Bacteria Isolated from Canada's White Rabbit Cave Revealed Antimicrobial Activities

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Abstract

Caves offer a unique habitat for microorganisms, which allow the adaptation of exclusive metabolic pathways to the resources available. This environment could enable the production of primary and secondary metabolites with unique antimicrobial or enzymatic properties. White Rabbit Cave is located in the Monashee Mountain range in south-central British Columbia, a metamorphic range not known for cave and karst development. The present study has recovered bacterial isolates from the White Rabbit Cave and assessed them for their antimicrobial properties by employing the agar plug assay. One hundred and six bacterial isolates were cultivated from the collected samples, among which, five bacterial isolates displayed antimicrobial properties against a methicillin-resistant *Staphylococcus aureus* (MRSA) strain. Furthermore, these five isolates were identified with 16S rRNA gene sequencing and through phylogenetic analysis. It has been observed that three of them (B076, B053, and B079) were identified as *Streptomyces spp*. (Phylum Actinobacteria) while the other two were recognized as *Paenibacillus spp*. (B039) and *Paenibacillus terrae* (B016) (Phylum Firmicutes). To the best of our knowledge, this is the first study that identifies bacterial species with antimicrobial properties from White Rabbit Cave in the Monashee Mountain range in British Columbia, Canada.

Introduction

Caves are unique resources that researchers have recently honed in on. They offer a biologically, chemically, physically, and geologically exceptional habitat for microorganisms to adapt, allowing them to possess unique properties (1-3). Generally, caves have four distinct zones: the entrance where the surface and the underground meet; the twilight zone where there is little light and plants are unable to grow; the transition zone where there is no light but, environmental conditions like temperature are still sensed; and the deep zone where there is no light, it is very humid, and the temperature is constant (1). In the subsurface areas of caves, specifically in the transition zone and in the deep zone, there is considerable influence from specific rock surfaces (1, 4). Each distinctive zone within a cave influences microhabitats for microbial communities. These microhabitats are dictated by microbial metabolic needs, abilities, tolerances, and rock chemistry (4). The twilight and transition zones also offer very constant environments (constant humidity and temperature, and the absence of light), allowing certain adaptable microbes to thrive here with few perturbations necessitating the microbial communities are relatively stable (1, 4). Some cave characteristics, including high humidity, little to no light, and little airflow, make energy production challenging for typical microorganisms Other characteristics such as low concentrations of organic nutrients, high concentrations of minerals, seasonally dependent air currents, air pressure changes to equalize with surface air pressure and water level changes, are also included for

such influence (1-3). These conditions and unusual resources provide a unique niche where most microorganisms are unable to survive (3); however, certain adaptable microbes are able to thrive because of their distinctive physiologies and their abilities to exploit cave environments (4). The cave environment causes microorganisms to adapt their metabolic pathways to the resources available, which could permit the production of primary and secondary metabolites that have antimicrobial properties (1-4). For example, *Streptomyces tendae*, strain HKI 0179 that has been isolated from a cave in Grotta dei Cervi, Italy was found to produce the antibacterial peptide cervimycins A-D effective against methicillin resistant *Staphylococcus aureus* (MRSA) (5). In another study, ATHUCY 3314, a strain of *Taxopsis calypsus*, found in Francthi Cave, Greece has been observed to produce an antibacterial lipid against MRSA, methicillin susceptible Staphylococcus aureus, vancomycin resistant enterococci (VRE) faecalis, VRE faecium and vancomycin susceptible *Enterococcus faecalis* (6).

White Rabbit Cave is situated in the Monashee Mountain range in south-central British Columbia, a metamorphic range not known for cave and karst development. This is an area of high annual precipitation, frequently referred to as the interior rainforest, occurring within the Interior Cedar Hemlock and Engelmann Spruce Subalpine biogeoclimatic zones. Named after the white marble in which it is formed, White Rabbit is one of the few known marble caves in Western Canada. The cave is formed in a narrow band of marble in the Mt. Grace Formation of the Shuswap Metamorphic Complex (7)

In this study, we would like to understand whether there are some bacteria that live in this Canadian White Rabbit Cave who can produce antimicrobial activity against some chosen multidrug resistant bacteria in the hope to explore and discovery more potential new antibiotic sources. Here, we describe the isolation of the White Rabbit Cave bacteria and screen them for antimicrobial activity against the MDR and non-MDR bacterial strains using the agar well diffusion assay. The identification of the positive bacteria with capacities to produce antimicrobial activities were revealed by using the 16S rRNA gene profiling.

Materials and Methods

Sample collection sites and cave measurements

The entrance of the White Rabbit Cave is in a polje (an extensive depression having a flat floor and steep walls) over 300 m in diameter at its base, 500 m wide at the upper edge and ranges from 60 to 250 m deep. It is among the largest surface karst features in the country. A stream flows year-round into the cave, and the suspected resurgence is over 2 km away as the crow flies and 400 m elevation below the main entrance. The first known record of the cave entrance is in a geological survey report from the late 1970's, where the entrance and resurgence are identified on maps (8). First explored and surveyed in 2009, it now stands at 4213 m surveyed length and 398 m deep as of September 2015, currently the sixth deepest cave in Canada with many leads still to be explored (Figure 1).

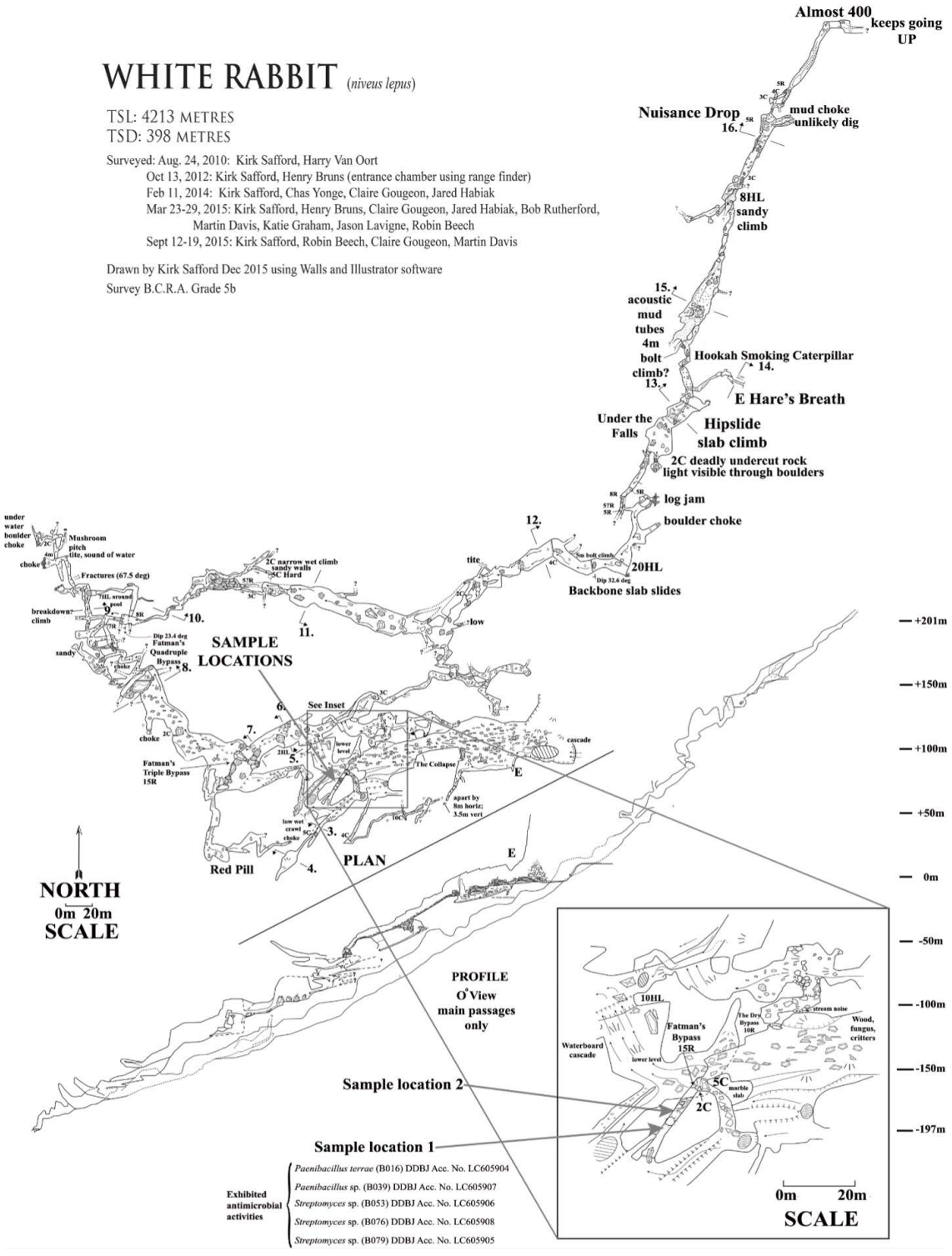

Figure 1. Map of White Rabbit Cave in the Monashee Mountain range in South-Central BC, Canada. Sample collection points are denoted as Sample location 1 and Sample location 2, which are indicated in fossil vadose passage. Samples from location 1 consisted of sediment and calcite and samples from location 2 consisted of calcite only. Distribution of bacterial isolates showing antimicrobial activities cultured from Sample location 1 is also presented here.

Sample collection and isolation of bacteria

The samples collected from various locations in White Rabbit Cave consisted of sediments, calcite, and calcite mineralized with copper (Figure 2). Sediments were collected from location 1 (Figure 2B), which is in fossil (i.e. inactive) vadose passage (Figure 2A), consisting of undisturbed silts and sands on a ledge 1.5 m above the floor. Calcite was collected at both locations 1 and 2, the latter being 10 m above location 1 in the fossil vadose passage in the same rift. Furthermore, the samples are categorized as: 1 and 2 containing sandy sediments; 3 and 4 containing. small pieces of a blue-green mineral formation; and 5 and 6 consisting of white minerals.

One hundred microliters of decimally diluted (10-1, 10-2, 10-3) samples were pour plated on Hickey- Tresner (HT) (Yeast extract 0.1%, Beef extract 0.1%, N-Z Amine 0.2%, Dextrin 1%, pH 7.3), R2A (Teknova, Hollister, CA, USA), Difco™ Actinomycete Isolation agar media (Thermo Fisher Scientific Inc., Waltham, MA, USA), and Soil agar media plates in order to isolate the bacteria as per previous studies (2, 3). The plates were incubated at 12.5°C for 15 days. Morphologically distinguishable colonies were selected and re-streaked for pure cultures.

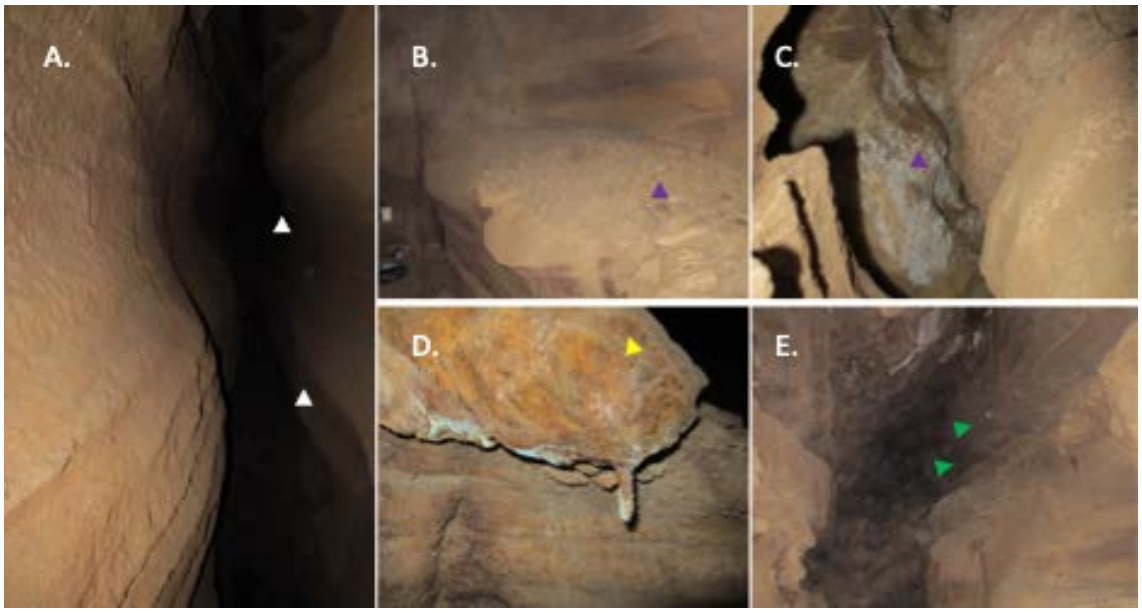

Figure 2. Location of sample collection in the White Rabbit Cave in the Monashee Mountain range in South-Central BC, Canada. A. Fossil vadose passage (white arrow heads). B. Sample location 1 for sediment sample collection. C. Calcite popcorn structures. D. Calcite mineralization. E. Entire fossil passage (green arrow heads). The purple and the yellow arrow heads correspond to the sediments and yellow microbial mats respectively.

Antimicrobial activity screening

The cave bacterial isolates were subjected to antimicrobial activities assay against the non-resistant strain of *E. coli* (*E. coli*-33) and MDR strains such as methicillin-resistant *Staphylococcus aureus* (MRSA)-43300, *E. coli* (NDM type carbapenemase) 15-102, *E. coli* (oxa48 type carbapenemase) 15-124, and *E. coli* (New Delhi Strain) 15-318. The non-resistant bacterial strain *Serratia marcescens* and yeast strain *Candida albicans* were also included in the antimicrobial assays. All the strains were procured from our previous studies (2). The tested bacterial strains were inoculated in 3 mL of the nutrient broth (Criterion™Dehydrated CultureMedia, Hardy Diagnostics, CA, USA) and incubated overnight on a shaking test tube rotator overnight at 37°C. The antimicrobial activities assay was conducted employing seeded agar method. All the tested bacterial strains were inoculated at a concentration of 106 cfu/mL in 250 mL molten agar media with a gentle shaking and poured in a Nunc® Bioassay Dish (245 mm x 245 mm x 25 mm) (Cole-Parmer Scientific Experts, Montreal, QC, Canada). The Agar plug assay for the antimicrobial activity screening was implemented in accordance with a previous study (2). The agar plugs (0.5 square centimeter) of each cave bacterial isolate after being properly incubated was aseptically cut using sterile scalpel. Each of the agar plug that contained the bacterial isolate was then placed on to the tested microbe's-seeded agar plates prepared. The antimicrobial assay plates were prepared in duplicates and incubated at 8°C for a period of 2-3 days. A disinfectant agent, Vikron (Vital Environmental Solutions, Alberta, Canada) and sterile water were served as positive and negative controls respectively. The antimicrobial activities were measured as the zone of inhibition around each bacterial colony. The diameter of the zone of inhibition was measured manually with electronic Vernier caliper (Guilin, Guangxi, China) in accordance with previous studies (2, 3).

Furthermore, bacteria isolates that displayed inhibitory activity were selected to test their antimicrobial production in the broth culture. The selected bacterial plugs from the cultured plate (day 7) were inoculated in 75 mL of HT and R2A broth and incubated at 12.5°C on a rotatory shaker (150 rpm) for 15 days. The incubated culture was tested on four occasions (Day 4, 6, 8, and 15) against three pathogenic bacteria (*Pseudomonas aeruginosa, S. aureus* (MRSA)-43300, and *E. coli* (NDM type carbapenemase)15-102) using the Kirby-Bauer Disk Diffusion Test (9) as used in a previous study (10). The plates contained the incubated-culture-soaked disks were incubated for 24 hours at 37°C followed by the measurements of the zone of inhibition as indicated above.

Molecular Identification of the Positive Candidates

Genomic DNA extraction and sequencing

The positive candidates exhibiting antimicrobial activities were inoculated in nutrient broth for 36 hours at 15°C. The

bacterial genomic DNA extraction was performed in accordance with previous studies (2, 3). The extracted genomic DNA were sent to MarcrogenTM Inc, Seoul, South Korea, for molecular identification of the bacterium through 16S rRNA gene sequencing. The forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used for the 16S PCR amplification reaction. The DNA sequences obtained were analyzed using the BLAST (Basic Local Alignment Search Tool) algorithm with the available sequences in GenBank at the National Center for Biotechnology Information (NCBI) http://www.ncbi.nih.nlm.nih.gov/genebank/index.html with the "Limit to type" option active and inactive, respectively. 16S rRNA gene sequences were identified with the >98% identity and coverage to the closest homologue in the GenBank. The sequences were assigned with the DNA Data Bank of Japan (DDBJ) gene accession numbers.

Sequence alignment and phylogenetic analysis

The first five sequences of each BLAST search were downloaded from GenBank and compiled into a dataset for phylogenetic analyses, with duplicates removed. Sequence alignment and phylogenetic analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) version 6 (11). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (12). The tree with the highest log likelihood (-1444.9044) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 51 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 541 positions in the final dataset.

Results

Cultured bacterial diversity

A total of one hundred six bacteria were isolated from cave sediments, calcite, and calcite mineralized with copper samples. Forty-four isolates were isolated on HT media and thirty-six were isolated on R2A media. Twenty and six bacterial isolates were cultivated on Actinomycete Isolation agar and Soil agar media plates respectively. Notably, ninety-four bacteria were isolated from cave sediments whereas the rest were from the mineralized samples.

Antimicrobial activities and molecular identification of positive candidates

Five bacteria from Sample location 1 of the White Rabbit Cave revealed antimicrobial activities (Table 1). The isolate B016 exhibited antagonistic effect against all the tested microbes. Notably, B016 exhibited inconclusive results against E. coli 15-318. Conversely, both B053 and B076 isolates

exhibited antagonistic effects against S. aureus MRSA 43300, *Serratia marcescens* and *Candida albicans* while B079 and B039 were only antagonistic against *S. aureus* MRSA 43300.

Bacterial Isolates	**Cultivated growth media**	**Sample Location/ Location description/ Sample type**	**Test microorganisms**						
			E. coli-33	*E. coli* 15-102	*E. coli* 15-318	*E. coli* 15 -124	*S. aureus* MRSA 43300	*Serratia marcescens*	*Candida albicans*
B016	HT	Location 1/Fossil vadose passage /cave sediments	+	+	+/-	+	+	+	+
B079	HT	Location 1/Fossil vadose passage /cave sediments	-	-	-	-	+	-	+
B053	R2A	Location 1/Fossil vadose passage /cave sediments	+	-	-	-	+	+	+
B039	R2A	Location 1/Fossil vadose passage /cave sediments	-	-	+/-	-	+	-	-
B076	R2A	Location 1/Fossil vadose passage /cave sediments	-	-	-	-	+	+	+

Table 1: Antimicrobial activities. Bacterial isolates cultivated from the White Rabbit Cave exhibited antibacterial activities against tested microorganisms. +/- = Inconclusive as the results could not be confirmed through further repetition of tests; + = Positive inhibitory activity; - = No inhibitory activity.

Furthermore, the five positive candidates underwent further testing of antimicrobial production in the broth culture. The bacterial strain B053 displayed antibacterial activities against all tested microbes when cultivated both in HT and R2A broth media. The HT broth media cultivated B053 exhibited antibacterial activities on all four days (Day 4, 6 , 8 and 15), while in the R2A broth only on the 15th day of incubation when grown in the R2A broth. Notably, the B076 exhibited an antagonistic effect against MRSA 43300 test bacteria on all four days of the assay, but only when cultivated in the HT media (Table 2).

Antimicrobial assay's day	**Bacterial isolates**	**Media used**					
		Hickey-Tresner (HT)			**R2A**		
		Test bacteria					
		S. aureus MRSA 43300	*E.* coli 15-102	*Pseudomonas*	MRSA	*E.* coli 15-102	*Pseudomonas*
4th	B016	-	-	-	-	-	-
	B039	-	-	-	-	-	-
	B053	+	-	-	-	-	-
	B076	+	-	-	-	-	-
	B079	-	-	-	-	-	-
6th	B016	-	-	-	-	-	n/a
	B039	-	-	-	-	-	n/a
	B053	+	-	-	-	-	n/a
	B076	+	-	-	-	-	n/a
	B079	-	-	-	-	-	n/a
8th	B016	-	-	n/a	-	-	n/a
	B039	-	-	n/a	-	-	n/a
	B053	+	-	n/a	-	-	n/a
	B076	+	-	n/a	-	-	n/a
	B079	-	-	n/a	-	-	n/a
15th	B016	-	-	n/a	-	-	n/a
	B039	-	-	n/a	-	-	n/a

Table 2: Antibacterial activities of the six positive candidates cultivated in the broth culture of HT and R2A media. Three test bacterial strains, ***S. aureus*** MRSA 43300, ***E. coli*** (NDM type carbapenemase) 15-102, and ***Pseudomonas*** were used for the antimicrobial assay which was conducted on the 4th, 6th, 8th and 15th day of incubation. (+ = Positive inhibitory activity; - = No inhibitory activity; n/a = conditions that were not tested)

16S rRNA gene identification exhibited three bacteria that belonged to the phyla Actinobacteria (*Streptomyces spp.*) and the other three belonged to Firmicutes. The Firmicutes bacteria were identified as *Paenibacillus terrae, Paenibacillus spp.* and *Bacillus safensis* (Figure 3). All sequenced bacteria exhibited >99% identity to their closest homologue. The DNA Data Bank of Japan (DDBJ) accession numbers were assigned to each of the isolate's sequences. The Samples A (5ffbb08d3a01a5000f85e942.B016), B (5ffbb08d3a01a5000f85e942.B079), C (5ffbb08d3a01a5000f85e942.B053), D (5ffbb08d3a01a5000f85e942.B039), E (5ffbb08d3a01a5000f85e942.B076) were assigned with the DDBJ Accession Numbers LC605904, LC605905, LC605906, LC605907 and LC605908, respectively.

Phylogenetic analysis

The evolutionary relatedness of the antimicrobial bacterial isolates demonstrated two key phyla containing our bacterial isolates: Actinobacteria and Firmicutes. Three bacteria are recognized from the genera Streptomyces which falls in the Actinobacteria clade while the remaining three, *Paenibacillus terrae, Bacillus safensis*, and *Paenibacillus* spp. fall in the Firmicutes clade. Each of the six bacterial isolates from White Rabbit Cave are shown in Figure 3 with an asterisk (*), visually indicating their evolutionary relatedness

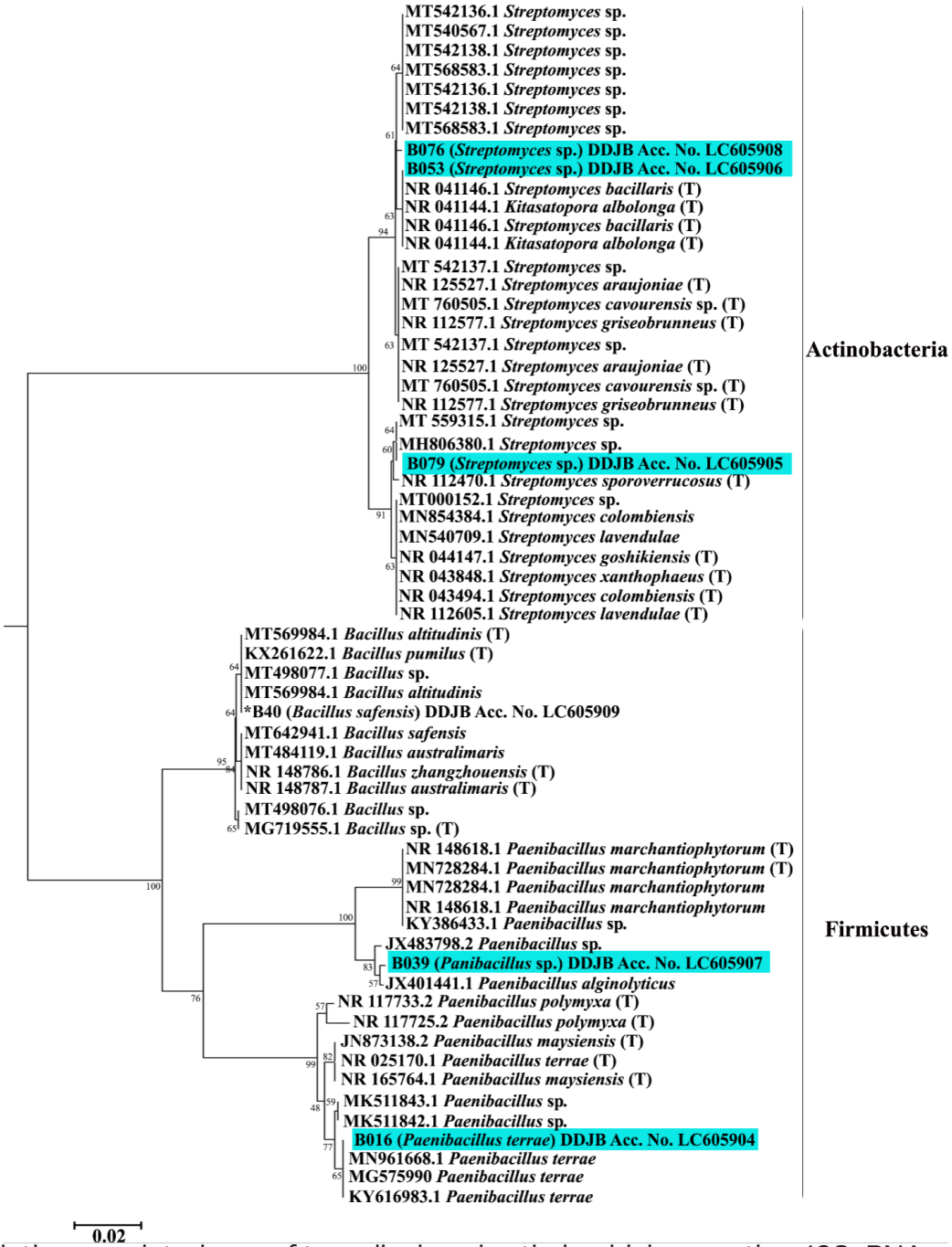

Figure 3. Evolutionary relatedness of taxa displayed antimicrobial properties. 16S rRNA sequences of the bacterial isolates B016, B039, B053, B076, and B079 were placed in the phylogenetic tree closest to their homologues obtained from the NCBI gene database. For each of the six bacterial isolates that showed antimicrobial properties the five closest homologues were chosen with % identity and % query cover >98%, this was repeated with typed material. These sequences were aligned by MUSCLE with default parameters. The final construction was done using MEGA6 by the Maximum Composite Likelihood method, with a bootstrap test of 1000 replicates. The bacterial isolates that showed antimicrobial activities are indicated with light blue shade. 'T' represents the typed isolates.

Discussion

White Rabbit Cave is a white marble, karstic cave found in the Monashee Mountain range in British Columbia, Canada. Two locations were identified in fossil vadose passage for sample extraction: sample location 1 provided sediment and calcite samples, while sample location 2 provided only calcite samples. However, there was insufficient bacterial load collected from the calcite samples; therefore, further investigations could not be conducted. Thus, all the bacterial isolates were obtained from the sediment samples from sample location 1. The current study has evaluated the antimicrobial activities of one hundred and six bacterial isolates cultured from White Rabbit Cave, among which only five have exhibited antimicrobial activities. 16S rRNA gene sequencing revealed 50% of the positive candidates were from the Actinomycetes phylum where the sole genus was Streptomyces spp. (B053, B076, B079). These findings were consistent with previous studies where it has been shown that the limestone cave environment has been predominantly inhabited by Actinobacteria (13-15) and *Streptomyces spp*., both of which were found to display antimicrobial activities (1, 3, 4, 16). A previous study from the Iron Curtain Cave, Canada isolated two *Streptomyces* spp., ICC4 and ICC1 where both isolates exhibited antimicrobial activities. ICC4 exhibited antagonism against the regular non-resistant strains of *E. coli*, *S. aureu*s and MDR strains of *E. coli* 15-318, E. coli 15-102, *E. coli* 15-124 and P. aeruginosa while ICC1 only showed activity against *E. coli* 15-102 and *E. coli* 15-124 (3, 4). Similarly, another study from Turkish karstic caves has specifically exhibited the antagonistic activities of Streptomyces spp. 1492 against MRSA, vancomycin resistant *Enterobacter faecium* (VRE), and *Acinetobacter baumannii.* (17). *Streptomyces spp*. are prolific antimicrobial agent producers (18). For instance, a previous study that examined the whole genome sequences of two *Streptomyces* spp., ICC1 and ICC4, revealed 37 and 35 biosynthetic gene clusters respectively (4). Furthermore, UHPLLC-HRMS analysis of the synthesized secondary metabolites from these *Streptomyces* spp. identified three diketopiperazines: cyclo (Leu- Pro), cyclo (Pro-Val) and cyclo (4-hydroxyPro)-Leu]) (4) which are considered to possess antimicrobial agents in accordance to a previous study (19).

The current study also recovered antimicrobial activities from the Phylum Firmicutes with *Paenibacillus* (B016, B039) as the foremost genera. Firmicutes have been frequently found in cave settings, particularly bacterial species from the *Bacillus* and *Paenibacillus* genera (3, 20, 21). For example, a former study has implemented Roche 454 pyro-sequencing of the 16S rRNA gene sequences that revealed the presence of Firmicutes from the 400 years old ice strata from an alpine ice cave (21). The genus *Paenibacillus,* especially *Paenibacillus terrae* B016 exhibited close homology, has been widely studied for its wide range of production of bioactive molecules such as polymyxin, xylanases protease, amylase, and cellulase (22). These bioactive molecules could potentially be attributed for their antimicrobial properties (23-26). Antimicrobial properties of *Paenibacillus* have been observed in other habitats as well. In one of the previous studies, *Paenibacillus* spp. GHS.8.NWYW.5 was found in active

layer permafrost soil and in saline spring sediment in Nunavut, Canada, and exhibited antimicrobial activity against MRSA, Listeria monocytogenes, Salmonella enterica, and *E. coli* O157:H7 (27). Similarly, in another study *Paenibacillus* spp. SMB1 isolated from a halo-alkaline lake in India exhibited antimicrobial properties against Gram-positive bacteria via the production of bacitracin A (25). Notably, neither of the isolates, GHS.8.NWYW.5 or SMB1, were found in a cave environment; however, they were found in unique, yet somewhat extreme habitats showing the resilience and adaptability of *Paenibacillus* as a genus, taking special notice of their ability to produce secondary metabolites to thrive and take advantage of each environment. Furthermore, *Paenibacillus* spp. was also known to possess an antibiotic resistance gene in their genome. A previous study has reported the isolation of *Paenibacillus* spp. LC231 from Lechuguilla Cave. The whole genome sequencing, functional genomics, and biochemical analysis of the isolate revealed its resistance towards a wide array of antibiotics (28).

The broth culture of the five positive candidates did not show any antimicrobial activities except for Streptomyces spp., B053 and B076. It has been observed that both these isolates exhibited antagonistic effects only when subjected to specific HT and R2A broth culture media and on specific days of incubation which is in line with previous studies (3, 29). A previous study has shown that two *Streptomyces spp.,* ICC1 and ICC4, isolated from the Iron Curtain Cave, Canada exhibited antagonistic effect only when grown in R2A and V8 & HT media respectively (3). Further investigations should emphasize whole genome sequencing, functional genomics, extraction and purification of potential bioactive compounds, optimization of seed media, production media, amount and age of seed inoculum, and growth and production condition to fully characterize these bacterial isolates for their antimicrobial activities.

The present study attempted to shed light on the antimicrobial activities of the bacterial isolates cultivated from White Rabbit Cave, Canada which has never been elucidated before. However, we believe that this preliminary approach shows the White Rabbit Cave habitat as a resource for potential bioactive molecules, but we recognize that this study is by no means a whole picture of the cave habitat. Future studies should explore the details of microbial diversities by implementing high throughput metagenomic approaches while also bio-prospecting novel molecules such as antimicrobial sand and enzymes of industrial and pharmaceutical importance. Additionally, studies should also look upon the potential microbial-mineral interactions in the formation of cave deposits in order to expand the present knowledge of biospeleology.

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Authorship statement

NC applied for research grant, designed and directed the study. SG helped supervised undergraduate students in the lab. KRS applied for research grants for sample collections and collected samples. CJ, AGP, GK, VY, TL performed lab experiments and analyzed the data. MW and SG prepared the manuscript with NC's supervision. KRS wrote the cave description section. CJ, AGP. KRS and GK read the manuscripts and offered inputs.

Declaration

The authors declare that there is no conflict of interests.

References

1. Ghosh S, Kuisiene N, Cheeptham N. 2016. The cave microbiome as a source for drug discovery: Reality or pipe dream? *Biochem Pharmacol* 134:18-34.

2. Ghosh S, Kam G, Nijjer M, Stenner C, Cheeptham N. 2020. Culture dependent analysis of bacterial diversity in Canada's Raspberry Rising Cave revealed antimicrobial properties. *International Journal of Speleology* 49:43-53.

3. Ghosh S, Paine E, Wall R, Kam G, Lauriente T, Sa-ngarmangkang PC, Horne D, Cheeptham N. 2017. In Situ Cultured Bacterial Diversity from Iron Curtain Cave, Chilliwack, British Columbia, Canada. *Diversity* 9:1-15.

4. Gosse JT, Ghosh S, Sproule A, Overy D, Cheeptham N, Boddy CN. 2019. Whole Genome Sequencing and Metabolomic Study of Cave Streptomyces Isolates ICC1 and ICC4. *Front Microbiol* 10:1-12.

5. Herold K, Gollmick, F. A., Groth, I., Roth, M., Menzel, K. D., Mollmann, U.,, al. e. 2005. Cervimycin A-D: a polyketide glycoside complex from a cave bacterium can defeat vancomycin resistance. *Chem Eur J*:5523–5530.

6. Lamprinou V, Tryfinopoulou K, Velonakis EN, Vatopoulos A, Antonopoulou S, Fragopoulou E, Pantazidou A, Economou-Amilli A. 2015. Cave Cyanobacteria showing antibacterial activity. *International Journal of Speleology* 44:231-238.

7. Yonge CJ. The Tupper Glacier Sink - Raspberry Rising Cave System, Glacier National Park, Canada: A Remarkable Example In Stripe Karst, p 158-163. In (ed), 2013 ICS Proceedings,

8. Woodcock JR, Booth. TD. 1978. Geological and Geochemical Report on the Seymour Property. Kamloops Mining Division.,

9. Hudzicki J. 2009. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. *American Society for Microbiology*:1-23.

10. Riquelme C, Enes Dapkevicius ML, Miller AZ, Charlop-Powers Z, Brady S, Mason C, Cheeptham N. 2017. Biotechnological potential of Actinobacteria from Canadian and Azorean volcanic caves. *Appl Microbiol Biotechnol* 101:843-857.

11. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30:2725-2729.

12. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425.

13. Lavoie KH, Winter AS, Read KJ, Hughes EM, Spilde MN, Northup DE. 2017. Comparison of bacterial communities from lava cave microbial mats to overlying surface soils from Lava Beds National Monument, USA. *PLoS One* 12:1-27.

14. Axenov-Gribanov DV, Voytsekhovskaya IV, Tokovenko BT, Protasov ES, Gamaiunov SV, Rebets YV, Luzhetskyy AN, Timofeyev MA. 2016. Actinobacteria isolated from an underground lake and moonmilk speleothem from the biggest conglomeratic karstic cave in Siberia as sources of novel biologically active compounds. *PLoS One* 11:1-12.

15. Northup DE, Melim LA, Spilde MN, Hathaway JJ, Garcia MG, Moya M, Stone FD, Boston PJ, Dapkevicius ML, Riquelme C. 2011. Lava cave microbial communities within mats and secondary mineral deposits: implications for life detection on other planets. *Astrobiology* 11:601-18.

16. Hamedi J, Kafshnouchi M, Ranjbaran M. 2019. A Study on actinobacterial diversity of Hampoeil cave and screening of their biological activities. *Saudi Journal of Biological Sciences* 26:1587-1595.

17. Yücel S, Yamaç M. 2010. Selection of Streptomyces isolates from Turkish karstic caves against antibiotic resistant microorganisms. *Pak J Pharm Sci* 23:1-6.

18. Maciejewska M, Adam D, Martinet L, Naome A, Calusinska M, Delfosse P, Carnol M, Barton HA, Hayette MP, Smargiasso N, De Pauw E, Hanikenne M, Baurain D, Rigali S. 2016. A Phenotypic and Genotypic Analysis of the Antimicrobial Potential of Cultivable Streptomyces Isolated from Cave Moonmilk Deposits. *Front Microbiol* 7:1455.

19. Kumar SN, Siji JV, Nambisan B, Mohandas C. 2012. Activity and Synergistic Antimicrobial Activity Between Diketopiperazines Against Bacteria In Vitro. *Applied Biochemistry and Biotechnology* 168:2285-2296.

20. Yasir M. 2018. Analysis of bacterial communities and characterization of antimicrobial strains from cave microbiota. *Brazilian Journal of Microbiology* 49:248-257.

21. Itcus C, Pascu MD, Lavin P, Persoiu A, Iancu L, Purcarea C. 2018. Bacterial and archaeal community structures in perennial cave ice. Scientific Reports 8.

22. Grady EN, MacDonald J, Liu L, Richman A, Yuan ZC. 2016. Current knowledge and perspectives of Paenibacillus: a review. *Microbial Cell Factories* 15.

23. Liang YL, Zhang Z, Wu M, Wu Y, Feng JX. 2014. Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by Paenibacillus terrae ME27-1. Biomed Research International doi:Artn 51249710.1155/2014/512497.

24. Shin SH, Kim S, Kim JY, Song HY, Cho SJ, Kim DR, Lee KI, Lim HK, Park NJ, Hwang IT, Yang KS. 2012. Genome Sequence of Paenibacillus terrae HPL-003, a Xylanase-Producing Bacterium Isolated from Soil Found in Forest Residue. *Journal of Bacteriology* 194:1266-1266.

25. Singh H, Kaur M, Jangra M, Mishra S, Nandanwar H, Pinnaka AK. 2019. Antimicrobial properties of the novel bacterial isolate Paenibacilllus sp. SMB1 from a halo-alkaline lake in India. *Scientific Reports* 9.

26. Ghosh S. 2015. Metagenomic Screening of cell wall hydrolases, their anti-fungal activities and potential role in wine fermentation. PhD thesis, Stellenbosch University, South Africa.

27. Marcolefas E, Leung T, Okshevsky M, McKay G, Hignett E, Hamel J, Aguirre G, Blenner-Hassett O, Boyle B, Levesque RC, Nguyen D, Gruenheid S, Whyte L. 2019. Culture-Dependent Bioprospecting of Bacterial Isolates From the Canadian High Arctic Displaying Antibacterial Activity. Frontiers in Microbiology 10.

28. Pawlowski AC, Wang W, Koteva K, Barton HA, McArthur AG, Wright GD. 2016. A diverse intrinsic antibiotic resistome from a cave bacterium. Nat Commun 7:13803.

29. Cheeptham N, Higashiyama T, Phay N, Fukushi E, Matsuura H, Tomita F, Lumyong S. 1998. An antifungal antibiotic (NCA-1) from Ellisiodothis inquinans L1588-A8, its identification and effects on growth of Saccharomyces cereviseae strain EC 19. *Ann Rep ICBiotech* 20:843-847.