AD overlap between amplicon datasets. The closest Jaccard distances observed between any two samples were obtained for samples CA07 and CA09 from the Helmcken Falls Cave, where 5.95 % of the AD OTUs were shared. Overall, samples within the Helmcken Falls Cave cluster more to each other than to the Raspberry Rising Cave sample (CA06) (Fig. 3a). The Raspberry Rising Cave (limestone) sample showed very limited relationship to any of the samples from the Helmcken Falls Cave (volcanic), sharing less than 0.6 % of its OTUs with any of the Helmcken Falls Cave samples.

Rarefaction and domain diversity analysis The number of AD OTUs present in each cave sample was predicted from the

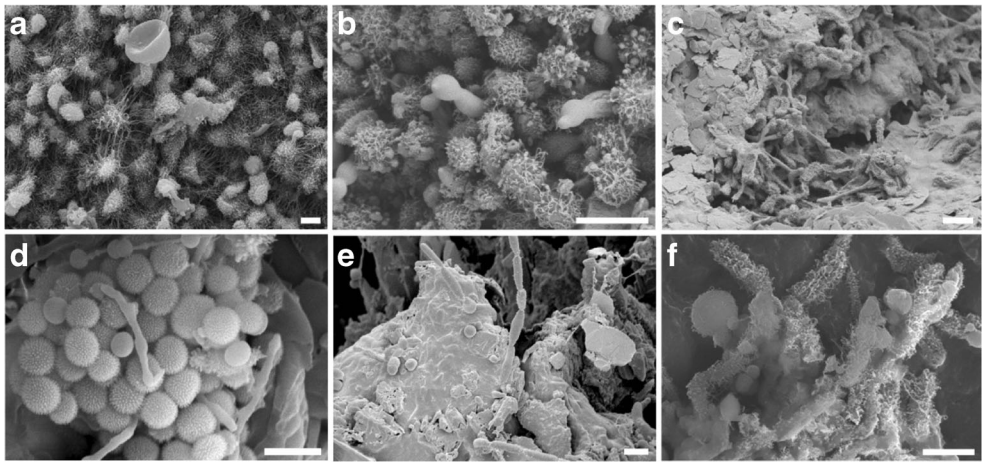

Fig. 2 Scanning electron microscopy images showing several morphological features of Actinobacteria-like structures from Canadian and Azorean cave samples. **a** Dense mass of spores with hairy surface and interwoven filaments found in Gruta dos Montanheiros (Pico Island, Azores, Portugal); **b** mass of spores with spiny and hairy surface from Helmcken Falls Cave (British Colombia, Canada); **c** Actinobacteria-like mat from Helmcken Falls Cave composed of chains of rod-shaped spores with spiny surface; **d** cluster of individual globose spores with spiny surface from Gruta das Torres Cave (Pico Island, Azores, Portugal); **e** rod-shaped spores with spiny surface developed in a long chain in samples from Helmcken Falls Cave; **f** chains of spores with hairy surface found within microbial mats from Gruta da Terra Mole (Terceira Island, Azores, Portugal). *Scale bars*, 2 μm

OTU table data using the Chao1 diversity metric (Fig. 3b). AD richness predictions for Helmcken Falls Cave samples range from ~5000 to 10,000 OTUs per sample, while the Raspberry Rising Cave sample is predicted to contain approximately 2500 unique AD OTUs. The Chao1 diversity metric can be heavily biased by singleton sequences, and therefore, additional diversity estimate experiments using the Shannon and Simpson diversity metrics were performed (Fig. 3c). Using either of these measures of richness, the relative richness among samples is similar, and the Raspberry Rising Cave sample is predicted to have lower AD sequence diversity than any other sample. Interestingly, in these analyses, very little differences are observed between the AD diversity estimate for the sample collected at the mouth of the Helmcken Falls Cave and the samples collected within this cave.

Tracking known natural product gene cluster families in cave samples Based on the metrics studied, we were able to map between 0.1 and 0.3 % of reads obtained from our amplicon data to known gene clusters (Fig. 3d). In the eSNaPD analysis, we detect AD sequences from both caves that have high sequence identity to a variety of gene cluster families known to encode for non-ribosomal peptides of diverse biomedical importance (Fig. 3e). Figure 3e shows that volcanic cave samples overall showed higher hits with the identified AD sequences from the eSNaPD analysis than those of limestone cave sample. Also, in comparison with a control sample (sample#11: forest sample outside the volcanic cave), two samples (samples#08 and 09 which were deeper in) showed higher number of the AD sequences similar to those known antibiotics from the eSNaPD analysis.

Screening for enzymatic and antimicrobial activity from Azorean volcanic caves

One hundred forty-eight isolates obtained from the walls and ceilings of volcanic caves from the Terceira and Pico Islands (Azores, Portugal) were identified as actinomycetes on the basis of their colony and cell morphology. A considerable proportion of the tested isolates (27 isolates; 18.1 %) displayed antibacterial activity against at least one of the target bacteria under study (Table 1). The active isolates were obtained from Furna do Lemos, Gruta dos Balcões, Gruta da Branca Opala, Gruta dos Montanheiros, Gruta das Torres, and Gruta da Terra Mole. No active isolates were obtained from Gruta dos Buracos and Gruta da Ribeira do Fundo (Supplementary Table S4).

From our findings, *Escherichia coli* was the most frequently inhibited target bacterium (21 isolates), whereas only one isolate (*Streptomyces mauvecolor* GM32B4) inhibited *Pseudomonas aeruginosa*. *S. mauvecolor* GMB32B4 was also the only isolate that was active against all of the tested pathogens, making it a promising source of broad spectrum antibiotics that should be investigated further.

Eight other isolates were active against most (7) of the tested target bacteria. None of them were active against *P. aeruginosa* and *Listeria innocua*. Two of them were identified as *S. avidinii* and two as *S. spiroverticillatus* (Table 2).

Most identified isolates belonged to the *Streptomyces* genus, with only two identified as *Arthrobacter* (Table 2). It was possible to assign all the isolates to a species, with five of them identified as *S. nojiriensis*, three as *S. spiroverticillatus*, three as *S. avidinii*, two as *A. nicotinovorans*, and one as *S. mauvecolor* (Table 2).

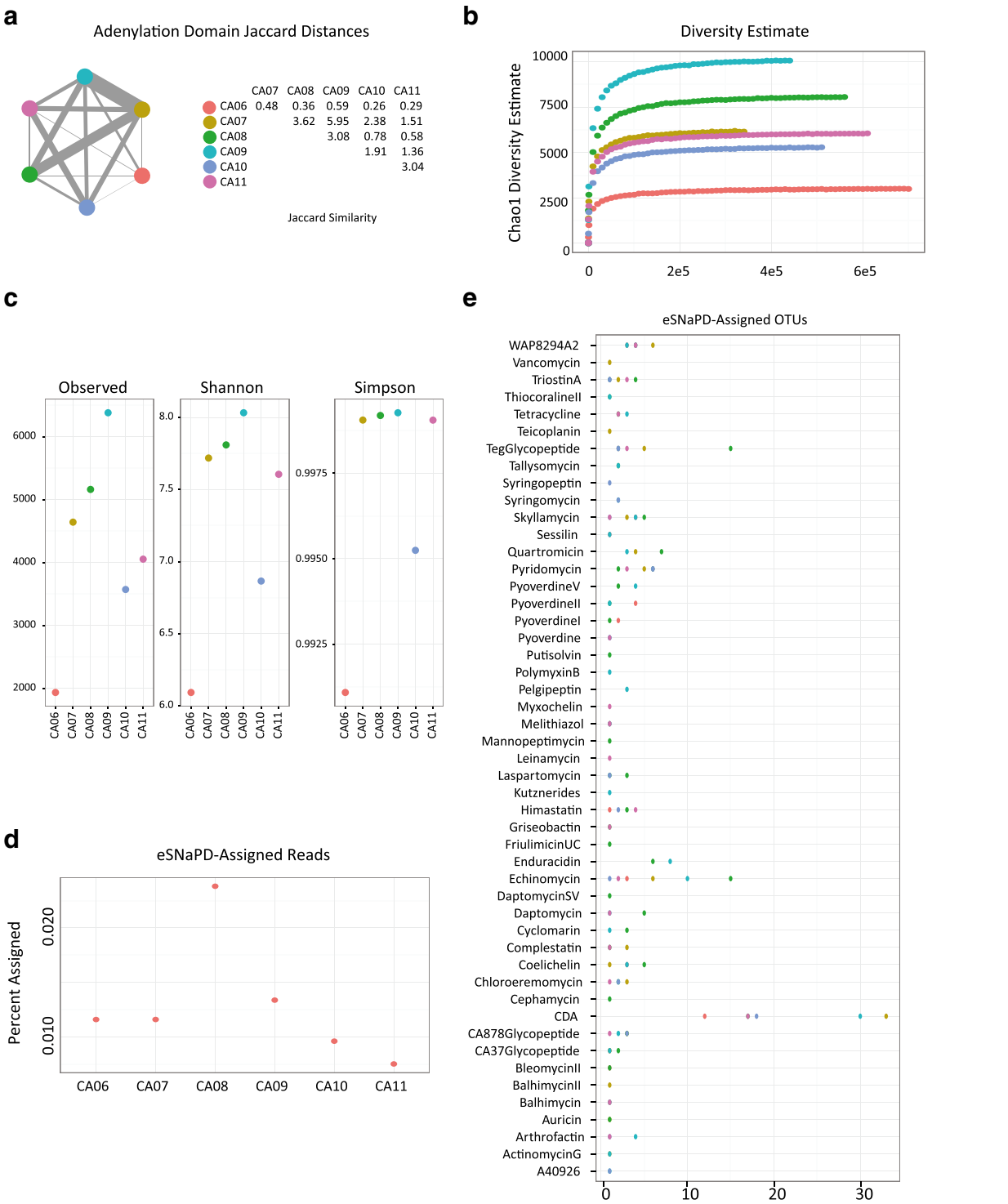

Fig. 3 Metagenomic analysis of cave sediment A-domains: **a** OTU tables generated from PCR-amplified and sequenced A-domains were used to calculate ecological distance between cave samples using the Jaccard distance metric. **b** A-domain diversity estimates using the Chao1 diversity metric. **c** A-domain diversity estimates using the Shannon and Simpson methods. D) Consensus sequences from 95 % OTUs were subjected to eSNaPD analysis and the fraction of reads assigned to a known cluster is displayed for each sample. **e** Number of OTUs that map to a known gene clusters using eSNaPD

Psychrotrophic enzyme activities (15 °C) were tested in 18 actinobacterial isolates (Table 3). All tested isolates displayed at least four of the tested enzymatic activities, but none was able to degrade phytate. Thirteen of the tested isolates degraded most (5–8) of the tested substrates. All isolates were able to degrade gelatin and inulin. Degradation of pectin (17 isolates),

Table 1 Antibacterial activity of actinobacterial isolates obtained from volcanic caves from Azores (Portugal) against *Salmonella* Typhimurium ATCC 14028 (ST), *Escherichia coli* ATCC 25922 (EC), *Pseudomonas aeruginosa* ATCC 2785 (PA), *Proteus* sp. (PT), *Listeria monocytogenes* ATCC 7466 (LM), *Listeria innocua* ATCC 33090 (LI), and *Staphylococcus aureus* (ATCC 9144 and 29,523; SA1 and SA2)

Isolate code	PT	ST	SA1	SA2	EC	PA	LM	LI
GBL 11D2	−	−	−	−	+	−	−	+
GBL 12 A	−	−	−	-	+	−	−	+
GBL 5B	+	−	−	−	+	−	−	−
GBL 5C	+	−	−	−	+	−	−	−
GBL 7 A1	−	−	−	−	+	−	−	+
GBO33B	−	−	−	+	−	−	−	+
GBO33F	−	−	−	+	−	−	−	+
GL02A4	−	+	+	+	+	−	−	−
GM32B4	+	+	+	+	+	+	+	+
GM32C1	−	−	+	+	−	−	−	−
GM35A2	+	+	+	+	+	−	+	+
GM35A4	−	−	−	−	+	−	−	−
GM35A5	+	+	+	+	+	−	+	+
GM35A6	−	−	−	−	+	−	−	−
GM35A7	+	+	+	+	+	−	+	+
GM35B11	-	−	+	+	+	−	−	+
GM35B14	−	−	+	−	+	−	−	−
GM47C3	−	−	−	−	+	−	−	+
GT12A3	−	−	+	−	−	−	−	−
GT12A7	+	+	+	+	+	−	+	+
GT12B3A	+	+	+	+	+	−	+	−
GT12B3B	+	+	+	+	+	−	+	+
GT12B3C	+	+	+	+	+	−	+	+
GT24C6B	+	+	+	+	+	−	+	+
GTM 1E2	−	−	−	+	−	−	−	+
GTM 5F	−	−	−	+	+	−	−	+
GTM 7C	+	−	−	+	−	−	−	+

Shown only the isolates that were inhibitory against at least one target bacterium

starch (15 isolates), xylan (11 isolates), and DNA (12 isolates) were frequent. Less than half of the isolates were found to degrade chitin and cellulose.

Screening for antibiofilm activity in Canadian volcanic caves

Three bacterial isolates (CM_A1A3, CM_PM58B, and CM_RA003), obtained from the sediment samples of the Helmcken Falls Cave, were used in this study because they showed the ability to inhibit biofilms of *P. aeruginosa* in a previous study (Mason 2015). These three isolates showed inhibitory activity against *P. aeruginosa* biofilms on day 8. Three different fermentation media (R2A, ISP2, and V8) were used in this study. The preliminary screening was used as a quick assessment of which cell-free extracts showed any signs of antibiofilm activity. In case of CM_A1A3, antibiofilm activity was observed only in the V8 juice medium, while CM_PM58B showed activity in both V8 juice and R2A media. CM_RA003, in all three media, showed antibiofilm activity. Only CM_RA003 and CM_PM58B in V8 had antibiofilm effects when exposed to UV light. After the preliminary screening, samples showing inhibition were further enumerated to calculate the colony forming units per microliter (CFU/ml). The observed CFU/ml for each growth control (R2A, ISP2, and V8) was compared to the TSB control using a 2 sample *t* test to confirm that the media had no effect on the *P. aeruginosa* biofilms. The *P* value for each comparison of media to TSB control was greater than 0.05 meaning that there was no significant difference.

Table 2 Closest relatives of bioactive actinomycetes isolated from volcanic caves from the Azores (Portugal)

Isolate code	Closest relative	Identity	% Coverage
GB10C	*Arthrobacter nicotinovorans*	0.995	100
GBL11D2	*Streptomyces nojiriensis*	0.994	100
GBL1B	*Arthrobacter nicotinovorans*	0.994	99
GBL5B	*Streptomyces nojiriensis*	0.996	100
GBL5C	*Streptomyces nojiriensis*	0.996	99
GM32B4	*Streptomyces mauvecolor*	0.991	99
GM35A2	*Streptomyces spiroverticillatus*	0.978	100
GM35A4	*Streptomyces nojiriensis*	0.990	99
GM35A7	*Streptomyces spiroverticillatus*	0.981	100
GM35B11	*Streptococcus nojiriensis*	0.986	99
GM47C3	*Streptomyces spiroverticillatus*	0.987	100
GT12A3	*Streptomyces avidinii*	0.972	100
GT12A7	*Streptomyces avidinii*	0.993	99
GT12B3A	*Streptomyces avidinii*	0.972	99

Significantly, the isolate CM_PM58B cultured in V8 juice medium demonstrated antibiofilm activity with and without UV exposure (Fig. 4). This would suggest that there was some active metabolite in the cell-free extract affecting the biofilm. In contrast, there was one sample out of 9 treatments that demonstrated activity after exposure to UV light, although a previous study conducted by Rule and Cheeptham (2013) also showed that UV exposure for enhancing/deactivating antimicrobial effects is inconclusive and therefore needs more work to understand mechanisms of UV exposure to these potential cave bacterial metabolites.

Discussion

Morphological observations

Morphologically, Actinobacteria are one of the richest groups of bacteria, sharing characteristics with both bacteria and

Table 3 Enzyme activities in actinomycetes isolated from volcanic caves from the Azores (Portugal)

Isolate code	Isolate identity	Dnase	Gelatinase	Xylanase	Chitinase	Cellulase	Amylase	Inulinase	Pectinase	Phytase
GB10C	*Arthrobacter nicotinovorans*	−	+	+	+	−	+	+	+	−
GBL11D2	*Streptomyces spororaveus*	+	+	+	+	+	+	+	+	−
GBL5B	*Streptomyces spororaveus*	+	+	−	−	−	−	+	+	−
GBL5C	*Streptomyces spororaveus*	+	+	−	−	−	−	+	+	−
GBL1B	*Arthrobacter nicotinovorans*	+	+	+	+	+	+	+	−	−
GM32B4	*Streptomyces mauvecolor*	+	+	+	+	−	−	+	+	−
GM35A2	*Streptomyces mauvecolor*	+	+	+	+	−	+	+	+	−
GM35A4	*Streptomyces spororaveus*	+	+	+	−	−	+	+	+	−
GM35A5	Not identified	−	+	+	−	−	+	+	+	−
GM35A7	*Streptomyces mauvecolor*	−	+	−	−	−	+	+	+	−
GM35B11	*Streptomyces spororaveus*	+	+	−	−	−	+	+	+	−
GM35B14	Not identified	−	+	−	−	−	+	+	+	−
GM47C3	*Streptomyces mauvecolor*	+	+	+	−	+	+	+	+	−
GT12A3	*Streptomyces avidinii*	−	+	−	?	−	+	+	+	−
GT12A7	*Streptomyces avidinii*	−	+	+	+	−	+	+	+	−
GT12B3A	*Streptomyces avidinii*	+	+	−	+	−	+	+	+	−
GT12B3B	Not identified	+	+	+	−	−	+	+	+	−
GT12B3C	Not identified	+	+	+	−	+	+	+	+	−

fungi (Li et al. 2016). The morphology of spore shape, spore surface, and spore structure is an important criterion for recognizing Actinobacteria by microscopy. Many microbial features observed by SEM in samples from Canadian and Azorean caves were mainly composed of Actinobacteria, suggesting that these bacteria are able to survive and develop within these volcanic caves, as demonstrated by Hathaway et al. (2014) and Riquelme et al. (2015a, 2015b). These observations have prompted us to furthering our research on cave-dwelling Actinobacteria in order to search for novel antimicrobial compounds and enzymatic activities.

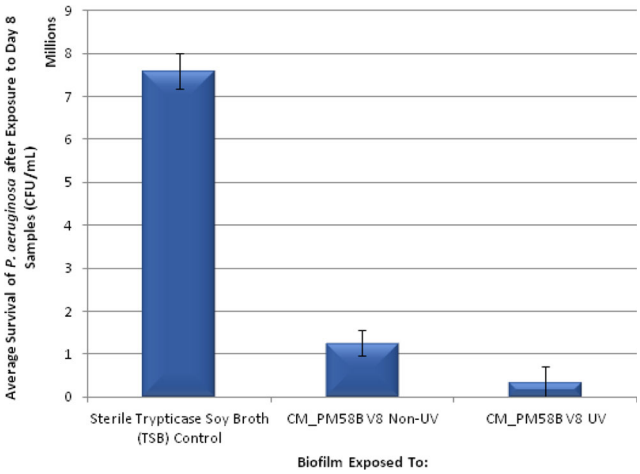

Fig. 4 The average survival of *Pseudomonas aeruginosa* cells (in CFU/ml) compared to the TSB control after exposure to day 8 samples ($n = 3$). The *error bars* indicate standard error of the mean

NRPS biosynthetic conserved domain pyrosequencing and assessment of NRP biodiversity of sediment samples from Canadian caves

Metagenomic profiling in caves of different origin and dimension may provide a means to probe the relationship between cave-depth and the bacterial and chemical diversity of the cave sediments. As has been observed in similar analyses of soils, only a small fraction of OTUs are shared among samples collected even in very close proximity to each other (Reddy et al. 2014). In this study, solely the samples CA07 and CA09 from the Helmcken Falls Cave clustered to each, possibly due to physical characteristics of the sediments. Unlike CA08 and CA10 (more like fine sand), the texture of CA07 and CA09 (coarse sand) sediments was observed to be very similar in texture and geological origin. In this particular cave, however, all of the samples within the cave are roughly equidistant to one another when using the 95 % jaccard distance metric, hindering the assessement of relationships between cave-depth and bacterial diversity. This observation is consistent with the culture-based studies of cave microorganisms from these caves, a hypothesis we sought to test by isolating organisms from cave samples. In previous work, 266 bacterial isolates were successfully isolated from 23 samples (collected at about 100 m from the cave entrance) from Raspberry Rising Cave (Golapkhan et al. 2013). Eighty of the cave isolates were screened and nine (11.25 %) showed antimicrobial activity against MDR-*Staphylococcus aureus* and seven against *Micrococcus luteus*. The detailed identification of these

positive bacteria is under investigation; however, judging by their microscopic and macroscopic features, these bacteria are not *Streptomyces*. Similarly, of the 400 isolates obtained from Helmcken Falls Cave, it was found that 1 % showed activity against extended spectrum ß-lactamase *E. coli*, 2.25 % against *Acinetobacter baumannii*, and 26.50 % against *Klebsiella pneumoniae*. In addition, 10.25 % showed activity against *Micrococcus luteus*, 2 % against methicillin-resistant *Staphylococcus aureus*, 9.25 % against *Mycobacterium smegmatis*, 6.25 % against *Pseudomonas aeruginosa*, and 7.5 % against *Candida albicans* (Cheeptham et al. 2013). Through 16S rRNA sequencing, 79.3 % of the positive 82 strains belong to the *Streptomyces* genus and 6.1 % were members of *Bacillus*, *Pseudomonas*, *Nocardia*, and *Erwinia* genera. Surprisingly, 14.6 % of the 16S rRNA sequences were similar to unidentified ribosomal RNA sequences in the library databases; the sequences of these isolates are under further investigation. Therefore, both metagenomic sequencing and culture-based approaches identify differences in the composition of cave microbiomes including antimicrobial activity (cultured) and the composition of putative antimicrobial biosynthetic genes (metagenomics).

When tracking known natural product gene cluster families in cave samples, once again, the sample from the Raspberry Rising limestone cave is an outlier, as it has a lower predicted AD diversity than any of the samples collected from the Helmcken Falls Cave. The little differences observed between the samples collected at the entrance of the Helmeken Falls Cave and within the cave, using both the Chao1 and Shannon and Simpson diversity measurements, may be explained by the physical characteristics and location of the cave, which consists of only one big room of about 72 m long and 20 m wide. Furthermore, a number of incoming drips of water can be seen in the cave, which suggests that there is a direct connection to the outside world. In addition, the presence of animals was also evident in some areas of the main room, while human visits are limited due to the difficult access (Cheeptham et al. 2013). We believe that this is suggestive of a relationship between cave depth and biodiversity.

In support of this idea, we found that a higher percentage of isolated cave bacteria could be isolated from samples#08 (CA08) and 09 (CA09) and that CA08 showed higher numbers of isolated bacteria demonstrating antimicrobial activity. Of all the samples tested, it was found that the limestone cave sample showed lower frequency of the known AD sequences from the eSNaPD analysis. Therefore, this data provides suggestive evidence in favor of the idea of both differences in biodiversity between cave microenvironments and the possibility that samples found deeper in the cave may be sources of natural product biodiversity. Although this is a small dataset, we believe that metagenomics may allow us to guide our cave-based antimicrobial search strategy by identifying sampling sites that harbor interesting or novel clades of biosynthetic genes.

Screening for enzymatic and antimicrobial activity from Azorean volcanic caves

The *Streptomyces mauvecolor* GM32B4 isolate showed promising results regarding antibacterial activity, inhibiting the growth of all target bacteria. The only reports on the antimicrobial activity of this species regard piperidamycins, which are coded in a silent gene cluster and are generally not expressed under usual cultivation conditions (Hosaka et al. 2009). *S. spiroverticillatus* is known to produce antimicrobial compounds (Cheng et al. 1987; Nogawa et al. 2010). No previous reports on antibacterial activity were found for *A. nicotinovorans*, *S. nojirensis*, and *S. avidinii*.

Psychrotrophic enzymatic activity was screened at low temperature (15 °C) for the 18 actinobacteria isolated from the Azorean caves. Cold-adapted enzymes are of interest for the modern industry because they are more productive at low temperature than their mesophilic or thermophilic homologs. Such enzymes allow for less energy consumption in industrial processes, are able to function in organic solvents, and easy to inactivate in the final product due to their thermolability (Cavicchioli et al. 2011). Most of the tested substrates were degraded by the actinobacterial isolates, with the exception of phytate. Gelatinases take part in collagen degradation and may be of use for the production of pharmaceuticals, cosmetics, and laboratory reagents from this protein (Watanabe 2004). Microbial inulinases find application in the enzymatic hydrolysis of inulin for the production of fructose (a sweetener for the food industry), inulin oligosaccharides (used in prebiotic food ingredients and supplements), and in bioethanol production (Chi et al. 2009; Kango and Jain 2011). Pectinases are widely used by the food industry to clarify fruit-based drinks, wine and beer, to macerate vegetables for puree production or oil extraction, in the processing of coffee and tea, as an animal feed additive and for the treatment of paper and pulp (Bhat 2000; Pedrolli et al. 2009). Amylases are of interest for the food (production of high-fructose syrups), textile, and detergent industries (Prakash et al. 2013). Xylan is the most abundant and principal type of hemicellulose. Xylan-degrading enzymes have a wide range of applications in the textile, pharmaceutical, paper and pulp, food and animal feed industries. In animal feed, they may improve the digestion of nutrients in the initial part of the digestive tract, resulting in a better conversion of the feed energy and reducing the nitrogen and phosphorus content of the excreta, thus helping to mitigate the environmental footprint of animal production (Harris and Ramalingam 2010). DNases are employed in genetic engineering processes (Sánchez-Porro et al. 2003), and their use in surface coatings to prevent biofilm formation has recently been considered (Swartjes et al. 2013). Chitinases have gained

tremendous importance in the past two decades, for the preparation of fungal protein, as biocontrol agents against human disease vectors and plant pathogens as well as for the production of chitin oligomers, which are important biomedical products. Cellulases are of interest for the detergent, textile, paper, pulp industries and as animal feed additives (Prakash et al. 2013).

The broad range of enzymatic activities found in this study is not surprising, since Actinobacteria (in particular the *Arthrobacter* and *Streptomyces* genera) are well-known sources of enzymes for industrial purposes (Narayana and Vijayalakshmi 2009; Fu et al. 2014). The scarcity of reports on industrially relevant enzymatic activities from the identified species (*A. nicotinovorans*, *S. avidinii*, *S. mauvecolor*, *S. nojiriensis*, and *S. spiroverticillatus*) may make them an interesting target for the isolation, purification, and characterization of these microbial products.

In spite of the limited number of isolates tested, our results show that actinomycetes from caves hold promise as sources of antibacterial compounds and industrially relevant enzymes. Because they still are an underexploited habitat, caves are more likely to yield novel microbial compounds than other well-known sources of Actinobacteria.

Screening for antibiofilm activity in Canadian volcanic caves

The KB disk diffusion showed interesting results regarding the antimicrobial efficiency of the cave bacterial isolates. No samples showed an inhibitory zone around any disk inoculated with cell-free extract. This would indicate that there are no any bioactive compounds present in the extracts that have biocidal effects against *P. aeruginosa*; if this is true, but there is an effect on the biofilm, this would suggest a biofilm disruption property. The cell-free extracts do not kill the cells, but rather disrupted the biofilm matrix they reside in (however, further experiments need to be conducted to confirm this). This observation is also supported given the possible identity of the cave bacterial isolates. The cave isolates CM_A1A3 and CM_PM58B were shown to be Gram-positive filamentous bacteria, which is consistent with *Streptomyces* species identification. Furthermore, the 16S rRNA sequencing also showed that both CM_A1A3 and CM_PM58B were *Streptomyces* species with 99 % shared similarity and 100 % query coverage (Mason 2015). The cave isolate CM_RA003 was a Gram-positive rod-shaped bacterium and the 16S rRNA sequencing agreed with this. The sequencing showed that the cave isolate CM_RA003 was in the *Bacillus* genus. Members of the *Bacillus* genus have previously been able to produce antibiofilm molecules against a wide variety of bacteria (Thenmozhi et al. 2009; Nithya et al. 2010; Alasil et al. 2014; Farmer et al. 2014). Soil bacteria similar to the ones used in this study have been shown to have biofilm inhibitory effects. The biofilms are quite often an important factor for the virulence of the bacterium in question. Recently, a study used bacteria from soil, cave, and rivers to produce cell-free extracts (Farmer et al. 2014). The extracts were then used against *Staphylococcus aureus* biofilms in a 96-well microtiter plate, similar to this study. The study found that 55/126 extracts significantly inhibited the biofilms due to a variety of chemical compounds found in the extracts. Also, 40 % of the extracts were found to contain DNase, which could break down extracellular DNA, a major structural component of some biofilms (Farmer et al. 2014). In another study, researchers collected bacterial samples from Magura Cave in Bulgaria (Lazarkevich et al. 2013). The samples were very diverse with respect to bacterial species, and all produced varying compounds such as proteases, xanthan lyase, and β-glycosidase. Again, the researchers found that 75 % of the collected samples showed antibiofilm activity against *Bacillus subtilis* ATCC 6633 and *P. aeruginosa* NBIMCC 1390 in a biofilm (Lazarkevich et al. 2013). Cave bacteria, including actinomycetes, continue to be important in the discovery of new bioactive compounds. The cave bacteria isolates CM_A1A3, CM_RA003, and CM_PM58B used in this study have continued to show inhibitory activity against other pathogenic bacteria in our previous studies (Cheeptham et al. 2013; Rule and Cheeptham 2013) and *P. aeruginosa* biofilms. Of the 90 total cell-free extract samples collected from culturing for 10 days (3 strains, 3 different media, 2 treatments, and 5 different days), 8 (approximately 9 %) of them showed inhibitory activity. These samples were all from the day 8 collection, and two of the samples showed significantly consistent inhibition of the *P. aeruginosa* biofilms. Since the samples had no effect on the planktonic cells, it is reasonable to think that there was a biofilm dispersal effect rather than a bactericidal effect. Lastly, the 16S rRNA sequencing identified the cave isolates CM_A1A3, CM_RA003 as members of the genera *Streptomyces*, and CM_PM58B as *Bacillus* (Mason 2015). Cells living in a biofilm are more resistant to antimicrobial compounds which has been problematic in health care system. Therefore, it is remarkable that the cave isolates could have inhibitory effects without any purification or concentrating. The next step would be to take samples from day 7 to day 9 in order to pinpoint where the most effective secondary metabolite is produced. The culture conditions such as fermentation media, temperature, pH, oxygen, inoculum size, and inoculum growth media could also be studied to optimize the bioactive compounds produced. As well, it would be important to purify and identify what specific molecules are present in the cave isolate samples in order to decipher the mechanism of action.

All in all, within the realm of actinobacterial community study, our work evidences secondary metabolite production in volcanic caves based on the presence of biosynthetic gene amplicons. Using degenerate PCR primers to target a conserved domain in the biosynthesis of non-ribosomal peptides,

we assessed the biosynthetic diversity of sediments derived from two Canadian caves (volcanic versus limestone). On the basis of sequence clusters generated from these amplicons, we can distinguish the single limestone cave sample from the samples obtained from the volcanic cave. We can also identify a number of AD amplicons that map back to biomedically relevant NRPS gene cluster families. However, in order to use metagenomics to predict the type of cave or location within a cave most likely to harbor novel secondary metabolite producers, we will need to investigate a larger number of caves and sample a larger number of sites within the cave. This data represents a starting point that can be used to compare and contrast cave biosynthetic diversity to help guide future culture dependent and independent natural product discovery efforts.

When it comes to bioactive compound screening from the isolated bacteria, the broad range of enzymatic and antimicrobial activities found across our study is not unexpected, since actinomycetes, in particular the *Arthrobacter* and *Streptomyces* genera, are prolific producers of secondary metabolites. In spite of the limited number of isolates tested, our results show that actinomycetes from caves hold promise as sources of antibacterial, antibiofilm compounds, and industrially relevant enzymes. Because they still are an underexploited habitat, caves are more likely to yield novel microbial compounds than other well-known sources of actinomycetes.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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