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Antibiotic Discovery: Extracting and Isolating a Novel Natural Product from *Herbaspirillum* sp.

Mars L. Zappia

Department of Chemistry and Biochemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Mentor: Amanda L. Wolfe

Abstract

There has only been one novel class of antibiotics introduced to the market since the late 1980s. This lull in antibiotic development is problematic because antimicrobial resistance is considered to be the largest global health crisis. If trends in antimicrobial resistant infections continue upward, drug resistance is projected to be responsible for approximately 10 million deaths per year globally by the year 2050. The isolation and modification of natural products is one of many antibiotic discovery methods with proven success. Natural products are compounds that are released by bacteria as a method of limiting the proliferation of other bacterial species. This study presents the extraction and isolation methods of a natural product elicited in monoculture from *Herbaspirillum* sp., a bacterial genus which has not previously been known to produce compounds with antibiotic activity. The natural product discussed here exhibits antibiotic activity against gram-negative and gram-positive bacteria. Past work on this project tested the compound's antibiotic activity through a cell death assay and began characterization through mass spectrometry. This work presents refined scale-up methodology, including the evaluation of media composition for bacterial culture, effective compound isolation through size exclusion extraction, and compound purification using reverse phase high performance liquid chromatography. Future research includes identifying the compound's structure before assessing its candidacy for clinical applications as an antibiotic or adjuvant.

Introduction

Antimicrobial resistance (AMR) in bacteria is considered to be the largest global health crisis due to the complexity and costs associated with treating these infections.[1] If trends in antibiotic resistant infections continue upward, there will be approximately 10 million deaths per year globally by the year 2050 (fig. 1).[2] In particular, the effects of antibiotic resistance cause high mortality and morbidity rates in vulnerable communities and countries on the basis of median income and age.[1] Widespread drug and multidrug resistance in bacteria has caused the development of effective treatments for infections in humans and animals to become increasingly complex.[1]

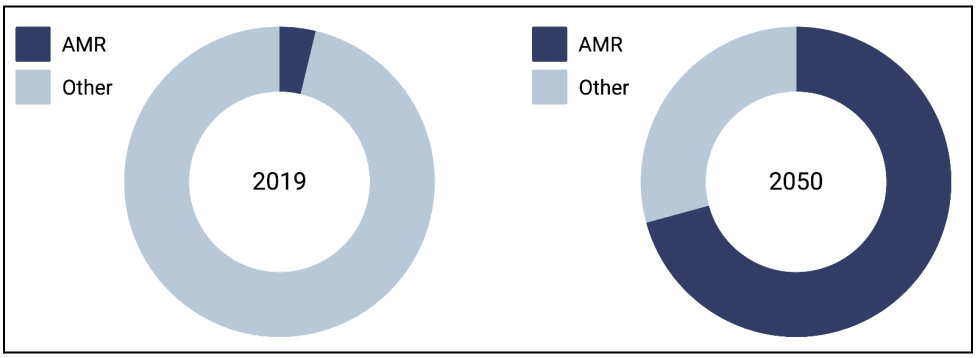

Figure 1. Global deaths projected per year due to antimicrobial resistance, 2019 vs. 2050. Note that graph size is not to scale; global deaths per year are projected to increase overall, with AMR being responsible for a much larger percentage of deaths over time. [2]

Bacterial drug resistance spreads quickly due to the increased survival benefits it confers.[3, 4] The overprescription and misuse of antibiotics in agriculture and medicine is the main contributor of widespread AMR.[1] Antibiotic resistance also develops to promote adaptability and survival in response to other bacteria.[3] AMR spreads among and within populations of bacteria through natural and acquired resistance. Acquired resistance includes horizontal gene transfer (transformation, transduction, and conjugation) as well as genetic mutations.[4] Transfer of plasmids, or extrachromosomal DNA, is the most common form of resistance gene transmission.[4, 5] Horizontal gene transfer among different taxa of bacteria has been documented for decades.[6] Modes of antibiotic resistance include: the formation of biofilms, which greatly reduces permeability of the bacterial membrane; efflux pumps, which are transport proteins that pump certain molecules like antibiotics from the cellular matrix into the extracellular space faster than the drug can take action within the celll; and the inactivation of drugs that enter into the cellular matrix by specialized enzymes that neutralize and inhibit the drug's activity.[7]

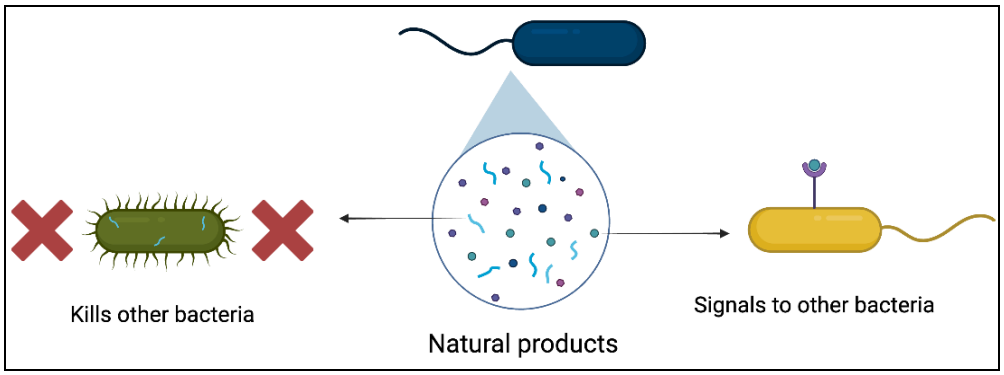

Figure 2. A few of many biological functions of natural products derived from bacteria.

Due to historic and continued public health threats imposed by drug resistance in bacteria, there is a considerable need for the discovery and development of novel antibiotics.[8] There are a number of common sources of novel antibiotics, including antibacterial antibodies, bioengineered genome expression, synthesis from known antibiotics, and natural products.[7] The use of natural products is a promising method for the development of antibiotics. From 1981 to 2014, 65% of all small-molecule drugs and 73% of all antibacterial agents that were introduced to the market and approved for clinical use were derived from natural products.[9] Natural products are compounds in nature that exhibit specific biological properties and are released by bacteria themselves (fig. 2).[9] Antibiotics are naturally produced by bacteria as a method of limiting proliferation of other bacteria in the instance of sympatric species outcompeting for space or nutrients, particularly in soil.[3] These natural products that possess antibiotic properties can be identified and isolated through screening bacterial libraries and growing bacterial species in mono- or co-cultures.

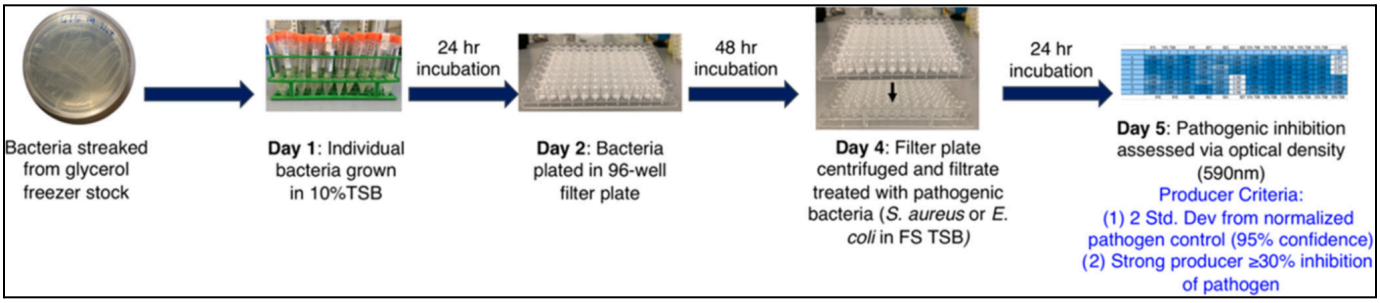

Figure 3. Wolfe Lab's high throughput screening method.[9]

The Wolfe Lab at the University of North Carolina Asheville developed a high-throughput screening method for bacterial libraries (fig. 3).[9] Using this technique, 400 strains of bacteria that were isolated from various locations across western North Carolina and the southeastern United States were grown in mono- and co-cultures with exposure to *Escherichia coli* and *Staphylococcus aureus* and evaluated for their ability to elicit natural products.[9, 10] In this screening, 115 bacteria were identified as being producers of natural products that exhibit antibiotic properties in monoculture.[9, 10]

This screening procedure identified a strain of bacteria from the genus *Herbaspirillum* that produced antibiotics.[10] Bacterial species from the genus

Herbaspirillum are found in the soil on the roots of plants, and this particular strain was isolated from soil in western North Carolina.[10, 11] It was found that this species produced compounds that exhibit antibiotic properties against gram-negative and gram-positive bacteria when grown in mono-culture during the screening process.[10] This finding is significant because bacterial species from the genus *Herbaspirillum* have not been previously known to produce compounds with antibiotic properties.[10]

After compounds were extracted and isolated from the screening process, a cell death assay was completed to identify any active compounds.[10] The active compound identified from this cell death assay was characterized using nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques.[10] Peaks produced from liquid chromatography-mass spectrometry (LC-MS) analysis of this compound were verified to be different than known antibiotics and known byproducts elicited by *Herbaspirillum* sp.[10] Further, MS allowed for the identification of the molecular ion peak of this compound and its various splitting capabilities, which allowed for the identification of nineteen possible chemical formulas of this compound.[10] MS analysis concluded that this compound's molecular ion peak is 254 m/z.[10] However, inadequate instrument resolution resulting in poor signal-to-noise ratio of ^{13}C NMR spectra for this compound did not allow for identification of the compound's specific structure.[10]

The study outlined below aims to identify the structure of this compound and evaluate its antibiotic or adjuvant potential for the clinical treatment of gram-positive and gram-negative bacterial infections. Refined scale-up methodology, including optimal media composition for bacterial culture and effective compound isolation, as well as purification through size exclusion extraction and reverse phase high performance liquid chromatography will be discussed. Future research includes identifying the compound's structure before assessing its candidacy for clinical applications as an antibiotic or adjuvant.

Methods

Safety Statement

Biosafety Level 2 (BSL-2) protocols were followed for the handling of all pathogenic bacteria and soil bacteria of unknown species.

Sterile Procedure

Aseptic technique was used for all procedures that involved bacteria or the preparation of media to be used for bacterial cultures. Gloves and working surfaces were wiped with ethanol (70%) before the procedure began, and repeated throughout the process as needed to maintain the sterility of supplies. Bacteria and media were handled under open flame. Working surfaces were wiped with bleach (10%) after handling bacteria. Pipette tips were autoclaved, and media was either autoclaved or filtered through a 0.2 µm filter. Any solid waste being disposed of that came into contact with bacteria was cleaned in bleach (10%) before entering the hazardous waste container. Bleach (10%) was added to any waste liquid that contained bacteria before it was put into waste.

Bacteria Storage

All bacteria samples, including *Herbaspirillum* sp. (UNCA Library ID: 614) and wild type (WT) bacteria *Escherichia coli* (EC) and *Staphylococcus aureus* (SA) were stored in glycerol stock (50%) at -80 °C.

Media

Citrate minimal media solution was previously found to be the most successful minimal carbon source for *Herbaspirillum* sp. growth.[10] Sterile citrate minimal media was prepared using 1 M citrate, 1 M $MgSO_4$, and 10x ppm salts [0.35 M K_2HPO_4 * 3 H_2O, 0.22 M KH_2PO_4, and 0.08 M $(NH_4)_2SO_4$].

Cultures and Scale-up

Herbaspirillum sp. cultures were prepared by placing a small amount of frozen *Herbaspirillum* sp. (UNCA Library ID: 614) stored in glycerol (50%) into 10 mL of citrate minimal media under sterile conditions. These cultures were allowed to incubate in a floor shaker at 150 rpm and ambient temperature for 48-96 hours, until visible growth had occurred. Samples were then stored in a refrigerator at 2 °C to slow down further growth. These 10 mL cultures were then placed into 3 L citrate minimal media, and incubated in a floor shaker at 150 rpm and ambient temperature for an additional 48-96 hours until growth was visible. Because *Herbaspirillum* is an aerobic genus of bacteria, bottle caps were left loose on all cultures to promote oxygen exchange and overall growth of the bacteria. Growth was determined by visible assessment of increased opacity and was not quantified using OD_{590} absorbances.

Extraction

Cellular debris was removed from cultures by centrifugation at 4,000 rpm and ambient temperature using a swinging bucket rotor. Poor pellet formation was achieved, so the supernatant was then filtered using a sterile 0.2 µm filter with a polyethersulfone membrane under vacuum to remove any remaining debris. Resin beads (Diaion HP20, 260 Å pores; 2% w/v of culture) were soaked in excess methanol for 20 minutes before being rinsed with 200-500 mL deionized (DI) water and added to the filtered 3 L culture. The bead and supernatant mixture was allowed to sit in the floor shaker for 150 rpm and ambient temperature for 24 hours before being placed in the refrigerator at 2°C for storage. The supernatant was separated from the beads using vacuum filtration and the beads were washed with 200-500 mL DI water to remove impurities. Compounds of interest that were adsorbed into the beads were eluted using 50 mL of methanol and water following a gradient of increasing methanol concentration by 20% each fraction. Solvent in the eluted samples was evaporated using assisted air drying. Beads were stored in 20% ethanol to be reused.

High Performance Liquid Chromatography (HPLC)

Reverse phase high performance liquid chromatography (Shimadzu LC-20AR, Kinetex 2.6 mm C18 100 Å LC Column 30 x 2.1 mm) was used to separate compounds eluted from the resin beads after extraction. Compounds are isolated based on their column retention time and activity in UV-Visible Spectroscopy (Shimadzu SPD-40V) under 254 nm wavelength light. HPLC was performed for 30 minutes with a 20-80% methanol to water gradient. UV active fractions were collected (Shimadzu FRC-40) and concentrated via assisted air drying followed by high vacuum drying.

Cell Death Assay

Compounds isolated by HPLC were tested for antibiotic activity in cell death assays using wild type (WT) bacteria *Escherichia coli* (EC) and *Staphylococcus aureus* (SA). Concentrated HPLC fractions were re-solubilized in minimal amounts of DMSO (<10 µL). A 96-well plate was designed in triplicate with bacteria and media controls as well as pathogens grown in concentrated tryptic soy broth (TSB). TSB media (89 µL) was added into each well, followed by pathogens (EC or SA, 10 µL) for all wells except the media only negative control. UV active fractions in DMSO (1 µL) were added into each well (1 µL). Plates were incubated for 20 hours before analysis using a microplate reader (BioTek Synergy HTX) to determine sample activity based on visible light absorbance to quantify bacterial growth (OD_{590}). Samples with relative lower absorbance are correlated with a lower pathogen growth rate, indicating that the compound's antibiotic properties inhibited pathogen growth.

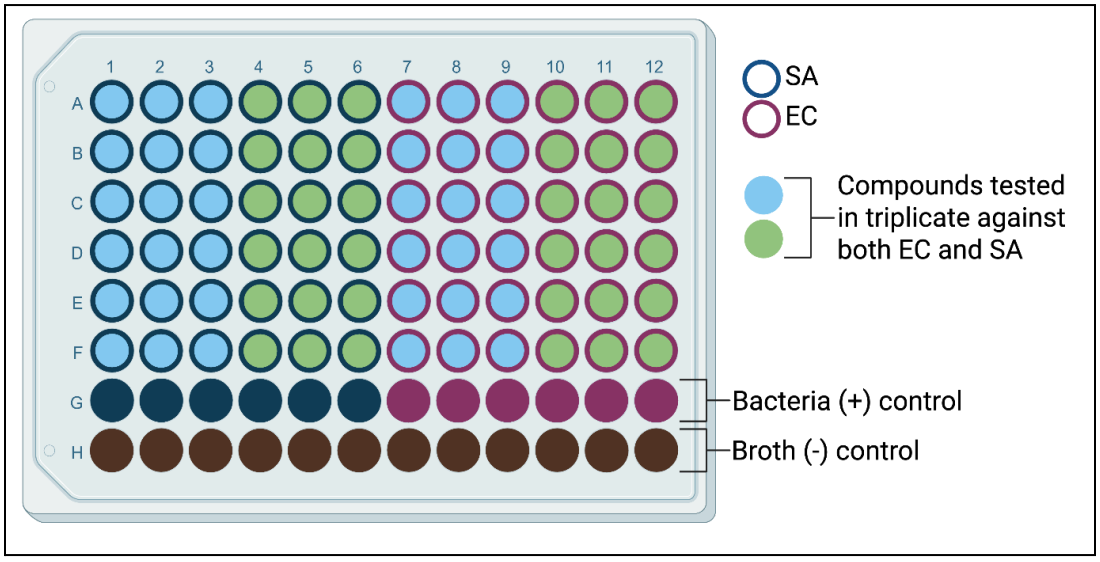

Figure 4. Example cell death assay plate setup.

Growth Curves

Overnight cultures of *Herbaspirillum* sp. (UNCA Library ID: 614) were grown in citrate minimal media and full strength TSB (10 mL). These cultures were diluted to low

concentrations of *Herbaspirillum* sp. (90 µL media:10 µL culture) and high concentrations of *Herbaspirillum* sp. (80 µL media:20 µL media) when plated in triplicate on a 96-well plate. Bacterial growth (OD_{590}) was recorded every 30 minutes for 70.5 hours using a microplate reader (BioTek Synergy HTX). Logistic growth curves were visualized in Excel.

Compound Identification

Past work on this project utilized mass spectrometry (single quadrupole Shimadzu LCMS-2020) to identify the compound's molecular weight and fragmentation patterns. MS samples were analyzed in LC-MS grade methanol. NMR (^{1}H or ^{13}C; Bruker Avance-400 MHz spectrometer, 298 K) was used to determine the functional groups present in the compound's structure. ^{1}H NMR was performed in deuterated methanol (MeOD) for 128 scans.

Results and Discussion

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7
Media conditions	citrate minimal media					10% TSB media	
Initial culture conditions	3 x 10 mL cultures grown for 72 hours (30 mL total)						
Scale up conditions	2 x 1.5 L cultures grown for 96 hours (3 L total, 15 mL initial culture added to each 1.5 L scale-up)						1 x 1.5 L culture grown for 30 hours
Growth observed	normal growth					dense growth	
Number of UV active HPLC fractions	6	6	19	12	14	9 (20 H_2O/80 MeOH and 100 MeOH extraction products combined between trials 6 and 7)	
Cell death assay results	no growth inhibition shown for EC or SA						

Table 1. Culture and purification conditions with assay results for all seven trials of *Herbaspirillum* sp. growth and natural product extraction.

Seven trials of *Herbaspirillum* sp. natural product extractions were performed, the conditions for which are summarized in Table 1. Past work identified citrate as being an adequate carbon source for minimal media that promotes optimal growth for this specific strain of *Herbaspirillum* sp. in liquid culture.[10] Because pathogen inhibition was not seen in trials 1-5, 10% TSB media was used for the cultures of trials 6 and 7 instead of minimal media to hopefully promote increased cell viability and therefore increased production of the compound of interest. In all trials, cell growth was visible after the initial 72 hour incubation for the small 10 mL cultures and after the 96 hour incubation for the larger 1.5 L cultures. After incubation, the cultures were filtered through a 0.2 µm pore membrane under vacuum to remove all of the cells. For trials 1-5, this filtering was successful in visibly removing all cellular structures, as the media went from cloudy to clear after filtering. However, for trials 6 and 7 where 10% TSB media was used for both steps of the liquid culture scale up, this filtering method was not successful in removing all cellular structures. The filtering itself was also difficult to complete, as the cells had grown so dense in the larger culture size, even when the incubation time was reduced from 96 hours to 30 hours for trial 7, that the pore membrane of the filter was quickly becoming clogged. Although altering the culture media to promote increased cellular growth might result in better production of the compound of interest, more work needs to be done to optimize the incubation times of these cultures to streamline the filtering and cell removal process.

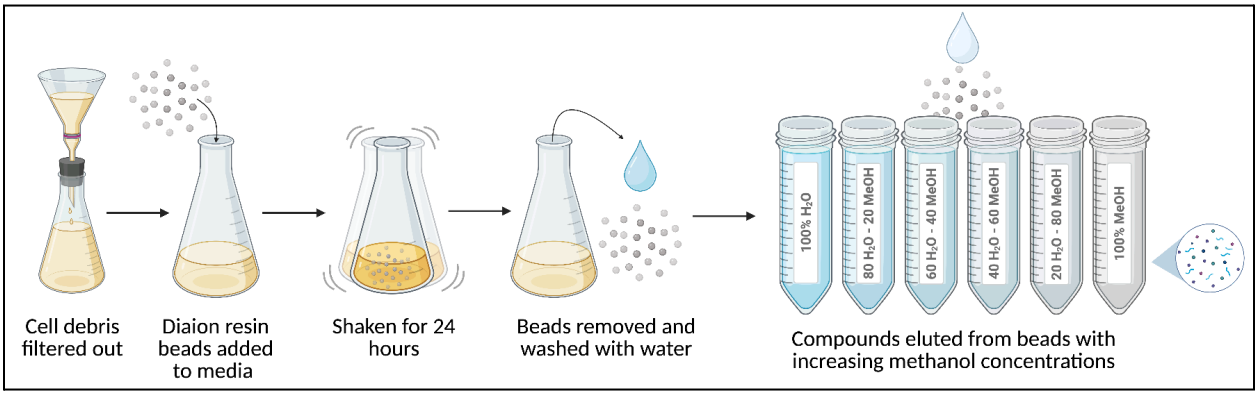

Figure 5. Compound extraction methodology.

Following cell removal via filtration, small molecules were extracted from the remaining liquid media by resin beads which operate on size exclusion principles as these beads contain micropores (~1.30 mL/g pore volume; 260 Å mean pore size) on their surface that allow small molecules to enter into the beads. When the beads are exposed to methanol, the small molecules are eluted as the methanol replaces the small molecules within the pores of the beads. After the resin beads were exposed to the remaining liquid media, compounds were eluted from the beads using six combinations of water to methanol starting with 100% water and increasing the methanol content by 20% each elution (fig. 5).

Molecules eluted in these methanol to water ratios were further isolated from one another using reverse phase HPLC, and fractions collected from HPLC showing UV activity were collected. Past work on this project discovered that the compound of

interest had a retention time of 13.5-15.2 minutes.[10] There were 4 UV active fractions eluted during the target retention window of 13-15 minutes combined between trials 1 and 2. However, none of these 4 compounds exhibited pathogen growth inhibition during cell death assay. Consistently in trials 3-7, minimal UV active fractions were eluted during the 13-15 minute time window. Instead, a majority of fractions were being eluted during the 0-3 minute and 19-22 minute time periods (figure 6). Compounds isolated during trials 1-7 also exhibited ^{1}H NMR spectra that were inconsistent with that of the target compound as determined by previous work.[10] No compounds from any of the seven trials exhibited pathogen inhibition of EC or SA in cell death assay. We suspect replication of previous results may have been challenging to achieve due to inadequate compound production.

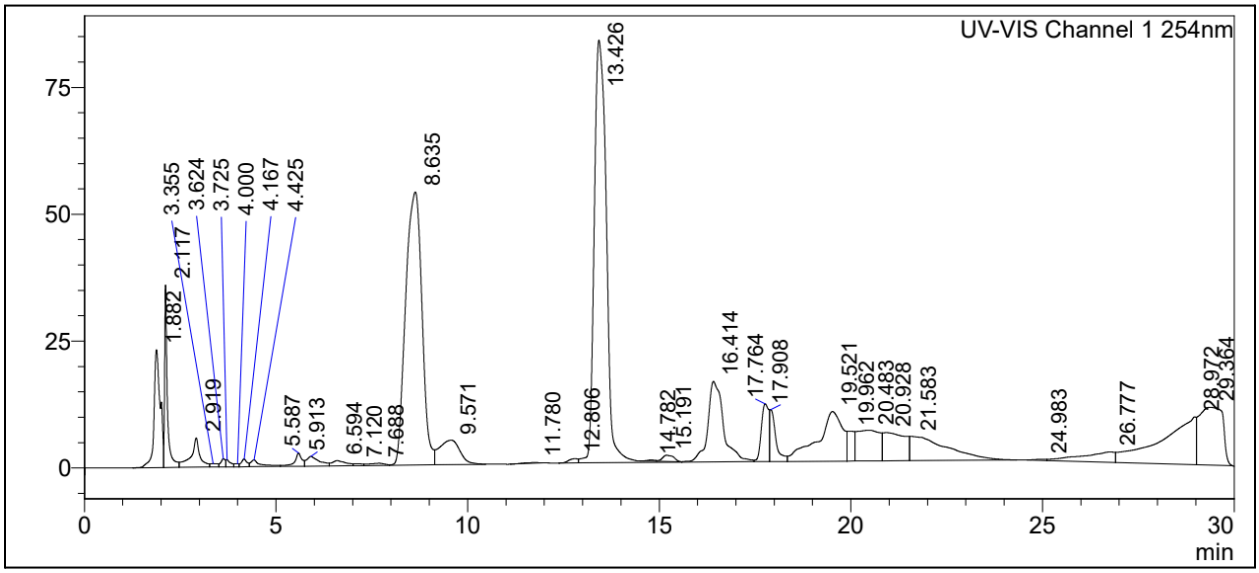

Figure 6. Sample HPLC spectra from trial 1 representing the absorbance of each compound and their retention time. Each number callout represents an individual compound with a unique absorbance that was collected by the HPLC fraction collector. Only compounds with clear peaks were retained and further analyzed in cell death assay. One compound was eluted during the 13-15 minute elution window of the target compound (retention time: 13.426 min). Instead, an unexpected number of compounds were eluted at the very beginning of the run, indicating potential inadequacy of mobile phase polarity.

To address possible scale-up complications, we created growth curves of *Herbaspirillum* sp. to gain a quantitative frame of reference to optimize our culture procedure. Growth curves visualize how the bacterial population is changing over time during the incubation period and can help optimize culture growth times, allowing for an informed incubation time that ends before the bacteria begin to enter the stationary phase and die.[12] Every replicate for both of the citrate growth curves resulted in no bacterial growth, with the exception of one replicate which demonstrated an oscillating amount of growth after about 10 hours of incubation, which is expected to be a plate reader error (Supplemental table 1). Every replicate for both of the TSB growth curves resulted in bacterial growth that follows an expected growth pattern.

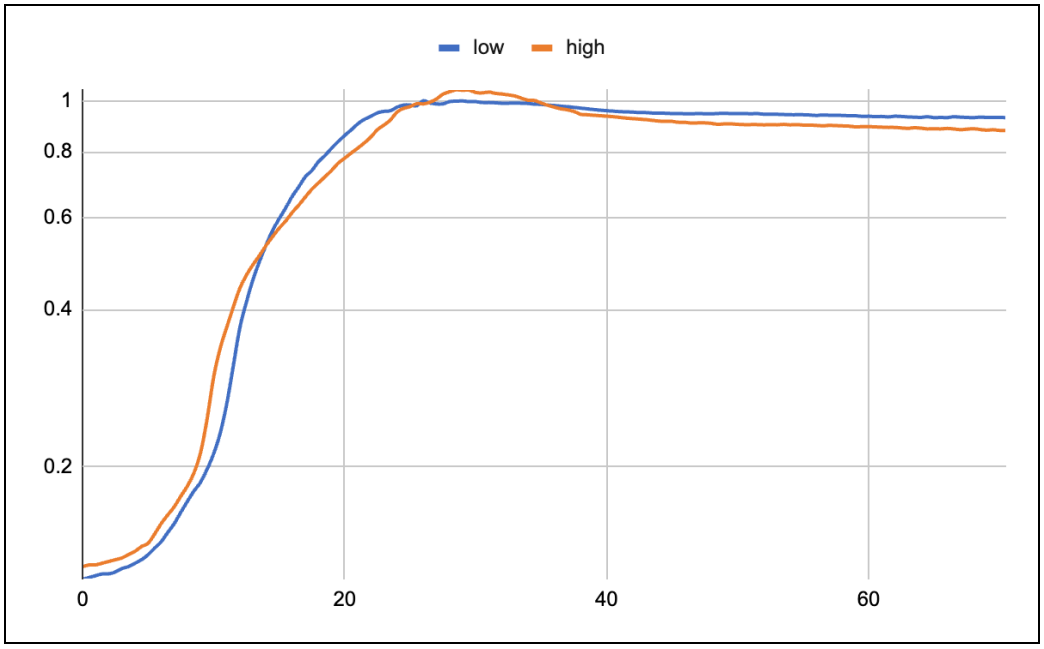

Figure 7. Growth curves of low and high concentration of *Herbaspirillum* sp. in full strength TSB media over a time period of 70.5 hours. The lag time for the low concentration was 10 hours, while the high concentration had a lag time of 9 hours. Interestingly, it took the high concentration about 2.5 more hours than the low concentration (28.5 hours and 26 hours, respectively) to obtain peak growth (OD_{590}=1) before bacterial growth began to decline.

One replicate from each of the low and high TSB growth curves that followed the most expected growth pattern was selected and plotted together (fig. 7). These two curves look to be in general agreement with each other, indicating that these growth curves could be used as a reliable reference for growing *Herbaspirillum* sp. in full strength TSB media. Specifically, this information allows us to optimize our incubation periods for scaled-up *Herbaspirillum* sp. growth in TSB media. Trials 6 and 7 of the compound extraction phase of this study were grown in 10% TSB– we maintained the same incubation times for this rich media as we did for minimal media, and both cultures grew too dense. Now, we will be able to More work needs to be done to produce reliable citrate minimal media growth curves.

Conclusions

The natural product extracted from *Herbaspirillum* sp. remains uncharacterized due to unresolved troubles with achieving successful product extraction. We believe that the trials reported in this work may not have shown pathogen inhibition on cell death assay due to inadequate concentrations of the product after extraction and purification. Minimal compound yields has been a continued issue that was also exhibited by initial iterations of this project.[10]

Bacterial growth quality is a common factor that influences natural product discovery, as poor bacterial growth can result in suboptimal product discovery.[13, 14] An

attempt was made to introduce an intermediate scale-up step of 750 mL between the 15 mL and 1.5 L culture sizes. However, the 750 mL culture did not demonstrate any visible growth despite cell density being visible in the initial 10 mL cultures. This finding, coupled with the failed citrate minimal media growth curves, necessitate further investigation into the use of citrate minimal media for *Herbaspirillum* sp. Activating the biosynthetic pathways responsible for producing valuable compounds is a common challenge faced in natural product isolation, and epigenetically relies on external and environmental cues.[15] Revisiting carbon utilization assays may identify a more effective minimal media for culturing this microbe that could result in improved compound collection. Once the compound of interest is successfully isolated, the compound will be structurally characterized. Characterization would then allow for this compound to be evaluated for its mechanism of action and candidacy as an antibiotic or adjuvant.

Acknowledgment

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References

Figures 1, 2, 4, and 5 were created with BioRender.com

(1) World Health Organization. 2021. Antibacterial Agents in Clinical and Preclinical Development: Overview and Analysis (2020).

(2) O'Neill, J. Antimicrobial resistance: Tackling a crisis for the health and wealth of nations. Wellcome Trust 2014.

(3) Clardy, J.; Fischbach, M.A.; Currie, C.R. The natural history of antibiotics. *Curr Biol* 2009, 19(11):R437-R441.

(4) Reygaert, W.C. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol* 2018, 4(3):482-501.

(5) Koraimann, G. Spread and Persistence of Virulence and Antibiotic Resistance Genes: A Ride on the F Plasmid Conjugation Module. *EcoSal Plus* 2018, 8(1).

(6) Trevors, J.T.; Berg, G. Bacterial conjugation between Escherichia coli & Pseudomonas. *Journal of Industrial Microbiology* 1989.

(7) Iskandar, K.; Murugaiyan, J.; Halat, D.H.; Hage, S.E.; Chibabhai, V.; Adukkadukkam, S.; Roques, C.; Molinier, L.; Salameh, P.; van Dongen, M. Antibiotic Discovery and Resistance: The Chase and the Race. *Antibiotics* 2022, Vol. 11 Issue 2.

(8) Salam, M.A.; Al-Amin, M.Y.; Salam, M.T.; Pawar, J.S.; Akhter, N.; Rabaan, A.A.; Alqumber, M.A.A. Antimicrobial Resistance: A Growing Serious Threat for Global Public Health. *Healthcare (Basel)* 2023, 11(13):1946.

(9) Murray, E.M.; Allen, C.F.; Handy, T.E.; Huffine, C.A.; Craig, W.R.; Seaton, S.C.; Wolfe, A.L. Development of a Robust and Quantitative High Throughput Screening Method for Antibiotic Production in Bacterial Libraries. *ACS Omega* 2019, 4 (13) 15414-15420.

(10) Ham, A.L. Extraction, Isolation, and Characterization of a Novel Compound Presenting with Antibiotic Properties from Herbaspirillum sp. *University of North Carolina Asheville Journal of Undergraduate Research* 2022, Spring 2022 edition.

(11) Batista, M.B.; Sfeir, M.T.Z.; Faoro, H.; Wassem, R.; Steffens, M.B.R.; Pedrosa, F.O.; Souza, E.M.; Dixon, R.; Monteiro, R.A. The Herbaspirillum seropedicae SmR1 Fnr orthologs controls the cytochrome composition of the electron transport chain. *Scientific Reports* 2013, 3.

(12) Buchanan, R.E. Life Phases in a Bacterial Culture. *The Journal of Infectious Diseases* 23 1918, no. 2.

(13) Zhang, H.; Wang, Y.; Pfeifer, B.A. Bacterial hosts for natural product production. *Mol Pharm* 2008, 5:212–225.

(14) Teijaro, C.N.; Adhikari, A.; Shen, B. Challenges and opportunities for natural product discovery, production, and engineering in native producers versus heterologous hosts. *J Ind Microbiol Biotechnol* 2019,46(3-4):433-444.

(15) Smanski, M.J.; Zhou, H.; Claesen, J.; Shen, B.; Fishbach, M.A.; Voigt, C.A. Synthetic biology to access and expand nature's chemical diversity. *Nat Rev Micobiol* 2016, 14:135–149.

Supplemental Information

Supplemental Table 1. Citrate minimal media growth curves (top) with low (left) and high (right) concentrations of *Herbaspirillum* sp. (denoted as 614); TSB media growth curves (bottom) with low (left) and high (right) concentrations of *Herbaspirillum* sp.