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In Situ Cultured Bacterial Diversity from Iron Curtain Cave, Chilliwack, British Columbia, Canada

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Abstract: The culturable bacterial diversity from Iron Curtain Cave, Chilliwack, British Columbia, Canada was examined. Sixty five bacterial isolates were successfully cultivated, purified, and identified based on 16S rRNA gene sequencing. Four distinguishable phyla, i.e., Actinobacteria (44.61%), Proteobacteria (27.69%), Firmicutes (20%) and Bacteroidetes (7.69%) were identified. *Arthrobacter* (21.53%) was identified as the major genus, followed by *Sporosarcina* (9.23%), *Stenotrophomonas* (9.23%), *Streptomyces* (6.15%), *Brevundimonas* (4.61%), and *Crocebacterium* (2.8%). Noteworthy, 12.3% of the population was recognized as unidentified bacteria. The isolates were evaluated for their potential antimicrobial activities against multidrug resistant microbial strains. Two species of the genus *Streptomyces* exhibited a wide range of antimicrobial activities against multidrug resistance (MDR) strains of *Escherichia coli* and *Pseudomonas* spp. along with non-resistant strains of *Staphylococcus aureus* and *E. coli*. However, all of the antimicrobial activities were only observed when the isolates were grown at 8 °C in different media. To the best of our knowledge, this is the first study conducted on the Iron Curtain Cave's bacterial diversity, and reveals some bacterial isolates that have never been reported from a cave. Bacterial isolates identified with antimicrobial properties demonstrated that the Iron Curtain Cave can be further considered as a potential habitat for antimicrobial agents.

Keywords: cave characteristics; cave bacterial diversity; 16S rRNA gene; antimicrobial activities

1. Introduction

The study of cave microbiomes has been at the center of biologists' attention for the last few decades, both because of the microbial diversity in cave habitats and the potential for the production of unique primary and secondary metabolites, which differ from those found in other extreme habitats [1]. Numerous microbial diversity studies have been performed using the samples taken from the sediment deposits found on the walls and ceilings of caves, aquatic sediments, and on speleothem surfaces [2]. These cave microorganisms were thought to be introduced from the surface by air currents, sediments, water, animal, or human vectors, which was unusual for subsurface habitats [3]. However, the cave microbial composition varied between the types and the nature of caves [4–6].

The cave microbiomes contain a wide array of bacterial groups such as Pseudonocardinae, Chromatiales, Xanthomonadales, Acidobacteria, Actinobacteria, Cyanobacteria, Ktedonobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Etaproteobacteria [3,7–9] and fungi such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Microdiplodia*, *Cladosporium* spp., *Mucor* spp. [10–12]. However, the cultivation of these microorganisms in the laboratory environment was proven to be challenging, as the typical incubation temperature often ranged between 25–37 °C. Moreover, the rich formulated media typically used to culture chemoorganotrophs could not support the growth of these microbes that were believed to be oligotrophs [1,2]. Later on, the scientists realized that most of these microbes are adapted to nutrient-poor, starving environments where they scavenge to obtain food. Therefore, a number of cultivation media at normal and diluted strengths such as R2A, Hickey-Tresner agar, humic acid-vitamin agar, actinomycete agar; and a low incubation temperature (8–12 °C) were used [1,2].

Initially and up until 1995, cave microbiomes were identified based on their cellular morphology using microscopy and staining techniques [2]. The implementation of molecular techniques have overcome the problem of identification of these microbes at the genus and species level. Several cave microbial diversity studies included denaturing gradient gel electrophoresis finger printing and 16S rRNA phylogenetic analysis. For instance, a study by Vlikonja et al. in 2014 [3] from a Slovakian Karstic cave adopted a 16S rRNA gene profiling showing that *Streptomyces* (25%), *Micrococcus* (16%) and *Rhodococcus* (10%) were major constituents of the population, followed by *Pseudomonas* (9%), *Agrobacterium* (8%), *Lysobacter* (6%), while *Paenibacillus* (5%) were present as minor members. The cave microbiome studies have drawn considerable interest in finding new antimicrobials from the microorganisms that live in these extreme habitats [5,13]. These environments are good habitats for exploration and research, as they potentially contain unknown/less studied bacteria and have not been previously explored for new drugs [2].

Here, we report on the cultivation-based bacterial diversity of Iron Curtain Cave, which has never been studied before. Media plates were swabbed with wall samples from each site in the cave and incubated in situ with lids closed for nine months. The isolates were identified using molecular biology tools based on 16S rRNA gene profiling techniques and the molecular phylogenetic analysis. Moreover, the bacterial isolates were screened for their antimicrobial properties against multidrug-resistant bacteria. Our study demonstrated that the investigated cave not only housed less studied/potentially new bacterial taxa, it is also an important resource for the discovery of bioactive compounds.

2. Materials and Methods

2.1. Cave Description

The Iron Curtain Cave is located near Chilliwack, British Columbia on the north side of Chipmunk Ridge (Supplementary Figure S1). It is a pristine carbonate cave and was discovered by Rob Wall in 1993. The cave's survey was performed and the map was chiefly drawn by Trevor Moelaert in April 1999 (Figure 1). The cave has a unique environment with high iron content sediment and limestone structures throughout. While sediment contains iron that gives it a unique reddish coloration (Figure 2E), it also has a clay consistency that indicates higher levels of moisture. Notably, there are six pools of water present from the entrance and throughout the cave (Figure 1), making the environment within the cave humid. The temperature ranges between 4–12 °C depending on the time of year. The cave is decorated in different types of speleothem structures formed by secondary mineral deposits from dripping water within the cave. The three main types of speleothems present in the cave are soda straws: long, hollow, calcium carbonate cylinders (Figure 2A,C), bacon: large, wavy, calcium carbonate structures (Figure 2B,D) and popcorn structures present in the "ancient room" (Figure 2G). The main decoration in the Iron Curtain Cave is the large curtain made of calcium carbonate (Figure 2D), which is about 10 ft long and hangs about 30 ft above a deep pool of water. The cave has been exposed to a limited number of people as it is gated and locked, and any access to it needs prior permission from the

cave custodian, Rob Wall. The limited access to the cave are constrained to a specific path to preserve the features and habitat of the cave.

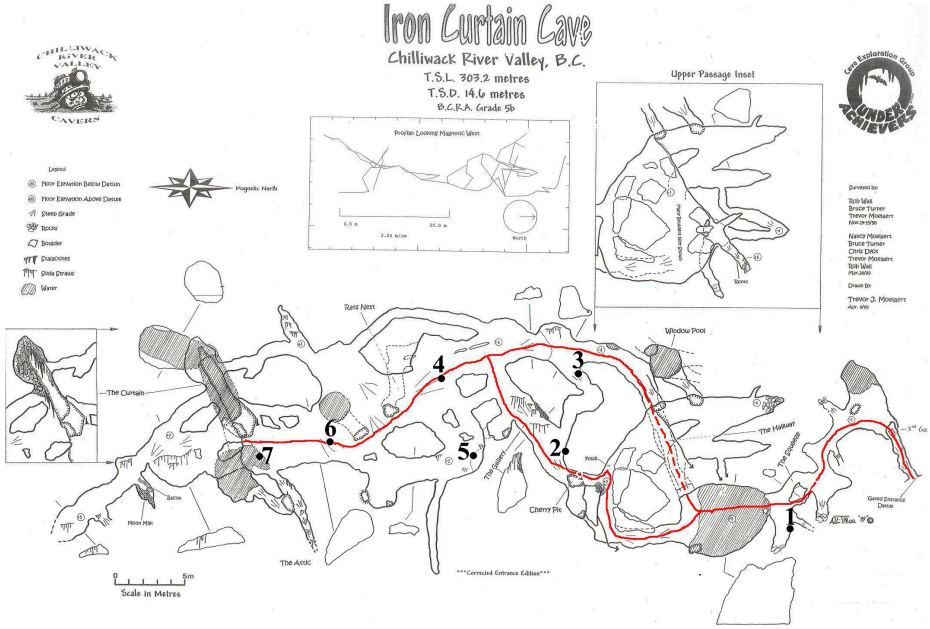


Figure 1. Map of Iron Curtain Cave in Chilliwack B.C. The numerals denote the locations of sampling points where media plates were left for nine months. #1 = Connection Room; near Squeeze dig around and Looking Pool, #2 = Upper section of The Gallery, near the climb to the Breakdown room, #3 = Entrance to side passage across from the Chocolate waterfall, #4 = Sitting area just beyond Lollipop Passage, #5 = Octopus Room, #6 = Mid passage to the Curtain, just past the Wishing Well, #7 = Chandelier Pit just below The Attic.

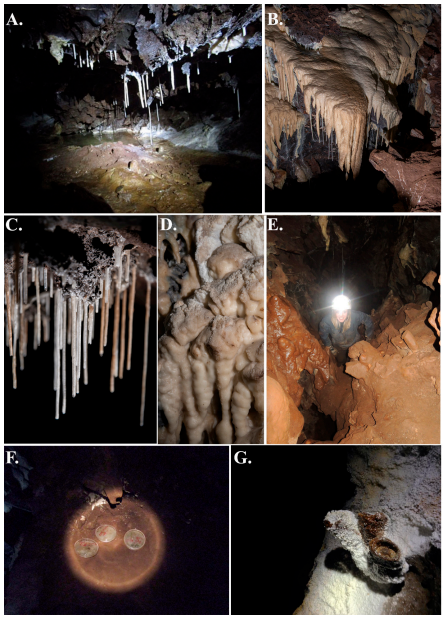


Figure 2. Iron Curtain Cave, Chilliwack, British Columbia, Canada. (**A**) Site #4: The Octopus Room; (**B**,**C**) Site #7: with two types of speleothem common to the cave; soda straws and bacon; (**D**) The Curtain made of calcium carbonate; (**E**) Soil sediment containing iron as well as some more geographical features unique to the cave; (**F**) The media plates are exposed for nine months in the cave; (**G**) The popcorn structures.

2.2. Sample Collection and Bacterial Isolation in the Cave

Seven different sampling points (1–7) were chosen arbitrarily in the cave in order to compare the microbiome diversities (Figure 1). At each site, the swabs of wall samples were inoculated on to the R2A (Teknova, Hollister, CA, USA) and Difco™ Actinomycete Isolation agar media (Thermo Fisher Scientific Inc., Waltham, MA, USA). These plates were then left with lids closed at these seven different locations in the cave (Figure 1) to mimic the original incubation cave temperature. The incubation temperature during these nine months ranged from 4–8 °C. After nine months from the day of inoculation, these plates were collected from the cave. Plates were transported to the laboratory aseptically for bacterial isolation.

The bacterial isolates from original media plates were cultivated on four types of culture media in order to obtain the best growth medium for each of these isolates: R2A, Actinomyces HiVeg™ (AHV) broth medium (HiMedia Laboratories Pvt. Ltd., Nashik, India), Hickey–Tresner (HT) (Yeast extract 0.1%, Beef extract 0.1%, N-Z Amine 0.2%, Dextrin 1%, pH 7.3) [5] and V8 original medium (200 mL/L; *v*/*v*) (Campbell Company, Toronto, ON, Canada)-$CaCO_3$ (3 g/L; *w*/*v*) (Thermo Fisher Scientific Inc., Waltham, MA, USA). Morphologically distinguishable colonies obtained from the exposed plates were streaked and incubated at 8 °C, for a period of 4–8 weeks, to obtain pure culture. The low incubation temperature was employed to mimic the cave temperature.

The pure culture obtained for each of the bacterial isolates were further inoculated in 3 mL volume of its respective (original plate) broth media (R2A, AHV, HT) and incubated for a period of 4–6 weeks at 8 °C, 15 °C and 25 °C for bacterial DNA extraction and antimicrobial activities assay. Three temperature conditions (8 °C, 15 °C, 25 °C) were employed in order to observe the best growth environments for these bacterial isolates and for their bioactive compound production.

2.3. Molecular Phylogeny

2.3.1. Genomic DNA Extraction and Sequencing

The broth culture of the bacterial isolates that exhibited maximum growth at the tested temperatures were used for genomic DNA extraction. The DNA extraction was performed as per the protocol followed in a previous study [14], with minor modifications at the cell lysis step. Along with the Proteinase K and Sodium dodecyl sulfate, lysozyme (10 mg/mL) was also added, followed by an incubation at 37 °C for 60 min. Following the extraction, the isolated genomic DNA was subject to polymerized chain reaction amplification of the 16S rRNA gene using 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primers [5] in a MyCycler™Thermalcycler (BIO-RAD, Mississauga, ON, Canada). A 50 µL PCR reaction mixture contained 100–200 ng of template DNA, 0.25 µM of each the primers, 1 µM deoxynucleotides, 1 mM $MgCl_2$, and 1 U of ExTaq™ Polyemarase (TakaRa Bio Inc., Olsu, Shiga, Japan) in ExTaq™ buffer (1X). The thermal cycling parameters were set with an initial denaturation at 94 °C for 2 min 30 s followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 40 s. The final extension was performed at 72 °C for 10 min. The PCR products were further resolved on an agarose gel (0.8%) containing ethidium bromide to confirm the DNA band profile of the bacterial isolates. The unpurified PCR products were sent for sequencing to Macrogen, Seoul, South Korea. The DNA sequences obtained were analyzed using the BLAST (Basic Local Alignment Search Tool) algorithm with the available sequences in the GenBank at National Center for Biotechnology Information (NCBI) http://www.ncbi.nih.nlm.nih.gov/genebank/index.html [15]. 16S rRNA gene sequences were identified with the >98% identity and >80% coverage to the closest homologue in the GenBank. GenBank accession numbers are given in Supplementary Table S1.

2.3.2. Sequence Alignment and Phylogenetic Analysis

Evolutionary analyses were conducted in MEGA6 [16]. The evolutionary history was inferred using the neighbor-joining method [17]. The optimal tree with the sum of branch length = 2.58482960 is

shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [18]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [19], and are in the units of the number of base substitutions per site. The analysis involved 65 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 744 positions in the final dataset.

2.4. Antimicrobial Activities Screening

The cave bacterial isolates were tested for their antimicrobial activities against the regular non-resistant and multidrug resistant (MDR) bacterial and normal yeast strains. The regular non-resistant *Escherichia coli* and *Staphylococcus aureus* were chosen, while MDR strains were *E. coli* (New Delhi strain)15-318, *E. coli* (NDM type carbapenemase)15-102, *E. coli* (oxa48 type carbapenemase)15-124 and methicillin-resistant *S. aureus* (MRSA)-43300. The normal bacterial strains of *Pseudomonas aeruginosa*, *Serratia marcescens* and the yeast strain *Candida albicans* were also included as test microbes in this study. The MDR *E. coli* 15-102, 15-124, 15-318 strains were provided by LifeLabs, Canada while non-resistant *E. coli* and *Candida albicans* were available at the Thompson Rivers University culture collections. *S. marcescens* and *S. aureus* MRSA-43300 were kindly donated by Dr. Julian Davies, University of British Columbia, Canada, and Royal Inland Hospital, Canada. All the test bacteria and yeast isolated colonies were inoculated in 3 mL nutrient broth (Criterion™ Dehydrated Culture Media, Hardy Diagnostics, CA, USA) and potato dextrose broth (Hi Media Laboratories Pvt. Ltd., Mumbai, India), respectively, with shaking at 37 °C and 25 °C on test tube rotator overnight.

The antimicrobial activities screening was performed by seeded agar method on nutrient agar, and potato dextrose agar for bacteria and yeast, respectively. All the test microorganisms were inoculated at an concentration of 10^6 cfu/mL in 250 mL of the media, mixed gently by shaking and poured in Nunc® Bioassay Dish (245 mm × 245 mm × 25 mm) (Cole-Parmer Scientific Experts, Montreal, QC, Canada). Five microliters of the previously grown broth culture of cave bacterial isolates were spotted on the solidified agar plate. Peroxigard (1.5% *v*/*v*) (Bayer, Toronto, ON, Canada), bleach 10% (*w*/*v*) (London Drugs, Richmond, BC, Canada) and ampicillin disks (10 µg) (In Vitro Diagnostic (IND Diagnostic Inc.), Delta, BC, Canada) were used as positive controls whereas uninoculated R2A broth medium was the negative control. All the plates were incubated at 15 °C in the upright position till the well-developed consistent lawn of the test microbe was observed. The antimicrobial activities was determined as the zone of inhibition around each bacterial colony. The diameter of the zone of the inhibition was measured manually with electronic Vernier calliper (Guangxi China, Mainland).

In order to examine which culture medium supports the antimicrobial activities maximally, the positive candidates that exhibited antimicrobial activities after screening were further grown in four different fermentation media: R2A, V8, HT, and Actinomyces HiVeg™ broth, incubated at 8 °C and re-spotted on the agar seeded test microorganisms media plate. The plates were incubated at 15 °C to observe the antimicrobial activities.

2.5. Scanning Electron Microscopy (SEM) of Bacterial Isolates with Antimicrobial Properties

The bacterial samples were grown on the agar media plate, and one colony from each plate were excised. The excised samples were osmium fumed in an airtight container with 4% OsO_4 (aq) mixed with an equal volume of acetone for approximately 30 min. Samples were post-fixed in 8% glutaraldehyde +1% tannic acid, under vacuum, using a microwave protocol (Pelco 3431 Laboratory Microwave, Redding, CA, USA). Following post-fixation, the samples were washed and taken through a staged ethanol dehydration, accelerated by microwave. The samples were kept at 70% ethanol overnight at 4 °C, and the staged dehydration was completed the following day. Critical point drying was accomplished using a Tousimis Auto-Sam Dri 815B with one 10 min purge and one 5 min purge separated with a standing time in liquid carbon dioxide for 30 min, followed by an overnight stasis and one last 5 min purge before heating the

sample to the critical point. The samples were mounted to aluminum scanning electron microscopy (SEM) stubs using double-sided, carbon conductive tabs and sputter coated with 8 nm PtPd (Cressington 208HR) and imaged on either Hitachi S 2600 VP-SEM or Hitachi S4700 cold-FESEM.

3. Results

The present study elucidated the preliminary cultivable bacterial community profile of the Iron Curtain Cave, Chilliwack, BC, Canada employing the cultivation based 16S rRNA profiling approach. Furthermore, the study also evaluated the antimicrobial properties of the bacterial isolates against the microbial drug resistant strains and pathogens.

3.1. Sampling Location in the Cave

The distances of each of the seven sites from the entrance of the cave were measured and given in the Figure 3.

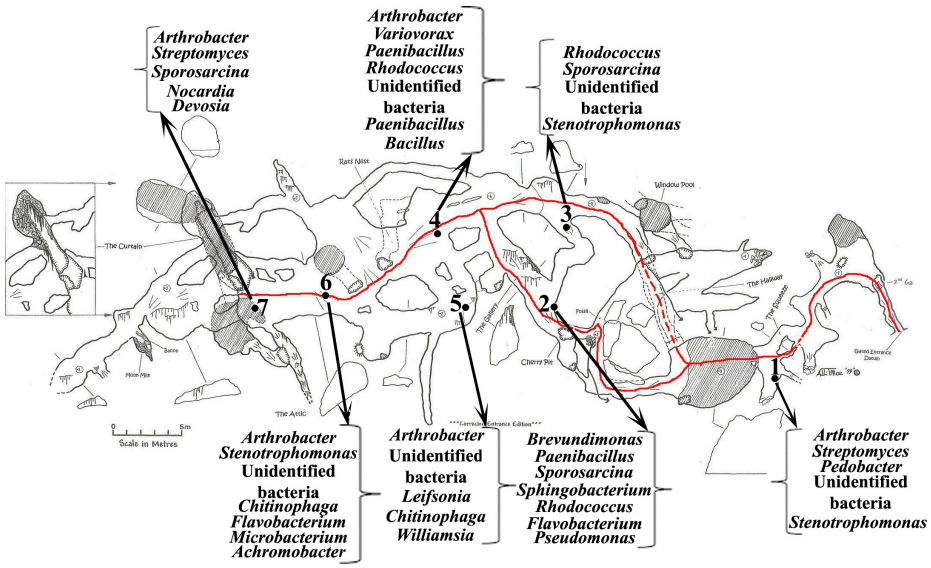
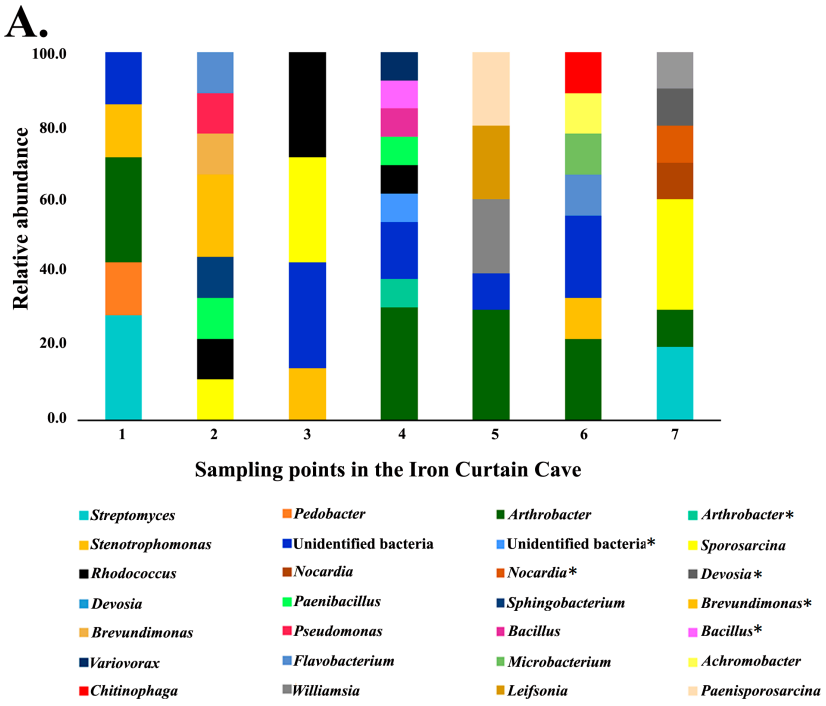


Figure 3. Distribution of bacterial isolates at different sampling points in Iron Curtain Cave, Chilliwack, BC, Canada.

3.2. Bacterial Community Composition

The bacterial isolates were subcultured on their respective culture media, R2A, Actinomyces HiVeg™ broth, HT, and V8 media both at 8 °C and 15 °C, while no growth were observed at 25 °C for most of the bacterial isolates.

Sixty five bacterial isolates were cultivated, followed by genomic DNA extraction, which were further subjected for the 16S rRNA gene amplification. The PCR product revealed amplicons ranging from 1300–1500 bp. The nucleotide sequencing of these amplicons categorized bacterial isolates into four major phyla, including Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes (Figure 4). Further investigations revealed genera of *Arthrobacter* (21.53%) as the major genus, followed by *Stenotrophomonas* (9.23%), *Sporosarcina* (9.23%), *Streptomyces* (6.15%), and *Brevundimonas* (4.61%). Species of *Rhodococcus*, *Bacillus*, *Paenosporosarcina*, *Paenibacillus*, *Williamsia*, *Leifsonia*, *Nocardia*, *Devosia* and *Flavobacterium* were identified as 3.07%, while the members of genera *Pedobacter*, *Sphingobacterium*, *Chitinophaga*, *Pseudomonas*, *Microbacterium*, *Achromobacter* and *Variovorax* accounted for the minor (1.53%) population of the cultivable diversity. *Crocebacterium* exhibited 2.8% of the bacterial population identified, and 12.30% were identified as unidentified bacterium. Notably, 26.15% of the bacterial isolates exhibited $\leq$98% when compared with available with 16S rRNA sequences in GenBank (Supplementary Tables S1 and S2).



B.

Percentage of isolates present in the cave	
Arthrobacter	21.53%
Unidentified	12.30%
Stenotrophomonas *Sporosarcina*	9.23%
Streptomyces	6.15%
Brevundimonas	4.61%
Rhodococcus *Nocardia* *Devosia* *Paenisporosarcina* *Flavobacterium* *Paenibacillus* *Bacillus* *Williamsia* *Leifsonia*	3.07%
Crocebacterium	2.81%
Variovorax *Chitinophaga* *Pseudomonas* *Sphingobacterium* *Pedobacter* *Microbacterium* *Achromobacter* *xylosoxidans*	1.53%

Figure 4. (**A**) Taxonomical distribution of the bacterial isolates from Iron Curtain Cave by sampling sites. The vertical rectangular stalked columns represent the relative bacterial abundance. The asterisk (*) denotes the percentage of the bacteria that are not assigned with the gene accession numbers. (**B**) Percentage of total isolates from all sampling sites.

The *Arthrobacter* spp. and the unidentified bacterium were widely distributed in most of the sampling points, including points numbered 1, 4, 5, 6, 7 and 1, 3, 4, 5, 6 respectively (Figure 4A,B). The species *Stenotrophomonas*, *Sporosarcina* and *Rhodococcus* were identified only at three points, while *Streptomyces*, *Paenibacillus* and *Flavobacterium* were found at two points only. The rest of the bacteria identified were minimally distributed and found at one spot only (Figure 4A,B).

3.3. Phylogenetic Analysis

The evolutionary relatedness of these bacterial isolates depicted in the dendrogram demonstrated *Actinobacteria* (44.61%) were the major phylum, followed by *Proteobacteria* (27.69%) and Firmicutes (20%), and the least common phylum were Bacteroidetes (7.69%). *Proteobacteria* and Firmicutes were identified as 27.69% and 20% respectively. Among the *Proteobacteria*, 55.55% were recognized in the class of Gammaproteobacteria, 27.77% as Alphaproteobacteria and 16.66% as Betaproteobacteria.

The unknown bacterial isolates fall in clades of *Arthrobacter* spp. (Phylum Actinobacteria), *Stenotrophomonas* spp. (Phylum: Gammaproteobacteria) and *Paenibacillus* spp. (Phylum Firmicutes) (Figure 5). Notably, both the antimicrobial bacterial isolates (ICC1 and ICC4) may be identified under the phylum Actinobacteria; however, with ICC1's sequence having low similarity (73%) to the *Streptomyces nojiriensis* (Table S2), it is hence referred to as an unidentified bacterium.

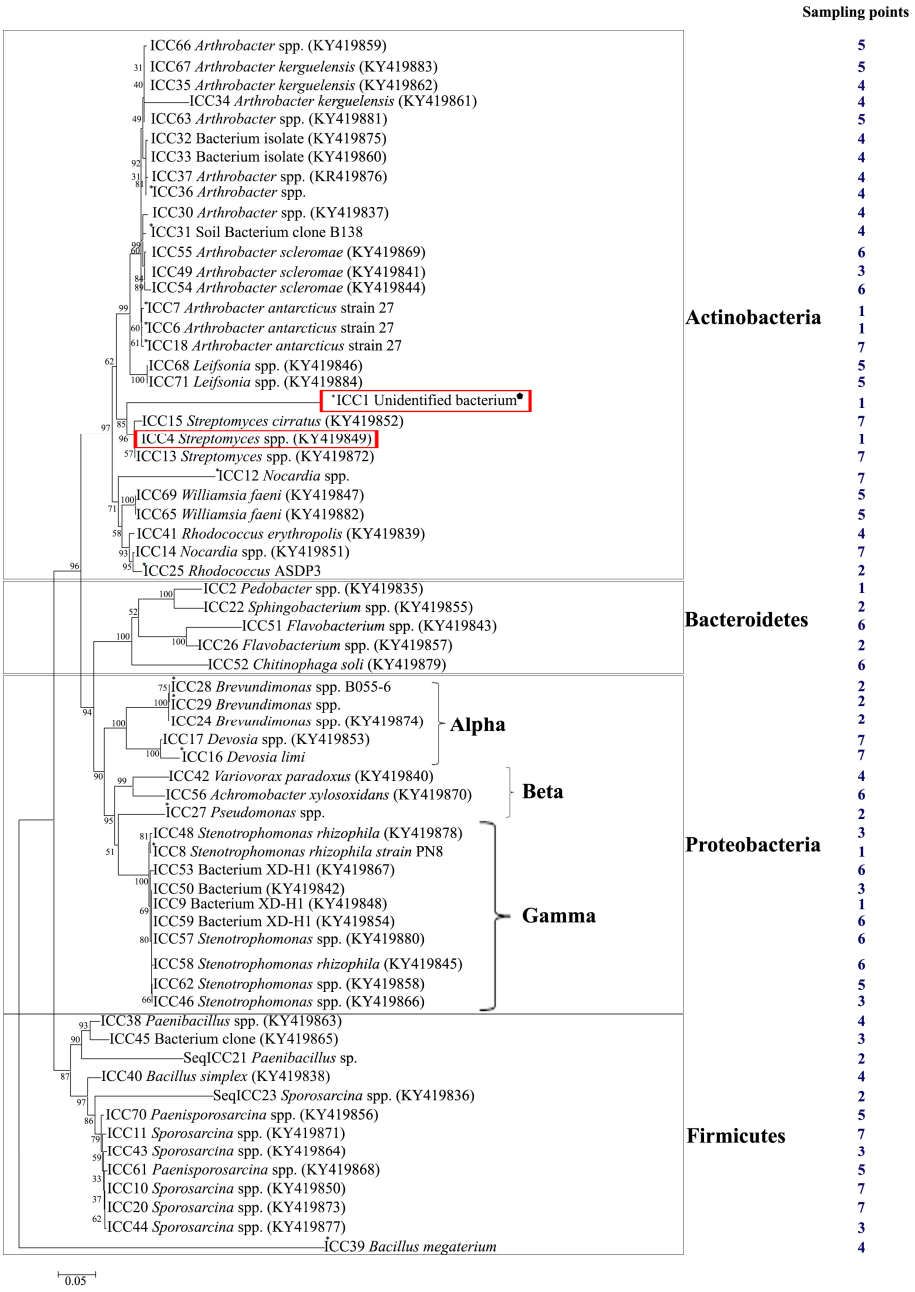


Figure 5. Evolutionary relationships of taxa. The 16S rRNA gene sequences obtained from the NCBI gene database were aligned by MUSCLE with default parameters. The phylogenetic tree was constructed using MEGA6 by neighbor-joining method, with a bootstrap test of 1000 replicates. The asterisk (*) denotes the sequences that were not assigned with a GenBank accession number. The red boxes represents the bacterial isolates that exhibited antimicrobial properties. The polygonal (⬣) represents the ICC1 having an identity of 73% to *Streptomyces nojiriensis*.

3.4. Antimicrobial Activities of the Cave Bacteria

Two bacterial isolates exhibited antimicrobial activities. Surprisingly, these activities were only observed when the bacteria were grown in V8 and HT media at 8 °C. While the ICC4 (closest relative: *Streptomyces* spp., 99% identity) isolate exhibited a wide range of antimicrobial activities against regular non-resistant strains of *E. coli*, *S. aureus* and MDR strains of *E. coli* 15-318, *E. coli* 15-102, *E. coli* 15-124 and *P. aeruginosa*, the ICC1 showed activity against *E. coli* 15-102 and *E. coli* 15-124 only (Table 1). ICC1 was not deposited to GenBank (Supplementary Table S2) because of its low identity (73%), although it showed 95% coverage. However, the closest relative was identified to be *Streptomyces nojiriensis*.

Table 1. Antimicrobial activities. ICC4 (Closest relative: *Streptomyces* spp.) and ICC1 (Unidentified bacterium). The numerical in the boxes indicated the diameter of zones of inhibition in mm. The antimicrobial activities were observed at 8 °C. The symbol (-) denotes 'no antimicrobial activities'. No antimicrobial activity was observed in all cave isolates tested against *Serratia marcescens* or *Candida albicans*.

Bacterial Isolates	Growth Media	Test Microorganisms						
		E. coli	***E. coli* 15-318**	***E. coli* 15-102**	***E. coli* 15-124**	***Staphylococcus aureus***	**MRSA-43300**	***Pseudomonas aeruginosa***
ICC1	V8	-	-	-	-	-	-	-
	R2A	-	-	14	8	-	-	-
	AHV	-	-	-	-	-	-	-
	HT	-	-	-	-	-	-	-
ICC4	V8	13	17	21	8	13	-	7
	R2A	-	-	-	-	-	-	-
	AHV	-	-	-	-	-	-	-
	HT	24	-	-	-	12	-	-

3.5. Scanning Electron Micrographs

The SEM images of the ICC1 and ICC4 isolates revealed the presence of possible actinobacterial-like structures. To confirm this observation, 16S rRNA sequencing was performed to obtain the isolates' identity. Morphological characteristics observed for these two isolates were found to coincide with *Streptomyces*'s common characters. These two isolates were rod-shaped in long interwoven filaments, with a diameter of about 0.3 to 0.5 µm (Figure 6).

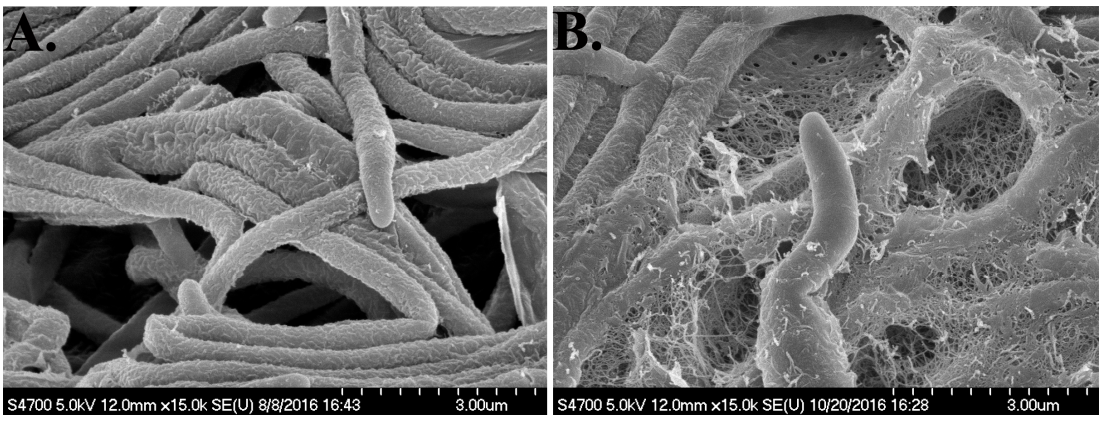


Figure 6. SEM images of actinobacterial-like structures from Iron Curtain Cave samples. (**A**) ICC1: Dense mass of rod-shaped and interwoven filaments with irregular rugose surface, Scale bars, 5 µm; (**B**) ICC4: Mass of hyphae with smoother surface, Scale bars, 3 µm.

4. Discussion

Our study of cultivation-/PCR-based microbial diversities, and evaluation of their antimicrobial properties, were performed for the first time from the Iron Curtain Cave, Chilliwack, Canada. The earlier microbial ecology studies conducted on these caves involved identifying the presence of bacteria on soda straw samples through isolation followed by SEM [20]. However, those studies essentially lacked the purification, molecular identification, and more importantly, antimicrobial activities screening of the purified bacterial isolates. Our investigations, though preliminary, encompassed culturable in situ microbial diversity and their potential in antimicrobial activity production. The study detected four phyla of the bacterial population with Actinobacteria as the major phylum, followed by Proteobacteria, Firmicutes and Bacteroidetes (Figure 5).

The dominance of Actinobacteria (44.61%) in our study is in accordance with previous microbial ecology studies of limestone caves where Actinobacteria was identified as the predominant bacterial population [21,22]. Our study showed that among the Actinobacterial phylum, *Arthrobacter* spp. occupied the vast majority (48.27%), which could not be identified in earlier studies [13,23]. About 10.10% of the Actinobacterial phylum contained unidentified bacterium that clustered together with *Arthrobacter* (Figure 5). Our findings suggested *Streptomyces* spp. as the second largest class (13.79%) in the Actinobacterial phylum. However, most of the previous microbial diversity studies both from Karstic and volcanic caves identified *Streptomyces* as the major bacterial class [22,24,25]. *Rhodococcus* spp. (6.89%) and *Nocardia* spp. (3.44%) were also recognized in our study. A study from Riquelme et al. 2017 has reported the presence of these genera in the Canadian and Azorean volcanic cave [24]. However, a study from Jurado et al. 2010 designated both *Rhodococcus* spp. and *Nocardia* spp. as opportunistic and possibly pathogenic cave bacteria [26]. Furthermore, our investigation has demonstrated the presence of *Leifsonia* spp. (6.89%) and *Williamsia* spp. (6.89%), which had never been reported in any previous studies of a cave environment. However, studies have reported *Leifsonia* spp. from different environments, such as teak rhizosphere soil [27], Japanese lichen [28] and ginseng root [29]. Similarly, *Williamsia* was also demonstrated from an oil-contaminated soil [30] and human blood [31].

Proteobacteria are the second most abundant phylum observed in our investigations. Earlier, a similar study from Blowing Spring Cave (Lauderdale County, Alabama) reported Gammaproteobacteria (23%) as the most prevalent group, followed by Alphaproteobacteria (19%) and Betaproteobacteria (1%) [32]. Among the Gammaproteobacteria, *Stenotrophomonas* spp. was only observed in the phylum. Four unidentified bacteria were also observed to be clustered in the same clade of *Stenotrophomonas* spp. (Figure 5). One of the earlier studies has isolated *Stenotrophomonas* from the Karstic Herrenberg Cave, Germany and identified it to be involved in calcite biomineralization [23]. Among the Alphaproteobacteria phyla, our study has observed *Brevundimonas* spp. as the major class (60%) along with *Devosia* spp. (40%). In correlation to our findings, a study by Busquets et al. in 2014 reported two strains and two species of *Brevundimonas* from the alkaline waters of the cave pools at the Cova des Pas de Vallgornera (Mallorca, Western Mediterranean, Spain) [33], while another study by Shabarova et al. in 2010 reported *Devosia* spp. from the water sample collected from three pools of the Bärenschacht cave system (Switzerland) [34].

Among the Betaproteobacteria phylum, three different species of bacteria were identified: *Variovorax*, *Achromobacter* and *Pseudomonas* (1:1:1). The presence of *Variovorax* in our findings could be correlated to an earlier microbial diversities study from Lechuguilla and Spider Caves (Carlsbad Caverns National Park, in Eddy County in Southeastern New Mexico, USA), where two bacterial clones identified as the nearest homologue to *Variovorax* [35]. Numerous studies reported *Pseudomonas* as the prevalent species in the cave environment. For instance, eight strains and five species of *Pseudomonas* were reported to play roles in the regulation of the biogeochemistry from the Majorcan Caves [33]. Similarly, another bacterial diversity study from the bacterial biofilms explored from five caves from the Meghalaya in India, and identified *Pseudomonas* spp. as the major genus [36]. We found the presence of *Achromobacter* spp., although other studies have rarely identified

it. For instance, a previous study on the Lascaux Cave (Vezere Valley, Montignac, France) detected 3.6% of bacterial clones as *Achromobacter xylosoxidans* [37].

Firmicutes were the third abundant phylum detected in our study. Although most of the other studies identified *Sporosarcina* as rare Firmicutes in the cave environment [38], our study identified 46.15% of *Sporosarcina*. In agreement with our study, a study by Kim et al. [38] has revealed two bacterial isolates, *Sporosarcina globispora* and *Sporosarcina* spp., from limestone cave soil samples that play crucial roles in calcite precipitation in the cave soil. *Bacillus* (15.38%) has also been observed as one of the genus in our study. An earlier study, both by cultivation-dependent and -independent approaches, identified *Bacillus* as a common microbe [39]. Surprisingly, both of the *Bacillus* spp. (ICC40 and ICC39) identified in our study have been found to be apart from each other on the phylogenetic tree (Figure 5). However, a study reported that the *Bacillus* species is an incoherent taxon that lacks a common evolutionary history [40]. Our study has identified two isolates of *Paenibacillus* spp. (occupying 15.38% of the total Firmicutes population). Furthermore, one of the unidentified bacteria also falls in the clade of the *Paenibacillus* spp. (Figure 5). Previously, the *Paenibacillus cavernae* spp. was reported to be isolated from soil in one of the natural caves in Jeju, Republic of Korea [41]. Moreover, *Paenibacillus* spp. was well known to possess antibiotic resistome on their genome. For instance, Pawlowski et al. [42] has identified a *Paenibacillus* spp. LC231 from Lechuguilla Cave and after a detailed whole-genome sequencing, functional genomics, and biochemical analysis, it has exhibited resistance to a wide array of antibiotics. Interestingly, 15.38% of *Paenisporosarcina* spp. were detected in our study; this species was never been reported before from any caves studied. However, studies have showed the identification of this genus from other habitats, such as glacier water (*Paenisporosarcina indica* sp. nov.) [43] and a landfill site (*Paenisporosarcina quisquiliarum* sp. nov.) [44].

Bacteroidetes were the least abundant phylum identified in the Iron Curtain Cave. The Bacteroidetes contained the genera that exhibited close homology to *Pedobacter* spp., *Sphingobacterium* spp., *Flavobacterium* spp. and *Chitinophaga* spp. (Figure 5). In relation to our study, *Flavobacterium* spp. was previously reported in abundance in Ferromanganese deposits from the caves of the Upper Tennessee River Basin, along with other bacteria indicating that this bacterium contributed Mn (II) oxidation [45]. The presence of this bacterium in the Iron Curtain Cave indicates that it might potentially participate in the iron oxidation of the cave, and impart a reddish coloration to the soil sediment (Figure 2E). Similarly, in 2013 Engel et al. identified both *Sphingobacterium* and *Flavobacterium* in the calcium carbonate speleothem samples from two Italian caves and both clustered in the same clade [46]. *Chitinophaga* spp. was also observed in our study, which was never reported before from a cave. However, other studies reported *Chitinophaga* spp. from other sources, such as *C. qingshengii* sp. nov. isolated from weathered rocks [47] and *C. ginsengihumi* sp. nov. from the soil of ginseng rhizosphere [48]. In our study, the microbial diversity study specifically revealed that the sampling points differed in their microbial content, although overlap of the microbial populations was observed between many of the sampling points (Figures 3 and 4). This varied microbial content observed among the seven points is an important revelation implying that each of the sampling point could be a potential microhabitat within a single cave environment. However, the bacterial taxonomic profile did not reveal a comprehensive pattern identified at each of the sampling points, and therefore further studies needed to be conducted.

The antimicrobial activity screening of the identified bacterial isolates (ICC1 and ICC4) detected two homologues of *Streptomyces* spp. that possess antagonistic properties against regular and MDR bacterial strains of *E. coli*. Although the ICC1 has exhibited a very low homology of 73% to *Streptomyces* spp., SEM images of the both the isolates displayed Actinobacterial-like structures (Figure 6). Both SEM images showed typical aerial hyphae that are densely packed without spores being observed. ICC1 showed interwoven filaments with an irregular rugose surface, while ICC4's hyphae was observed with a rather smoother surface. The antimicrobial activities of these bacteria could be correlated to a previous study on Turkish Karstic caves, where *Streptomyces* spp. strain number 1492 (*Actinomycetes* bacteria) displayed antagonism against *S. aureus* (MRSA), vancomycin resistant *Enterobacter faecium*

(VRE), and *Acinetobacter baumannii* [49]. A similar study from the Helmcken Cave in Wells Gray Provincial Park, BC (volcanic caves) identified 400 bacterial isolates, where a preliminary screening of these bacteria for antimicrobial activities identified 26.50% against *K. pneumoniae*, 10.25% showed inhibitory activity against *M. luteus*, 9.25% against *M. smegmatis*, 7.5% against *C. albicans*, 6.25% *P. aeruginosa*, 2.25% against *A. baumannii*, 2% against *S. aureus*, 1.75% against *E. coli* and 1% against ESBL *E. coli* [5]. In alignment to our findings, a recent review article [1] indicated that cave microbiomes has recently been considered as potential resources for novel drugs and antibiotics. Notably, both ICC1 and ICC4 isolates were from sampling point one in the cave. We intended to use quantitative analysis and comparison of the cultured bacterial diversity among the seven sites to see whether there is any connection between site(s), its microbial habitants, and antimicrobial activity potential. However, we think more detailed studies need to be conducted before we can conclude on such relationships.

Our study has also identified that the antimicrobial activities of ICC1 and ICC4 were fermentation-media specific (Table 1), which was never being elucidated before from the cave environment. However, other studies demonstrated antimicrobial activities of microbial isolates in a fermentation-medium dependent manner. For instance, a study reported that *Ellisidothis inquinans* L1588-A8 exhibited antifungal activities against *Saccharomyces cerevisiae* strain EC19 [50]. *E. inquinans* L1588-A8 antimicrobial activities were only observed when it was incubated in media A and No.2 and not in F1A and MW [50]. However, our study has identified the two bacterial isolates (ICC1 and ICC4) possessing antimicrobial activities, and hinted that the antimicrobial components could be a possible proteinaceous compound (unpublished data). Further investigations, such as whole genome sequencing, functional genomics, biochemical assays, fermentation structure elucidation, active component extraction and mode of action need to be performed in order to fully characterize these antimicrobial activities.

5. Conclusions

Our present study is the first report attempting to shed light on microbial diversity and their potential in antimicrobial activity on in situ cultured bacterial diversity. This study is by no means a whole picture of this habitat; however, we believe that this finding can still contribute a small piece of the puzzle of the big picture of cave microbial diversity. Not surprisingly, cultivation-based approaches have certain limitations; nevertheless, our study has documented the Iron Curtain Cave as a rich reservoir for potentially unique metabolites. Therefore, future studies should embark on the metagenomic exploration, which will be a holistic approach for the taxonomical and functional profiling (bioprospecting potential molecules such as enzymes/and of antibiotics of industrial and pharmaceutical relevance) of the Iron Curtain Cave. Additionally, the potential microbial and mineral interactions and the cave population dynamics could also be elucidated.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/9/3/36/s1, Figure S1: The area shaded with red lines shows the Chipmunk Ridge. The Iron Curtain Cave is located on the north of this ridge, Table S1: Bacterial isolates identified to their closest homologue, with their GenBank accession number assigned, from different locations in the Iron Curtain Cave, Chilliwack, BC, Table S2: Bacterial isolates that have not been deposited to GenBank hence without accession numbers. These are identified to their closest homologue. The asterisk (*) denotes the bacteria that show identity below 90%.

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Author Contributions: Naowarat Cheeptham obtained funding, conceived the study and designed the experiment. Soumya Ghosh, Elise Paine, Gabrielle Kam, Tanna Lauriente and Pet-Chompoo Sa-ngarmangkang conducted all the experiments under the close guidance and supervision of Naowarat Cheeptham. Soumya Ghosh analyzed the data and drafted the manuscript. Elise Paine, Rob Wall and Naowarat Cheeptham collected all

samples from the caves. Derrick Horne performed the SEM of the microbial samples. Naowarat Cheeptham and Soumya Ghosh read and edited the manuscript.

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