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CHARACTERIZATION OF THE ANTIMICROBIAL SECONDARY METABOLITES PRODUCED BY THE CAVE BACTERIA *STREPTOMYCES* SP. STRAIN ICC1

2021 | APRIL LEIGH-ANN READ

B.Sc. Honours thesis – Biology

THOMPSON RIVERS UNIVERSITY

CHARACTERIZATION OF THE ANTIMICROBIAL SECONDARY METABOLITES PRODUCED BY THE CAVE BACTERIA *STREPTOMYCES* SP. STRAIN ICC1

by

APRIL LEIGH-ANN READ

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE (HONS.)
in the

DEPARTMENT OF BIOLOGICAL SCIENCES
(GENERAL BIOLOGY)

This thesis has been accepted as conforming to the required standards by:

Dr. Heidi Huttunen-Hennelly (Ph.D.), Thesis Supervisor, Dept. Physical Sciences
Dr. Naowarat Cheeptham (Ph.D.), Co-Supervisor, Dept. Biological Sciences
Dr. Donkor Kingsley (Ph.D.), Co-Supervisor, Dept. Physical Sciences
Dr. Dipesh Prema, Co-Supervisor (Ph.D.), Dept. Physical Sciences
Dr. Eric Bottos, Co-supervisor (Ph.D.), Dept. Biological Sciences
Dr. Jonathan Van Hamme (Ph.D.), External Examiner, Dept. Biological Sciences

Dated this 27th day of April, 2021, in Kamloops, British Columbia, Canada

ABSTRACT

Streptomyces is one of the most abundant microbial genera in cave environments. The characteristic most notable of *Streptomyces* is its ability to produce bioactive secondary metabolites – such as antifungals, antivirals, antitumorales, anti-hypertensives, immunosuppressants, and antibiotics. Through the present research, secondary metabolites produced by the cave-dwelling *Streptomyces* sp. strain ICC1 were examined; a strain which is prevalent in the isolated environment of the Iron Curtain Cave in Chilliwack, British Columbia. Secondary metabolites secreted by *Streptomyces* sp. ICC1 have demonstrated antimicrobial properties, effective against both multi-drug resistant strains and common laboratory strains of *Escherichia coli* and *Staphylococcus aureus*. Liquid organic solvent extractions as well as preparative and analytical reverse-phase high performance liquid chromatography suggest that the bioactive secondary metabolites are polar with hydrophobic substituents. Further instrumental analysis is required to determine the antimicrobial active secondary metabolites structure and mode of action.

Thesis Supervisor: Naowarat Cheeptham

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to my supervisor Dr. Heidi Huttunen-Hennelly for her help even during her absence. To Dr. Naowarat Cheeptham for her continued support, patience, understanding, as well as laboratory space and supplies. I would also like to extend thanks toward professors and lab technicians who provided me guidance throughout my research. To Dr. Eric Bottos for his knowledge on microbiology and problem-solving abilities. To Trent Hammer for his assistance in instrumental set up and analysis, particularly with HPLC techniques. To Dr. Dipesh Prema and Dr. Kingsley Donkor for their instruction with NMR analysis and valuable chemistry expertise. Further, to Jon Van Hamme for his role as an external examiner.

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INTRODUCTION

Drug resistance microbes have presented an issue to human healthcare since the first observed resistance from a strain of *Staphylococcus* against penicillin in 1942 (Lobanovska and Pilla 2017). Since then, the discovery of new antibiotics has been necessary to combat the spread of bacteria that are resistant to available pharmaceuticals. Understanding the evolution and origins of antibiotic resistance genes is vital to predicting, preventing and managing this global health problem. Studies over the past decade have revealed that antibiotic resistance is common in contemporary and ancient environmental bacteria, and that the associated genes are similar or identical to those circulating in pathogens (Perry et al. 2014). Most notable was a study performed by Bhullar and colleagues (2016) where microbial samples from a region of the Lechuguilla Cave in New Mexico, which was thought to have been isolated from surface input for over 4 million years, displayed resistance to commercially available antibiotics. While resistance is a natural phenomenon, what has changed since the beginning of the antibiotic era is a marked increase in selection for mobile resistance elements and consequently, increase of drug resistant pathogenic and non-pathogenic bacteria (Pawlowski et al. 2016). According to the World Health Organization at least 700,000 individuals die each year due to drug-resistant diseases (Sarkar et al. 2017). Therefore, an overwhelming demand for novel treatment presents itself; the likelihood of multi-resistant microbes surpassing the development of antibiotics escalates in a coevolutionary race between humans and bacteria.

The Iron Curtain Cave

The Iron Curtain Cave is located near Chilliwack, British Columbia. It was discovered by caver Rob Wall in 1993 and named for its iron-rich sediment. The cave is decorated with large curtains

made of calcium carbonate and limestone structures. While the sediment contains iron that gives it a unique reddish coloration, it also has a clay consistency that indicates higher levels of moisture (Ghosh et al. 2017). Studies of unique environments have demonstrated that metals play a pivotal role in determining subsurface living communities (Parnell et al. 2015). Iron is one of the most abundant metals in the Earth's crust and is present in various biomolecules (Parnell et al. 2015). Iron is essential for the function of many fundamental, conserved biochemical processes, such as the electron transport and Krebs cycle (Bonis and Gralnick 2015). Many cave bacterial species use iron in a variety of ways, for instance the genus *Thiobacillus* can obtain energy by oxidizing sulfur and ferrous iron compounds (De Mandal et al. 2017). The oxidation state of iron also influences soil structure, dissolved carbon stability, and enzyme activity, affecting microbial communities and soil fertility (Bonis and Gralnick 2015).

There are six pools of water present from the entrance and throughout the Iron Curtain cave, which creates a humid environment. The temperature ranges between 4–12 °C depending on the time of year (Ghosh et al. 2017). 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. Within the cave there is a specific path to limit human impact as well as to preserve the features and habitat of the cave. The microbe of importance in this study, *Streptomyces* sp. ICC1, was originally isolate 22.47 m from the entrance of the cave, depicted as point 1 in Figure 1 illustrated by Ghosh et al. (2017). The unique conditions of the Iron Curtain Cave – including high humidity, relatively low but stable temperatures, and low nutrients, create a highly selective environment. Energy-starved conditions of caves often lead to the production of various secondary metabolites that enhance their producers' likelihood of survival (Gosse et al. 2019).

Streptomyces sp. ICC1 is able to thrive in such desolate conditions due to their unique physiology and specialized metabolic pathways.

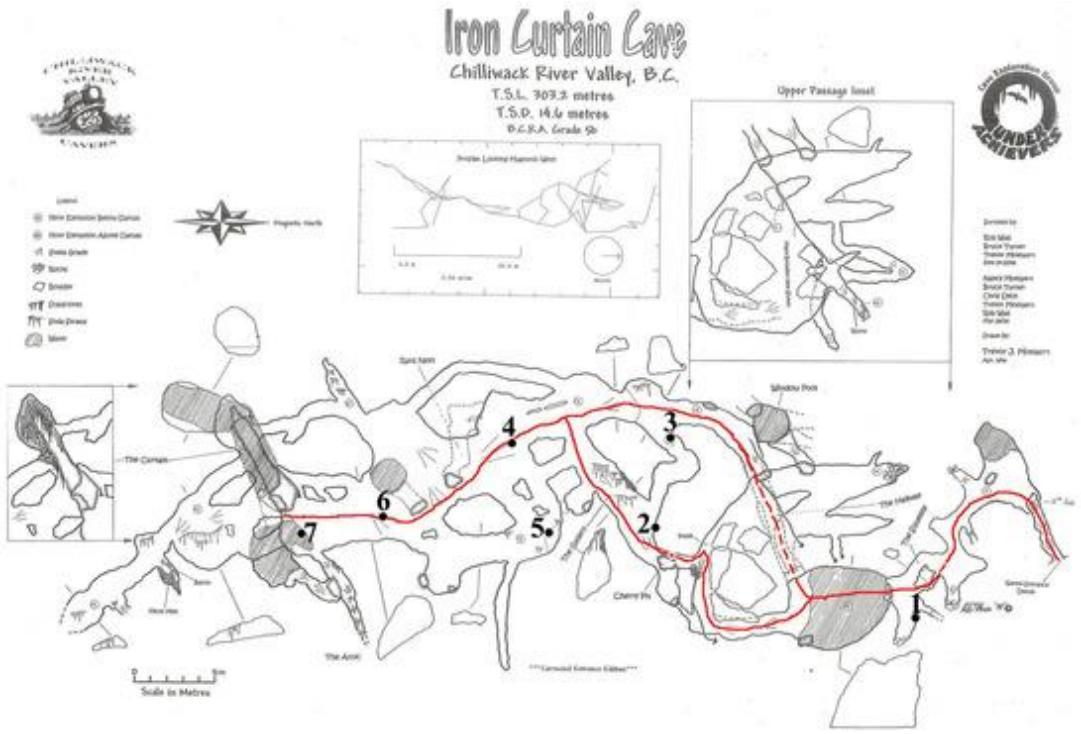


Figure 1. Map of the Iron Curtain Cave in Chilliwack, BC, where *Streptomyces* sp. ICC1, which is the strain of interest, was retrieved from Point 1 (Ghost et al. 2017).

Genus *Streptomyces*

Streptomyces is a genus of the family Streptomycetaceae belonging to the order Actinomycetales and the class Schizomycetes. *Streptomyces* are Gram-positive bacteria that have a DNA GC content of 69–78 mol% (Korn-Wendisch & Kutzner, 1992). This genus grows predominately in soil but is also found in various other locations including marine environments and caves (Flärdh and Buttner 2009). The morphology of most soil dwelling *Streptomyces* resembles filamentous fungi due to the production of an extensive branching substrate and aerial mycelia. The substrate hyphae are approximately 0.5 to 1.0 μm in diameter and often lack cross-walls during the vegetative phase (Anderson and Wellingtonn 2001). Growth occurs at the hyphal

apices and is accompanied by branching, thus producing a complex tightly woven matrix of hyphae during the vegetative growth phase. As the colony ages, aerial mycelia are produced, which develop into chains of spores by the formation of cross walls in the multinucleate aerial filaments. This is followed by separation of individual cells directly into spores. The morphological features of aerial mycelium are regarded as more significant for characterization than vegetative mycelium. The aerial mycelium include the mode of branching, the configuration of the spore chains, and the surface of the spores.

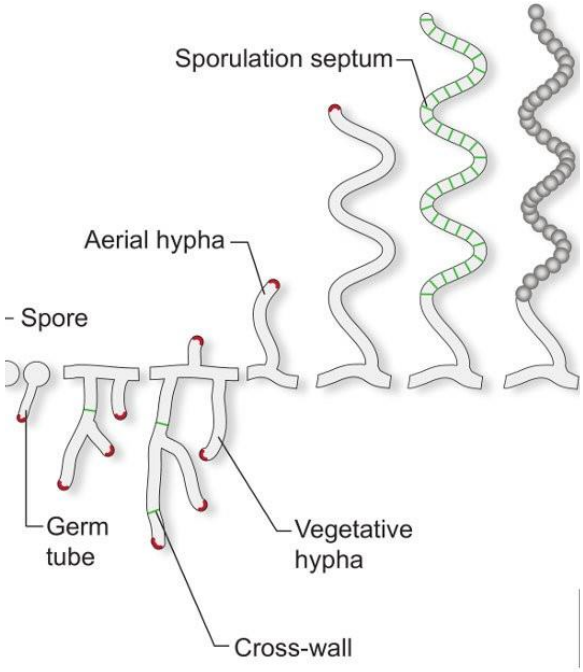


Figure 2. The developmental life cycle of *Streptomyces* from spore to aerial hypha (Flärdh and Buttner 2009).

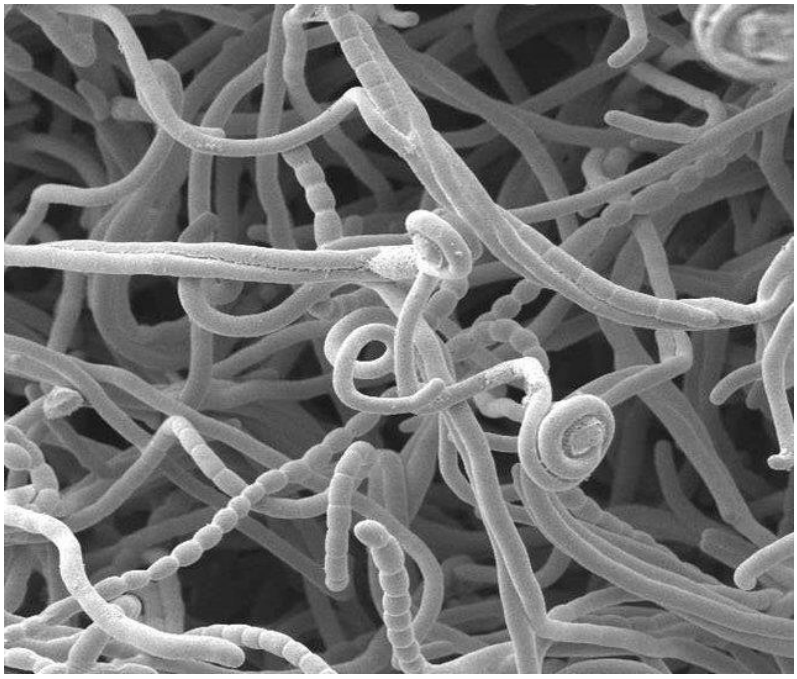


Figure 3. Scanning electron micrograph of hyphae and spores of *Streptomyces coelicolor* (Tschowri 2015).

The spores in soil germinate to produce mycelium under favorable conditions. Upon germination, the cells show complex vegetative hyphal growth, which aids the microbes' ability to colonize the soil. Under conditions of high concentration of nutrient and energy sources, the hyphae are highly branched and excrete a number of extracellular enzymes in order to obtain the required nutrients for growth (Avignone-Rossa et al. 2013). In contrast, conditions of nutrient scarcity restrict cell growth and aerial mycelium is formed, eventually differentiating into spores and therefore restarting the cycle. This morphological change is accompanied by metabolic changes, which result in the induction of secondary metabolism. The formation of spores, a semi-dormant stage in the life cycle of the bacterium, serves the double purpose of resisting unfavorable environments and spreading the organism. Cave dwelling actinomycetes, such as *Streptomyces,* have been studied less compared to soil strains.

The strain of interest in the present study is *Streptomyces* sp. ICC1, which was collected from the Iron Curtain Cave in Chilliwack, British Columbia. Preliminary 16S rRNA gene sequencing of ICC1 confirmed that the microbe belongs to the genus *Streptomyces*, which is supported by morphology and growth characteristics of the bacteria (Gosse et al. 2018). The scanning electron micrograph captured by Ghosh et al. (2017) shown below as Figure 2 demonstrates that *Streptomyces* ICC1 shares the general trends displayed across the genus, with its dense rode-shaped filaments and colonial appearance. Whole genome sequencing of *Streptomyces* sp. ICC1 by Illumina MiSeq (Illumina, United States) with *de novo* genome assembly using PATRIC generated a draft genome of 9,034,309 nucleotide base pairs containing a guanine and cytosine content of 72%. BLAST was used to compare the genomic profile of *Streptomyces* sp. ICC1 with other genomes on the database. The closest genome match was *Streptomyces lavendulae* strain CCM 3239 with 54% sequence alignment to *Streptomyces* sp. ICC1 and an average nucleotide identity of 87.9% between the organisms. (Gosse et al. 2018). In addition, RAST annotation predicted 8235 coding regions for *Streptomyces* sp. ICC1, which indicates very similar predicted protein content and function between the strain of interest and *S. lavendulae* (Gosse et al. 2018). While *Streptomyces lavendulae* was found to have the closest genomic match to *Streptomyces* sp. ICC1, due to a low percent similarity of 87.9% it remains undetermined if they are the same species. Current research regarding different strains *Streptomyces lavendulae* is being performed by Dr. Christopher Boddy at the University of Ottawa in the Department of Chemistry and Biomolecular sciences. Dr. Boddy was involved in the whole genomic sequencing and metabolomic study of *Streptomyces* sp. ICC1 as well as another bacterium originally isolated from the Iron Curtain Cave, *Streptomyces* sp. ICC4.

Figure 4. Scanning electron micrograph of *Streptomyces* sp. ICC1 showing a dense mass of interwoven filaments with irregular rugose surface collected from the Iron Curtain Cave (Ghosh et al. 2017).

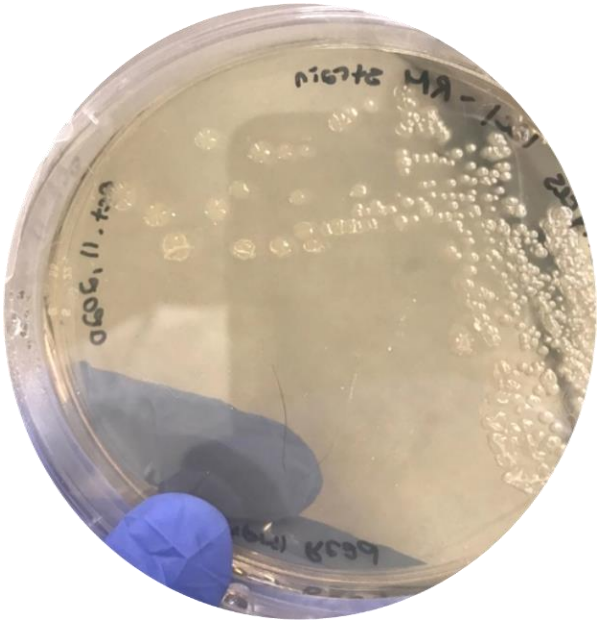


Figure 5. Macroscopic morphology of *Streptomyces* sp. ICC1 on nutrient agar after fourteen days of growth in a 15°C incubator.

Secondary Metabolite Production

A large variety of compounds are produced by microorganisms through means of specialized biosynthetic pathways. Secondary metabolites are low-molecular-mass organic compounds that, unlike primary metabolites, are not directly involved in the growth, development, or reproduction of the producing organisms. The small diffusible molecules disperse from the microorganism and may provide its producer an advantage by adapting to the extracellular conditions to some degree (Cihak et al. 2017). They may also aid in defense, competition, signaling, or interspecies interactions, depending on the environmental cues, thus increasing the likelihood of survival in an inhospitable environment. In natural conditions, secondary metabolites are primarily present at low concentrations (Netzker et al. 2015). Secondary metabolites may also play a role in transferring information between cells. Secondary metabolites create signaling networks that aid in intra- and inter-species communication, as well as in regulation of many aspects of metabolism, virulence, physiology, competence, motility, symbiosis, and other functions (Dufour and Roa 2011). Chemical communication occurs between bacteria-bacteria or bacteria-fungi since they form close communities in nature. Successful co-existence can be mediated through these signaling molecules in low concentration where the fungal mycelia and bacterial filaments of both microorganisms are connected together (Abdalla et al. 2017). For instance, α-Butyrolactone is present in most *Streptomyces* and was the first *Streptomyces* sporulation factor discovered. α-butyrolactones are analogous to homoserine lactones and have been deduced to play an important role as signals as well as markers for the setting-in of morphological and physiological differentiation (Safari et al. 2014). It was first characterized as an activator of streptomycin production and spore formation in *Streptomyces griseus* (Miguélez et al. 2010).

An interesting property of *Streptomyces* is its ability to produce bioactive secondary metabolites, such as antifungals, antivirals, antitumorals, anti-hypertensives, immunosuppressants, and antibiotics (Procópio et al. 2012). *Streptomyces* are regarded as the most prolific source of bioactive compounds in particular commercially available antibiotics since they produce approximately two-thirds of commercially available antibiotics (Barka et al. 2016). The production of most antibiotics is species specific, and these secondary metabolites are important for *Streptomyces* species in order to compete with other microorganisms that come in contact, even within the same genus. The bioactivity of the small molecules is mostly achieved by affecting transcription in receiving cells. The genes for the biosynthesis of *Streptomyces* secondary metabolites are mostly clustered and their expression is highly regulated (Tanaka et al. 2013). The biosynthetic genes of most secondary metabolites have previously been shown to be expressed during germination (Strakova et al. 2013). Secondary metabolite production predominately occurs at the aerial hyphae developmental stage and are isolated during stationary phase of growth in liquid cultures (Seipke et al. 2012). The setoff of those physiological states, and therefore of the associated synthesis of secondary metabolites, is linked to the depletion of nutrients. A decrease in growth rate may be the signal for triggering secondary metabolism.

The secondary metabolites produced by *Streptomyces* often have dark pigmentation due to the synthesis and excretion of melanins or melanoid pigments. Melanin compounds are irregular, dark brown polymers that are hydrophobic and negatively charged. Various microorganisms produce melanins through fermentative oxidation or oxidative polymerization of phenolic or indolic compounds. These compounds play important roles in microorganisms against thermal, chemical, and biochemical stresses. Specifically, melanins have radioprotective and antioxidant properties that can effectively protect the living organisms from ultraviolet radiation, heavy

metals, and oxidizing agents (Nagger and Ewasy 2017). In addition, the melanin synthesized by microbes help maintain their water and ion storage through increasing the structural rigidity of their cell wall (Allam and Zaher 2012).

Figure 6. Brown pigmentation characteristic of *Streptomyces* sp. ICC1 secondary metabolites grown in nutrient broth for nine days at 15℃**.**

In the present study secondary metabolites produced by *Streptomyces* sp. strain ICC1 have been examined. Secondary metabolites secreted by *Streptomyces* sp. ICC1 have shown antimicrobial properties, effective against both multi-drug resistant strains and common laboratory strains of *Escherichia coli* and *Staphylococcus aureus*. The bioactive secondary metabolites produced by *Streptomyces* sp. ICC1 strain were separated out from culture via diethyl ether liquid-phase organic solvent and water extractions. The metabolite containing solution was purified and separated via analytical and preparative reversed-phase high performance liquid chromatography (HPLC) techniques. Each peak from the HPLC was tested for antimicrobial activity through disk diffusion and well assay methods. One-dimensional proton nuclear magnetic resonance (NMR) spectroscopy was used in attempt to elucidate the structure of each active fraction that was identified by HPLC. Organic extractions with diethyl ether suggest the

bioactive compounds are polar but also contain hydrophobic substituents due to how the activity stays within the less polar organic layer relative to water. In addition, the reverse-phase HPLC results suggest that the secondary metabolites are polar due to short retention times with the elution of a polar mobile phase. Together, the findings of the present study indicate that the secondary metabolites secreted by *Streptomyces* sp. ICC1 are polar and with some hydrophobic substituents. Future work to further the understanding of the bioactive compounds involve additional instrumentation techniques, such as carbon-13 NMR spectroscopy and mass spectrometry, as well as comparisons to secondary metabolites produced by other members of the *Streptomyces* genus.

MATERIALS AND METHODS

Storage and Growth of *Streptomyces* sp. ICC1

Dormant cells of *Streptomyces* sp. ICC1 strain were stored in glycerol stock at -80°C. For recovery, a sterile loop was used to scrape material from the top of the frozen stock and streaked, in triplicate, onto nutrient agar plates. The plates were incubated at 15°C for growth and secondary metabolite production. Visible colonies were present after seven days of incubation and were irregular shaped, dry texture, rugose surface, and opaque cream in color. For liquid cultures, 150 mL of nutrient broth was inoculated with a single colony of *Streptomyces* sp. ICC1, followed by incubation in a shaker incubator at 150 rpm and 15°C for 5 to 40 days. Plates were sealed with parafilmed and kept at 4°C until fresh stocks were made.

Growth of *Escherichia coli* #15-124

Multi-drug resistant *Escherichia coli* #15-124 was prepared with antibiotic mixed into the nutrient agar in order to prevent mutations that decreased resistance. A stock solution of chloramphenicol was produced by weighing 0.8078g into 5 mL of ethanol for a final concentration of 0.05 M. From this stock solution, 100 µL was added to 100 mL of molten nutrient agar for a final concentration of 50 µM. The nutrient agar was poured into four petri dishes and allowed to solidify before thawed freezer stock of multi-drug resistant *Escherichia coli* #15-124 was four-way streaked. The plates were incubated at 37°C for 24 h prior to storing in a 4°C fridge.

Bioassay Screening for Antimicrobial Activity

Target organisms were cultured overnight at 37°C to 0.60-0.90 OD_{600} to suggest active logarithmic division was occurring. The target organisms used include *Escherichia coli* #15-124,

Escherichia coli #59, laboratory *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* ATCC-43300.

A 10ml aliquot of the *Streptomyces* sp. ICC1 broth culture was retrieved from 15°C incubator after 5-40 days at 150 rpm. Brown pigmentation was apparent after 5 days of incubation with bioactivity occurring at this time or anywhere up to 40 days after. The sample was centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected and filtered through a sterile hydrophilic low-protein-binding polyether sulfone membrane syringe filter with 0.22 μm pore size. The filtered supernatant contained the *Streptomyces* sp. ICC1 secondary metabolites. Liquid extractions were performed with filtrate aliquots of 1:1 broth to diethyl ether by mixing them in a separatory funnel for one minute with frequent pauses for ventilation.

Kirby-Bauer disk diffusion assays were used to determine whether a sample had antimicrobial activity by the presence or absence of a zone of inhibition around a solution infused disk. Bioassay screening was performed in 15 mm x 150 mm polystyrene Petri dishes. Eighty mL of autoclaved molten nutrient agar was inoculated with a ratio of 1% volume to volume of the exponentially dividing target organism, OD_{600} between 0.600-0.900. Autoclaved 6 mm paper disks (Toyo, Advantech) were saturated with filtered broth, diethyl ether, post-extraction aqueous filtrate or organic diethyl ether layer and dried completely prior to addition to the bioassay plate. For the Kirby-Bauer disk diffusion method, disks containing different controls and experimental conditions were evenly placed onto nutrient agar that had been inoculated with a target organism. Antimicrobial activity was determined by whether the target organism was able to grow around the disk. The Kirby-Bauer disk diffusion method was supplemented with Well-assays to determine antimicrobial activity. For the Well-assay technique, Pasteur pipettes were used to

make wells in the molten agar. The depth of the wells were approximately midway between the bottom of the top and bottom of the agar, 100 µl of filtered secondary metabolite broth as well as controls was pipetted into the wells.

Diethyl ether was used as a control to ensure diethyl ether did not inhibit the growth of the bacteria lawn. In addition, a disk containing10 µg of ampicillin was used to confirm that the target bacteria were resistant to common pharmaceuticals such as ampicillin. All bioassay screening was performed within a biosafety cabinet to reduce contamination. Five minutes of ultraviolet radiation was used to sterilize glassware, picks, loops, and plastic equipment prior to use. The bioassay plates were incubated at 37°C for 24 h to ensure optimal growth of the target bacterial lawn. The diameter or absence of zones of inhibition were recorded for each experimental condition.

Active Compound(s) Separation and Preliminary Structural Analysis

The organic layer of the diethyl ether extraction was known to possess antimicrobial activity against the previously mentioned target organisms based on the bioassays performed. Liquid extractions with diethyl ether were performed on aliquots of the *Streptomyces* sp. ICC1 broth culture collected between 5 and 40 days of incubation at 15°C at 150 rpm. One portion of broth culture was extracted with equal portions of diethyl ether three times. The organic layers from each extraction performed on the same day, which were one third of the total extraction volume, were combined and evaporated. Extracts were dissolved from the dry flasks in 10 mL of HPLC-grade methanol and 2 mL of water prior to injection into the Agilent 1220 Infinity II high performance liquid chromatography (HPLC) system. Separation was performed according to the analytical reversed-phase HPLC conditions summarized in Table 1. Following method

development, individual fractions were collected via the preparative HPLC conditions listed in Table 1. The resulting four separated components were divided among Eppendorf tubes and the eluent evaporated on a Vacufuge® 5301 Centrifugal Vacuum Concentrator at 45°C. One sample from each separation was resuspended in deionized 18MOhm water and analyzed for antimicrobial activity via Kirby Bauer disk diffusion assays. Remaining samples were resuspended in deuterium oxide for structural elucidation with one-dimensional proton nuclear magnetic resonance (NMR) spectroscopy via a 500 MHz Bruker Avance AMIII 500 Spectrometer.

Table 1. Analytical and preparative HPLC parameters for compound separation.

	Analytical HPLC Parameters	**Preparative HPLC Parameters**
Instrument	Agilent 1220 Infinity HPLC	Waters HPLC 486
Column	Kinetex® 2.6 µm EVO C18 100Å	Phenomenex® Jupiter 10µ C18 300Å
Dimensions	100 x 3.0 mm	250 x 21.20 mm
Detector	272 nm	272 nm
Flow Rate	0.4 mL/min	5 mL/min
Injection Volume	5.0 µL	1.0 mL
Eluent	A: 5 mM NH4OAc in 18 MOhm H_2O B: Acetonitrile	
Program Gradient	1-21 min 90% A 10% B 21-35 min 100% B 35-47 min 90% A 10% B	

Table 2. NMR parameters for structural elucidation.

Instrument	Bruker Avance AMIII 500 Spectrometer
Operating Frequency	500 MHz
Solvent	D_2O
Number of Scans	1024

RESULTS

Bioassay for Antimicrobial Activity

Well assays and Kirby-Bauer disk diffusion assays were performed on nutrient agar with all target organisms listed. Zones of inhibition occurred 5 days post-inoculation and decreased in diameter when samples were incubated for longer durations. Zones of inhibition ranged from 8 to 6.5 mm in diameter for the organic layer after diethyl ether extraction and filtered broth culture. After antimicrobial activity was confirmed, liquid organic solvent extractions were performed to elucidate the relative polarity of the bioactive antimicrobial secondary metabolites. Extractions were performed with diethyl ether, which was previously shown by Alysha Milward (2019) to be the most successful organic solvent compared to ethyl acetate, hexane, and 1-octanol. Solvent success was determined by the antimicrobial activity being maintained in either the aqueous or organic layer post extraction. Solvents were deemed unsuccessful if bioactivity against the target organism was either inconsistently observed or absence. As depicted in Figure 7, activity was present in the organic layer of the extraction as well as filtered broth when all conditions including diethyl ether control, ampicillin control, and aqueous layer of the extraction were tested using a Kirby-Bauer disk diffusion assay. Also, it should be noted that bioactivity was inconsistent throughout the present research, for example the same culture would be sporadically successful and unsuccessful against the same target bacteria at different points of incubation. Furthermore, some liquid cultures produced from the same plate of *Streptomyces* sp. ICC1 would have detectable bioactivity while others did not.

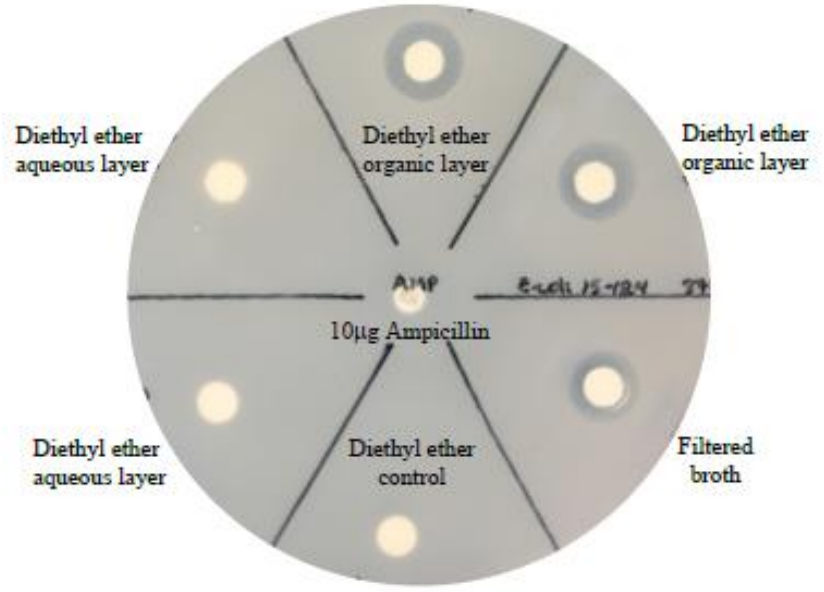


Figure 7. Kirby-Bauer disk diffusion test for *Streptomyces* sp. ICC1 secondary metabolites against multi-drug resistant *Escherichia coli* #15-124 showing bioactivity in the organic layer after diethyl ether extractions.

Compound Separation via Reverse-Phase HPLC

In order to begin separating compounds in the diethyl ether extract from *Streptomyces* sp. ICC1, reverse-phase HPLC was used to produce fractions of the sample based on differing polarities. The program gradient for both the analytical and preparative reverse-phase HPLC was as follows: during the first 21 minutes the initial 90:10 ammonium acetate buffer to acetonitrile ratio moved to 100% acetonitrile, which remained at 100% between 21 to 35 minutes, after 35 minutes the program gradient transitioned slowly back to 90:10 buffer to acetonitrile ratio that occurred at 47 minutes. Absorbance was measured at 272nm for reverse-phase HPLC techniques since this wavelength was previously found by Alysha Milward (2019) to provide a chromatogram with a stronger baseline and sharper peaks. Analytical revers-phase HPLC was used for method development to estimate when the sample eluted and provide information regarding how long the preparative reverse-phase HPLC should be run for. Blank runs with water were first run to

determine baseline activity. Figure 8 shows the chromatogram from the bioactive secondary metabolites resuspended in methanol. The majority of the sample eluted quickly from the column while the rest eluted at 38.434 minutes when the program gradient returned to a 90:10 buffer to acetonitrile ratio.

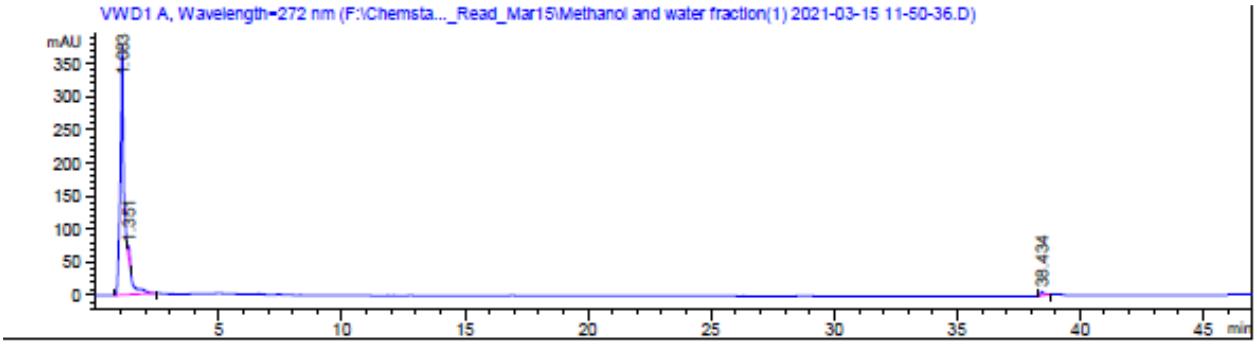


Figure 8. Analytical reverse-phase HPLC chromatograms of bioactive secondary metabolites in methanol measured at 272 nm.

The preparative reversed-phase HPLC chromatogram, shown below in Figure 9, had different absorbance readings and separation from that of the analytical chromatogram as a result of the different column volumes. The Phenomenex® Jupiter 10u C18 column has a non-polar stationary phase, therefore polar substituents are the first to elute due to their high affinity for the polar mobile phase. Five runs were performed on the same culture sample to ensure the results were repeatable. Fractions covered a broad range of materials Fraction 1 was a broad short peak, which had a retention time between 8 to 10 minutes in each of the five trials run. Fraction 2 was the tallest peak of the four observed and had a consistent height between samples. The trail of peaks following Fraction 2 were denoted Fraction 3 since the number and height of each peak within this cluster varied between runs. Fraction 4 was a relatively small peak that occurred approximately at 42 minutes after injection. Due to the high-volume capacity of the preparative

Phenomenex® Jupiter 10u C18 column, it can be reasoned that all of the fractions collected were likely eluted in 90% 5 mM ammonium acetate buffer and 10% acetonitrile.

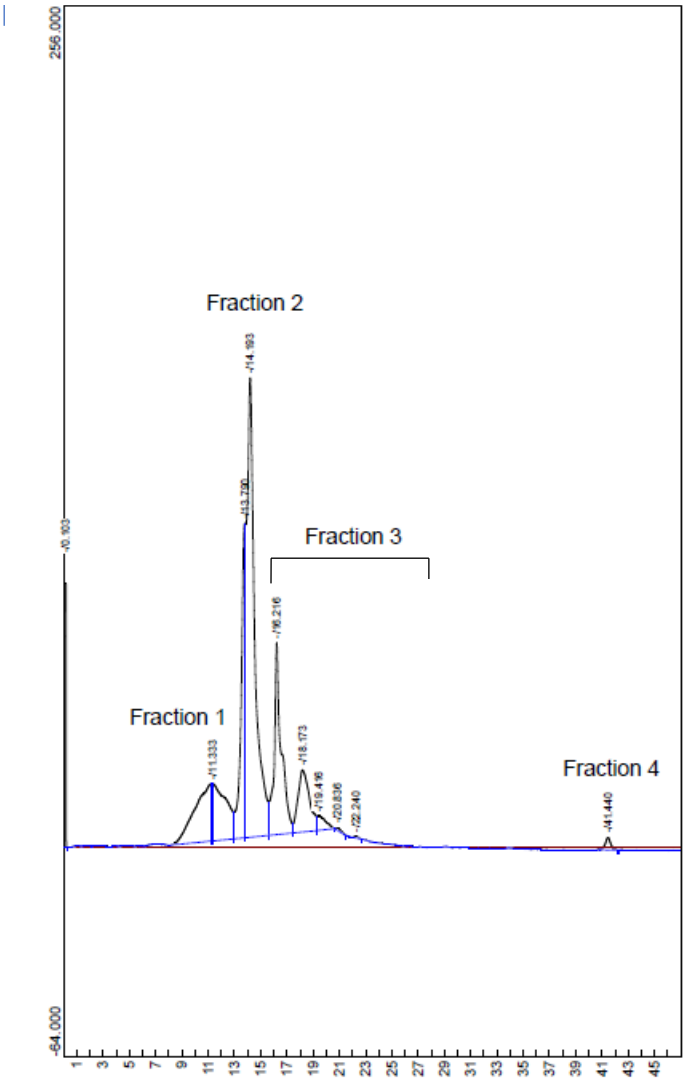


Figure 9. Preparative reverse-phase HPLC of bioactive secondary metabolites in methanol.

The four fractions were evaporated to remove all the remaining HPLC solvent, which left a brown pigmented solid residue in the collection tubes. Following eluent evaporation, the bioactivity of each of the four factions collected, which were dissolved in methanol, were tested via Kirby-Bauer disk diffusion assays. As shown below in Figure 10, the bioactive secondary metabolites were separated into Fraction 2 and Fraction 4 using the preparative reversed-phase

HPLC conditions used. The clear zones of inhibition against the multi-drug resistant strain *Escherichia coli* #15-124 are indicative of antimicrobial agents having been absorbed into the applied paper disk. Fraction 2 was further analyzed for structural elucidation via one-dimensional ^{1}H NMR analysis. Whereas Fraction 4 did not produce enough sample to be effectively used for 1-D ^{1}H NMR analysis.

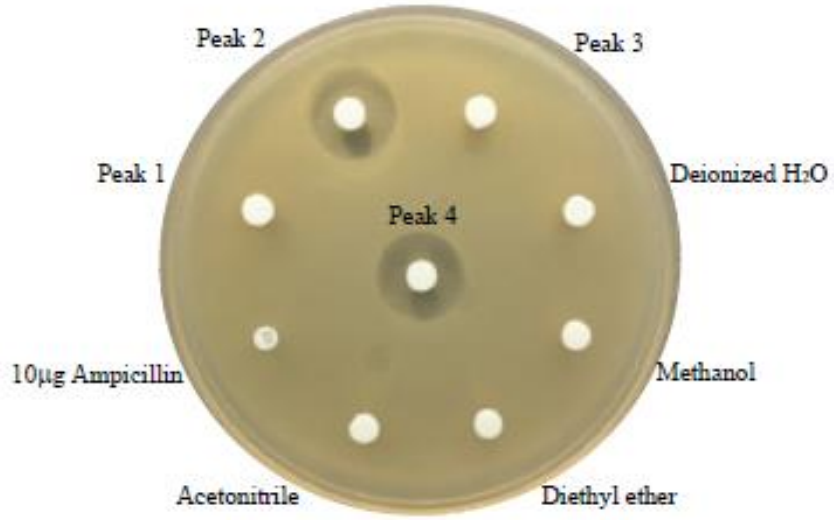


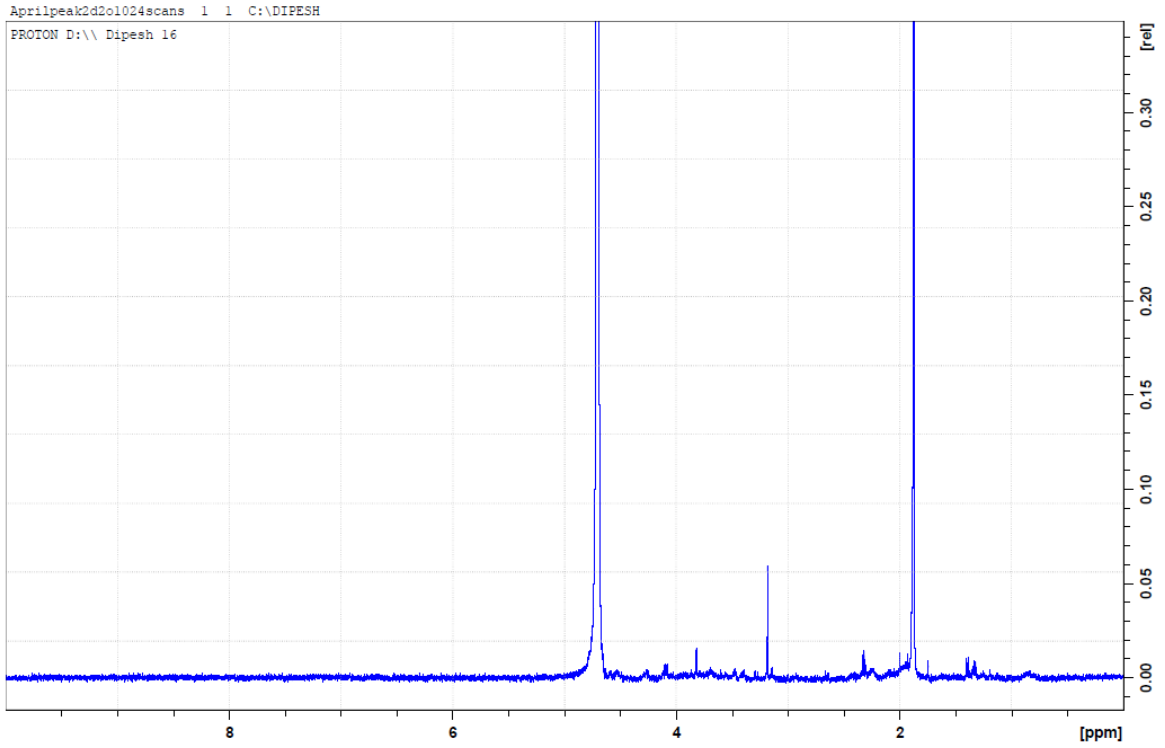
Figure 10. Kirby-Bauer disk diffusion test against multi-drug resistant *Escherichia coli* #15-124 showing bioactivity on Peak 2 and 4 of preparative HPLC collections.

Structural Elucidation of Bioactive HPLC Collections by 1-D ^{1}H-NMR

The spectrum obtained from analyzing Fraction 2 in D_2O at 1024 scans by the 500 MHz Bruker NMR spectrometer can be seen below in Figure 11. Chloroform was initially used in attempt to dissolve solid Fraction 2 after evaporation; however, this solvent was ineffective so D_2O was used to dissolve the Fraction for the remaining trials. The large singlet at 4.75ppm was the solvent residual peak of D_2O. The signal peak at 2.0 ppm was likely acetonitrile residue, which was used in preparative HPLC. The peaks present at 0.8 and 1.2 ppm are likely grease carried over from greasing the joints of the evaporation apparatus. The following interpretations of the

various splitting observed in Figure 11 should be confirmed by running 1-D ^{1}H NMR spectroscopy with a larger quantity of sample. The singlet at approximately 3.20 ppm may correspond to a *tert*-butyl methyl ether group due to its position in the spectrum. Additionally, the complicated splitting observed in the peaks appearing following the OCH_3 from 3.5-4.0 ppm may be a result of protons being nearby electron-withdrawing groups, such as alkenes, or electronegative oxygen or nitrogen atoms. The quadruplet at approximately 4.2 ppm is possibly an ethyl proton adjacent to a methyl group due to its' relatively deshielded position. Also apparent on the spectra are a quadruplet and triplet at 1.35 ppm and 4.30 ppm, respectively. No peaks were observed downfield of the D_2O singlet at 4.75 ppm. The sample did not appear to decompose after being left in a 4 ºC refrigerator for 48 hours in D_2O since the sample run for 128 scans the first day showed the same distinctive peaks after being run for 1024 scans two days later. A larger number of scans was used to produce sharper peaks that were more easily distinguished from the baseline.

A



B

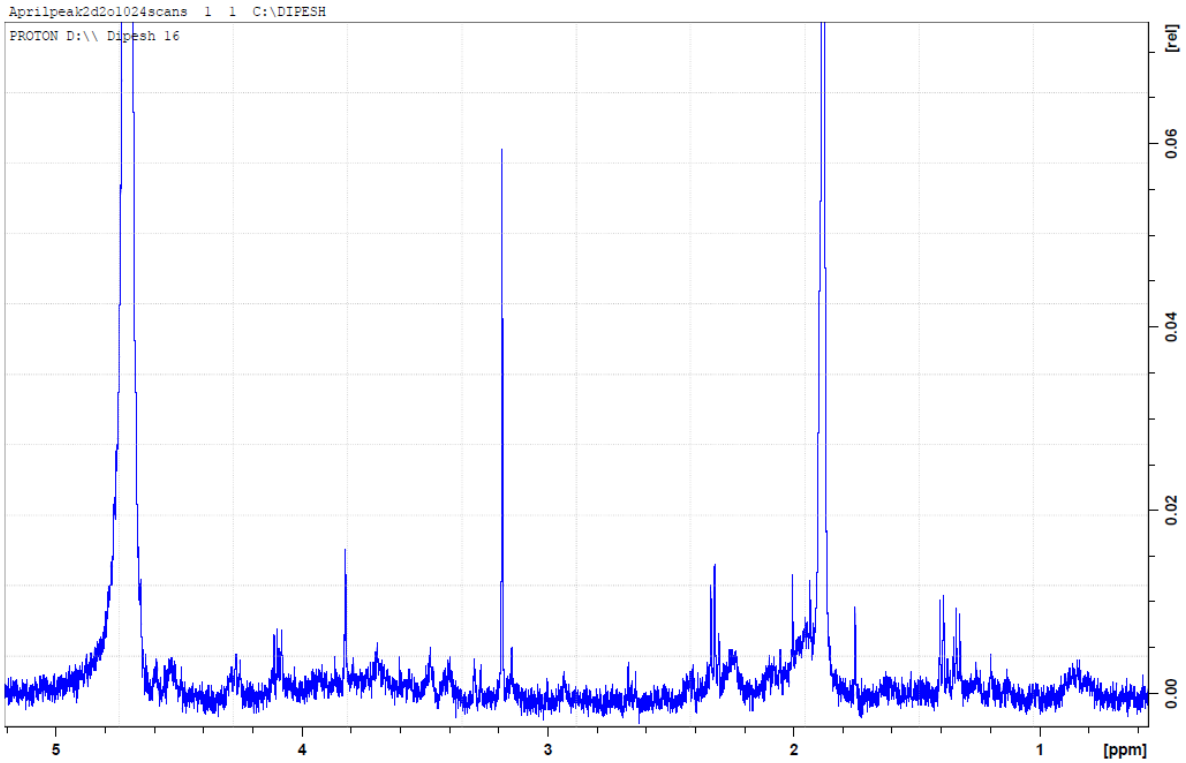


Figure 11. A) 1-D^{1} H NMR spectra of Peak 2 dissolved in deuterium oxide, NS=1024. B) zoomed in portion of A from 0 to 5 ppm.

DISCUSSION

Through antimicrobial assays, the secondary metabolites produce by *Streptomyces* sp. ICC1 have been notably effective against Gram-negative and Gram-positive target bacterium, namely laboratory and multi-drug resistant strains of *Escherichia coli* and *Staphylococcus aureus*. The antimicrobial activity demonstrated by *Streptomyces* sp. ICC1 is comparable to other members of the *Streptomyces* genus, which are among the most numerous and most versatile soil microorganisms. The genus *Streptomyces* is characterized by their large metabolite production rate, biotransformation processes, capability of degrading lignocellulose and chitin, and their fundamental role in biological cycles of organic matter (Procopio et al. 2012). The genome of *S. coelicolor*, for example, encodes many secreted proteins, including 60 proteases, 13 chitinases/chitosanases, eight cellulases/endoglucanases, three amylases, and two pactato lyases (Procopio et al. 2012). In addition, the results relate to other cave studies for instance Turkish karstic caves were reported to harbor Actinobacteria, for which over half of the isolates, were active against several microbial pathogens including Gram positive bacteria, Gram negative bacteria, yeast, and filamentous fungi (Yücel and Yamac 2010). One of the Actinobacteria, *Streptomyces* sp. 1492, had strong activity against clinical strains of MRSA, VRE, and *Acinetobacter baumannii*. Furthermore, *Streptomyces* E9 isolated from Helmcken Falls cave in British Columbia could inhibit the growth of *Paenibacillus larvae*, a causative agent of American foulbrood disease in honeybees (Kay et al. 2013). The bioactive compounds examined in this research should be tested against additional infectious microbes to determine their limitations and effectiveness.

Following liquid organic extractions with the aqueous metabolite-containing broth culture, bioactivity was present with in the organic, less polar layer of the diethyl ether-water extraction. Diethyl ether has a bent molecular structure that produces a small dipole moment, this property of diethyl ether allows the essentially polar liquid to be used to isolate both non-polar and polar compounds in aqueous extractions. The secondary metabolites were maintained in the organic layer following extractions, which suggests that the compounds are polar and may contain hydrophobic functional groups.

Analytical and preparative reversed-phase high performance liquid chromatography analysis revealed mainly polar Fractions within the diethyl ether extracts. The compounds within Fractions 1, 2 and 3 likely possess more of a hydrophilic, polar nature due to how they eluted when the program gradient contained ammonium acetate in the polar mobile phase. Ammonium acetate buffer is more polar relative to acetonitrile, which means compounds that eluted during this program gradient were polar since they were able to elute in the more polar mobile phase compared to 100% acetonitrile. Fraction 4 eluted once the program gradient returned to a 90:10 ammonium acetate buffer to acetonitrile ratio. It can be inferred that Fraction 4 was less polar since it adhered to the column until the mobile phase returned to a polarity it was able to dissolve in. From additional Kirby-Bauer disk diffusion assays, it was determined that the antimicrobial secondary metabolites from *Streptomyces* sp. ICC1 were separated into Peaks 2 and 4. It unclear whether these peaks contain entirely different bioactive compounds or if there was carry-over from the high-volume capacity of the preparative Phenomenex® Jupiter C18 column. The chromatogram suggests there may be incomplete chromatographic separation due to the close proximity of the Fractions observed. A longer program gradient at a higher aqueous

concentration could potentially establish whether these peaks possess different bioactive molecules.

1-D^{1}H NMR spectroscopy should be repeated with higher concentrations of the analyzed sample. Further analysis may intensify present peaks or reveal additional peaks, such as the highly deshielded signals found in past work by Alysha Milward (2019). The 1-D^{1}H NMR spectrum demonstrates insufficient sample may have been used due to the small size of sample peaks compared to solvent residual peaks. The high level of splitting and peaks present could suggest that the *Streptomyces* sp. ICC1 bioactive compounds are complex, however they are unlikely proteinaceous in nature due to the absence of amine groups on the spectrum. The 1-D^{1}H NMR spectrum also provides supports that the bioactive compounds are polar with hydrophobic substituents due to the hydrophobic functional groups, which contain hydrogen atoms, illustrated in the spectrum. In future work sodium phosphate should be added to the deuterium oxide to mimic physiological conditions.

Comparing the compounds produced by *Streptomyces* sp. ICC1 to those synthesized by *streptomyces* from the literature does not offer much insight since they exhibit extreme chemical diversity. The compounds range from simple amino acid derivatives to high molecular weight proteins, and macro lactones from simple eight membered lactones to different condensed macro lactones (Harir et al. 2018). For example, actinomycin was first isolated from *Streptomyces antibioticus* and is produced by many *Streptomyces* strains. The actinomycins are a family of bicyclic chromopeptide lactones sharing the chromophoric phenoxazinone dicarboxylic acid to which are attached two pentapeptide lactones of nonribosomal origin (Singh et al. 2020). Actinomycin D acts as a transcription inhibitor, binding to DNA duplexes at

the transcription initiation complex and preventing RNA polymerase elongation (Koba and Konopa 2005).

Throughout the present research bioactivity was lost in the secondary metabolite solution, further analyses are required to understand the loss of activity. The inability to inhibit the target bacteria may be due to *Streptomyces* sp. ICC1 mutation during growth in the 15 ºC incubator, contaminants being present, or the bioactive compounds not being excreted in sufficient concentrations. In addition, after centrifuging the *Streptomyces* sp. ICC1 nutrient broth solution, the pellet could contain the bioactive compounds due to adherence to the bacteria cell walls. The pellet was discarded after centrifugation and was not tested for antimicrobial activity.

CONCLUSIONS AND FUTURE WORK

The progression of antibiotic-resistant microorganisms has hindered the therapeutic efficiency of various commercially available pharmaceuticals. Therefore, an overwhelming demand for novel treatment presents itself as the likelihood of multi-resistant microbes surpassing the development of antibiotics escalates in a coevolutionary race between humans and bacteria. Extreme environments are starting to be considered as valuable sources of microbial capability, particularly in the production of pharmaceutically significant metabolites (Rangseekaew and Pathom-aree 2019). The findings of the present study aids in furthering knowledge regarding bioactive compounds produced by cave dwelling microbes.

Laboratory growth conditions should be optimized for *Streptomyces* sp. ICC1. Previous research performed by Alysha Milward (2019) found the cave-dwelling strain produced the largest amount of bioactive secondary metabolites when grown in 15 mL of nutrient broth compared to Hickey

Tesner broth and Reasoner's 2A broth. However, other media should be examined since media used for the isolation of cave actinobacteria range from routine cultivation media such as International *Streptomyces* Project medium 2 (yeast malt extract agar, ISP2) or tryptic soy agar (TSA) to selective media including humic acid vitamin agar (HV), starch casein agar (SC), starch casein nitrate agar (SCN), peptone-yeast extract/brain-heart infusion medium (PY-BHI), R2A medium, actinomycete isolation agar (AI), and Gauze's medium No.1 (Rangseekaew and Pathom-aree 2019). Moreover, isolation media that mimic the conditions of low concentration nutrients in caves such as tap water agar, 1/100 ISP2 and oligotrophic medium (M5) were also successfully used for the isolation of actinobacteria. (Lee et al., 2000b; Velikonja et al., 2014; Covington et al., 2018; Passari et al., 2018). High concentration of nutrients in standard cultivation media were reported to cause cell death in cave-associated bacteria due to osmotic stress (Ghosh et al. 2017).

In addition, optimization for the seed should be determined. The seed refers to stock cultures from which samples are taken and move into another media for selection of the stationary phase of the growth cycle and production of secondary metabolites. Optimal growth conditions likely differ to maintain the culture at logistic versus stationary growth phase. The optimal pH, temperature and oxygen content should also be established for *Streptomyces* sp. ICC1. Pervious work performed by Alysha Milward (2019) examined the bioactive present after incubation in 8 ºC, 15 ºC, and 37 ºC. A temperature of 15ºC was found to be most effective at promoting the production of secondary metabolites as determined by larger diameters of zones of inhibition against target bacteria compared to the other temperatures. However, the Iron Curtain Cave ranges between 4–12 °C depending on the time of year, which suggests a larger variety of temperatures should be tested to determine the optimal growth conditions for *Streptomyces* sp.

ICC1 (Ghosh et al. 2017). *Streptomycetes* are regarded to prefer neutral to alkaline environmental pH, the optimal growth pH range being 6.5–8.0 (Kutzner 1986; Locci 1989). However, acidophilic and alkalophilic *streptomycetes* have also been found, such as those causing potato scabies have represented phylogenetically diverse group, and included alkalophilic strains (Kutzner 1986; Takeuchi et al. 1996). Among *streptomycetes* common habitats, soil and hay have a low mean pH of 3.5–6.8 and pH of 5.5–6.5, respectively (Kutzner 1986; Erviöet al. 1990). In buildings suffering water damage and mould growth, pH varies from acid and neutral values of building materials to highly basic pH of concrete (Nevalainen et al. 1991; Andersson et al. 1998; Kontro et al. 2005).

The methodology practiced should be repeated to produce greater quantities of bioactive compounds to validate and ensure the repeatable of the results found here. Future instrumental analyses should be executed on the purified samples, primarily ^{13}C NMR and mass spectrometry, to elucidate the bioactive molecular structure of the compounds. Additionally, the minimum inhibitory concentration of the produced bioactive molecules should be established and investigated against several target strains, both Gram-positive and Gram-negative. Further bioactive screening on the pellet of the centrifuged product could explain the lost bioactivity. The findings should be compared to commercially available secondary metabolites produced by the *Streptomyces* genus to assess the efficiency of a cave-dwelling strain to the more common soil species. Further analyses should be performed on the secondary metabolites secreted by cave dwelling *Streptomyces* sp. ICC1 to determine its potential as an antibiotic producing organism for clinical use.

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APPENDIX

Table A1. The diameters of zones of inhibition =from Kirby-Bauer disk diffusion assays using *Streptomyces* sp. ICC1 broth culture before and after diethyl ether extraction, with *E. coli* #15-124 as the target organism.

Experimental Condition	Incubation time (days)	Diameter (mm)
Filtered broth	7	7.0 mm
Filtered broth	7	6.5 mm
Filtered broth	10	6.9 mm
Filtered broth	10	7.0 mm
Filtered broth	10	6.6 mm
Filtered broth	15	6.6 mm
Organic layer	15	6.8 mm
Organic layer	15	6.8 mm
Filtered broth	21	6.6 mm
Filtered broth	21	6.6 mm
Filtered broth	23	6.7 mm
Organic layer	23	6.5 mm
Organic layer	23	6.6 mm
Peak 2	40 +	8.0 mm
Peak 4	40+	7.8 mm