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Extraction of Antibacterial Compounds Produced by *Pseudomonas* and *Serratia* Species, and Induction of Antibiotic Production by Bacterial Competition

Kayla R. Blair
Department of Chemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Amanda L. Wolfe

Abstract

New avenues for antibiotic drug discovery are in demand due to increasing emergence of multidrug-resistant bacterial pathogens. Even as multidrug resistant bacteria become an increasing threat to global health, the discovery of new antibiotics with novel mechanisms of action has slowed over the last 20 years. The use of mixed cultures of bacteria is a relatively new method shown to induce the production of cryptic antibacterial natural products, many of which may represent novel compounds that can be isolated for further research. Here, we explore how co-culturing of diverse natural bacteria may influence expression of secondary metabolite antibiotics unseen under standard laboratory conditions. Using a high throughput assay against *Staphylococcus aureus*, pure bacterial cultures isolated from pitcher plants in western North Carolina were evaluated for their ability to produce antibiotic compounds independently or in co-culture with other species. Antibiotic producers, including *Pseudomonas* and *Serratia* species, have been isolated and identified and active fractions have been extracted and purified via preparative thin layer chromatography. Co-culture screening has identified a number of bacterial pairs with enhanced antibiotic activity, yet none with solely co-culture induced antibiotic production. ^{1}H and ^{13}C NMR, IR, and mass spectrometry data is discussed as characterization of the antibacterial compounds is ongoing.

Keywords: Co-culture, Natural Product, Extraction

1. Introduction

Antibiotics have revolutionized medicinal treatment by curing life-threatening diseases over the past 50 years.[1] However, due to increasing amounts of drug-resistant bacterial strains, there is a great need for the discovery of new classes of antibiotics. One pervasive human pathogen strain in particular is methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is resistant to β-lactam antibiotics (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporins.[2] *Staphylococcus* bacterial infections are becoming more difficult to cure due to mutations resulting in resistances to current antibiotics which makes MRSA one of the most critical emerging health issues.[3] New antibiotic compounds must be developed or discovered to combat the evolved, drug-resistant *S. aureus*.

Typically, antibacterial compounds either inhibit bacteria cell growth and proliferation or cause bacteria cell death. One mechanism of action can be to interfere with the formation of the bacterium's cell wall. The two types of cell walls that classify bacteria are Gram-positive and Gram-negative. Gram-positive have a thick peptidoglycan layer with one plasma membrane whereas the Gram-negative class have a thinner peptidoglycan layer with two lipid bi-layer membranes as seen in Figure 1.[4]

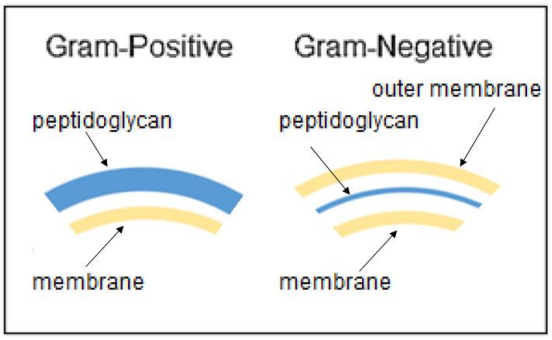


Figure 1. Gram-positive and Gram-negative cell membranes

These cell differences have allowed an evolved response of bacteria to recognize another species and produce antibacterial compounds that are cell wall-specific as to not kill the producing species but the intruder instead, minimizing competition for nearby resources.[4] These competitive environments can be mimicked *in vitro* through the use of co-culture assays in which secondary metabolic pathways can become expressed. Secondary metabolites may aid in survival against competitors as some have developed antibacterial activity towards competitive species. [5] Secondary metabolite pathways produce the bioactive constituents and their analogues that are used as antibiotic drugs.

The Food and Drug Administration (FDA) has approved both natural products and synthetic compounds for use as antibiotics such as the penicillins, macrolides, and steroid antibacterials. Natural products and their analogues account for two-thirds of new antibacterial compounds approved from 1980 to 2010.[6] Natural products dominate in the drug market due to evolutionary processes which give the producing organisms an advantage.[6] Organisms can evolve to elude the effects of man-made synthetics, however natural products can also evolve and continue to target the organism. Natural products have had a significant impact in antitumor, antimicrobial, and antihypertensive research areas.[7] Despite the promising potential of natural products, chemical redundancy in drug discovery has led to the decrease in discovery and development of new antibacterial drugs by large pharmaceutical companies.[6,7] Rediscovery as well as economic and regulatory approval challenges in antibiotic research has led to the abandonment of antibiotic development to more profitable markets.[3]

The lack of new antibiotics in development may also be due to exhaustion of previously used antibiotic sources. Many new antibiotics on the market today are synthetic analogs of already existing drugs leaving less room for the development of more antibiotic compounds. Genomic studies indicate that some groups of bacteria have dozens of secondary metabolite pathways that are not expressed in standard laboratory conditions.[7] Due to the ability of microbes to sense and respond to changes in their environment, the environmental conditions in vitro are also critical to the synthesis of microbial secondary metabolites.[7] Accessing previously hidden secondary metabolites may lead to new antibiotic production and isolation. Therefore by optimizing environmental nutrients, temperature, pH, aerations, and incubation time can greatly influence productivity of new and already existing antimicrobial compounds.[7]

An alternate approach to access metabolite pathways that are not expressed in standard laboratory conditions is by co-cultivation. Many biosynthetic genes remain silent and are not expressed in vitro and therefore the chemical diversity of microbial compounds is limited through fermentation alone.[8] In mixed fermentation, or co-cultivation, two different microorganisms are combined to try to mimic the ecological situation in which microorganisms co-exist in nature.[8] Organisms in these artificial microbial environments may express new secondary metabolic functions and produce antibiotic compounds in laboratory conditions in order to compete against or destroy the other bacteria.[4] Mirroring highly competitive environments like those in nature where space and nutrients are limited may induce the synthesis of bioactive secondary metabolites (Figure 2).[5,7]

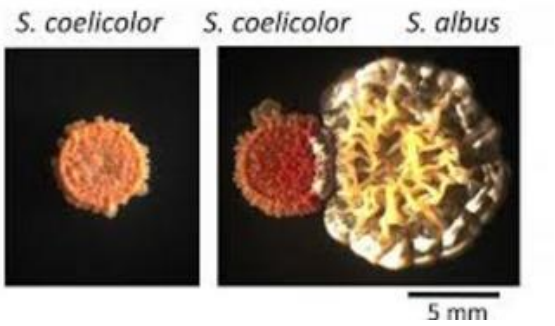


Figure 2. *Streptomyces coelicolor* (left) responds to *Streptomyces albus* by producing a red antibiotic compound in a co-cultivation setting (right).[9]

Increased antibiotic activity is only one of the benefits of co-cultivation. Studies have also seen amplified yields of metabolites, increased amounts of previously undetected metabolites, analogues of known metabolites produced, and stimulation of previously unexpressed pathways for inducing antibiotics due to co-cultivation. [7] Inducer strains in a co-cultivation setting may inadvertently create antimicrobial compounds that target other, absent species. One co-cultivation study by Mearns-Spragg et al. involving epibiotic bacteria exemplifies an isolate product from competing strains exhibiting inhibitory activity when previously exposed to *S. aureus*. Results suggest that antimicrobial compounds produced by surface-associated marine bacteria can be induced by unrelated terrestrial bacteria.[4] Mearns-Spragg et al. indicated exposure to live *Staphylococcus aureus* stimulates antibiotic activity in seven out of 12 algal epiphyte strains.[4] Tyc et al. also published activation of antibiotic production against *S. aureus* in 6% of all co-cultured combinations.[10] Antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MRSA) was also seen to be enhanced by co-cultivation.[4] With numerous possible microbe combinations and new methods in progress, the potential for co-cultivation in natural product drug discovery is promising.

Methods that provided Mearns-Spragg et al. data involved utilizing a semi-permeable dialysis membrane physically separating the species, suggesting antimicrobial production is due to a chemical secretion into the medium by the inducer strain. Alternative methodology was practiced to elicit antibiotic activity in a later case co-cultivating marine surface bacteria with *S. aureus*.[5] It was found that without use of a separating membrane, direct introduction of live cultures and the free mixing of signal compounds also induces enhancement of activity in the marine bacteria.[5] The enhancement of antibiotic activity by direct contact may be a valuable strategy for discovery of novel metabolites as seen in 2001 by Cueto et al. where a previously unexpressed pathway was induced by microbial competition.[7] Pestalone, a new benzophenone antibiotic, was produced by Cueto et al. and was shown to have activity against MRSA.

The method used by Mearns-Spragg et al. to screen for antimicrobial activity in co-cultured combinations utilized a standard paper disc assay (Figure 3).[4]

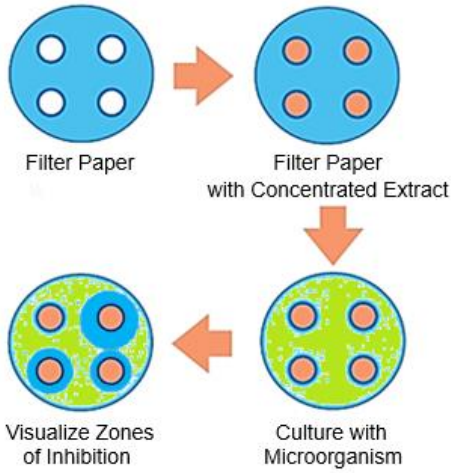


Figure 3. The Disc Diffusion method is used to determine antibiotic activity in the isolate.

This methodology involved analysis of the cell-free supernatants (CFS) after the co-culture incubation period.[11] The ability of the CFS bacterial isolates to inhibit the growth of indicator microorganisms was assayed using disc diffusion also known as the standard paper disk method. Sterile filter paper disks were infused with the CFS and placed on agar plates previously spread with the indicator strains and incubated.[11] Zones of inhibition were then measured and compared against control tests of the individual cultures' CFS.[11] The zones of inhibition were used to determine the minimum inhibitory concentration (MIC), or the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. Using the same disc diffusion method, a novel cyclic decapeptide antibiotic, loloatin B, was found to inhibit the growth of MRSA (Figure 4).[1]

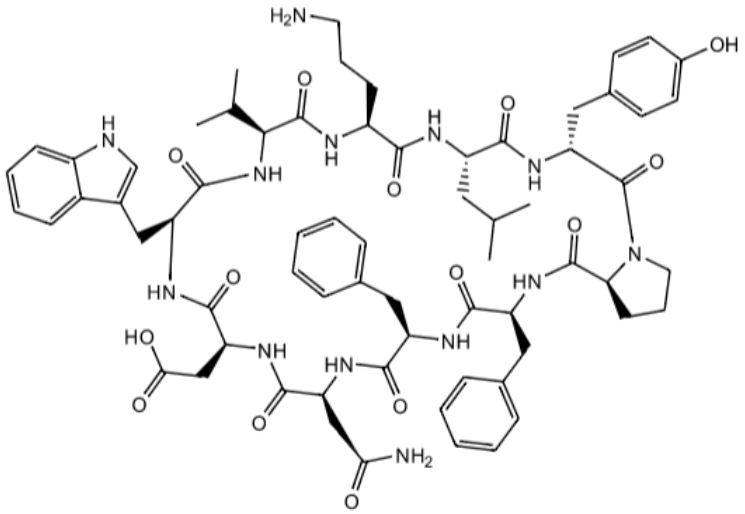


Figure 4. Loloatin B is a cyclic decapeptide antibacterial molecule with the formula [L-Val-L-Orn-L-Leu-D-Tyr-L-Pro-L-Phe-D-Phe-L-Asn-L-Asp-L-Trp].

In this work, both independent and co-cultures of various Gram-positive and Gram-negative bacteria were screened for an antibiotic compounds active against *Staphylococcus aureus* proliferation. The goal of co-culturing bacteria was to induce novel antibacterial products with inhibitory activity against *S. aureus*. Once a zone of inhibition was assessed, the bacteria was produced on a larger scale and the antibacterial compound was isolated based on previously described methodology by Seyedsayamdost et al. Antibiotic activity for each isolate was assessed against *S. aureus* using a modified disk diffusion method.[12] An isolate with antibiotic activity was further characterized in an effort to elucidate novel antimicrobial compounds combative against *S. aureus* and MRSA. Producing and isolating these natural products may aid the endeavor to find new antimicrobial drugs combative against MRSA and eliminate it as one of the multidrug-resistant infectious diseases worldwide.

2. Experimental Methods

2.1 Sterile Technique

All methods were carried out under sterile conditions. All unsterilized equipment was decontaminated by flame, including inoculating loops and container closures, before and after contact with bacteria. All media and glassware were autoclaved under pressure at 120 ºC for 20 minutes before use.

2.2 Growth And Culture Conditions

Tryptic soy broth (TSB) was diluted to 10%, 3g in one liter of dH_2O, and combined with 20g of agar powder to create low-nutrient tryptic soy agar (TSA) plates for optimum soil bacteria growth. Antibiotic positive (AB+) and antibiotic negative (AB-) *Pseudomonas fluorescens* were grown for controls on the diluted tryptic soy agar (dTSA) plates at room temperature. Various culture conditions were assessed to achieve optimal antibacterial production.

Ninety-six unidentified soil bacteria strains were grown on dTSA plates at room temperature. The Gram-positive bacteria *S. aureus* was purchased from the American Type Culture Collection (ATCC 29213). *S. aureus* was grown on full-strength Muller Hinton (MH) plates (24 h, 37 °C) and stored at 4°C. Liquid cultures of the soil bacteria were grown in tryptic soy broth (TSB) that was diluted, three grams in one liter of dH_2O. Bacteria soil samples were grown in 2.5 mL of diluted tryptic soy broth (dTSB) (48 h, room temperature). Liquid cultures of *S. aureus* were grown in 10 mL full-strength TSB (48 h, 37 °C).

2.3 Assay Method

Ninety-six soil bacteria samples were assayed for antibiotic activity for *S. aureus* in co-culture as well as assayed for independent-culture production capabilities. For co-culture, 25 µL of each of the liquid bacteria samples was combined separately with 25 µL of a different soil bacteria liquid culture. One microliter from each co-cultured combinations, independent culture samples of each soil bacteria, and AB+ and AB- controls was transferred onto replicate dTSA plates previously overlaid with a thin layer of liquid *S. aureus* culture which was grown for 24 hours at 37 °C (Figure 5). These plates remained at room temperature for 48 hours. Zones of inhibition were assessed by visual observation and the diameter of each zone was documented.

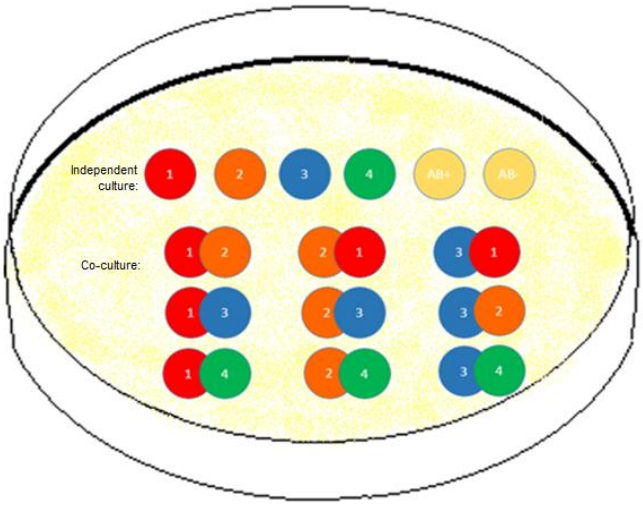


Figure 5. Schematic of an assay on an agar plate overlaid with *S. aureus*. Independent-culture bacteria, AB+ and AB- controls, and co-cultured bacteria samples are inoculated onto the plate.

2.4 Time Trial And Well Method

A 10 mL culture of antibiotic-producing bacteria was gown for 24 hours at room temperature and was then sub-cultured into one liter of broth and placed in a culture shaker at 110 RPM (Eppendorf, Excella E25). Each culture was monitored over time for optimal antibiotic production and for cell growth using absorbance at 600 nm (BIO-RAD Benchmark Microplate Reader). Antibiotic production was determined by a time trial: testing cell-free supernatant of the bacteria cultures at various time intervals for antibiotic activity against *S. aureus*. Cells were removed from the bacteria culture to create the cell-free supernatant using a 0.22 µm filter. Wells were drilled in the agar of a dTSA plate previously overlaid with liquid culture of *S. aureus* (see Assay Method) and the concentrated supernatants were pipetted into the wells. After incubation at 37 ºC, any filtered samples that had antibiotic small molecules were indicated by zones of inhibition in the *S. aureus* around the wells.

2.5 Extraction And Purification

To remove antibiotic small molecules from the bacteria culture, large scale cultures were centrifuged at 5,000 RPM for 12 minutes to remove cellular debris. The supernatant was then extracted with ethyl acetate (3x, 500 mL). The organic extracts were combined and washed with saturated aqueous NaCl. The solution was then dried with Na_2SO_4 and concentrated under reduced pressure. The crude extracts were then separated by Preparative Thin Layer

Chromatography (Prep TLC, SiO_2) or by normal phase, gradient column chromatography (SiO_2, 60% EtOAc in hexane for SS400 and SS407). Extracts were tested for antibiotic activity using the well method. Extracts were dissolved in dimethyl sulfoxide (DMSO), diluted to 1% in culture media, and pipetted into a well drilled in a dTSA plate overlaid with *S. aureus* (see Assay Method). After incubation for 24 h at 37 ºC, zones of inhibition were then visualized. Antibiotic active fractions were dissolved in $CDCl_3$ and spectra were obtained from an Oxford 400 MHz Nuclear Magnetic Resonance (NMR).

2.6 Minimal Media

The following molar quantities were dissolved in deionized water to a total of one liter and autoclaved (121 ºC, 15min): 0.035M K_2HPO_4, 0.022M KH_2PO_4, 0.008M $(NH_4)_2SO_4$, 0.0012M $MgSO_4$, and 0.025M sodium succinate.

3. Results and Discussion

Using AB+ and AB- controls, the optimal culture conditions to achieve zones of inhibition of *S. aureus* proliferation were found to be growth on dTSA plates at room temperature for 48 hours. Out of the 96 bacterial strains, 22 were reconfirmed independent producers. Figure 6 illustrates independent producers in a trial of 19 unidentified soil bacteria samples in independent culture along with AB+ and AB- *Pseudomonas fluroescens* controls. Zones of inhibition were indicated by clear circles encompassing a bacteria sample site. These zones appear at the bacteria sample numbers 2, 3, 4, 6, 12, 16, and 18. There is also a zone of inhibition at the antibacterial positive-producing *P. fluroescens* sample (top right).

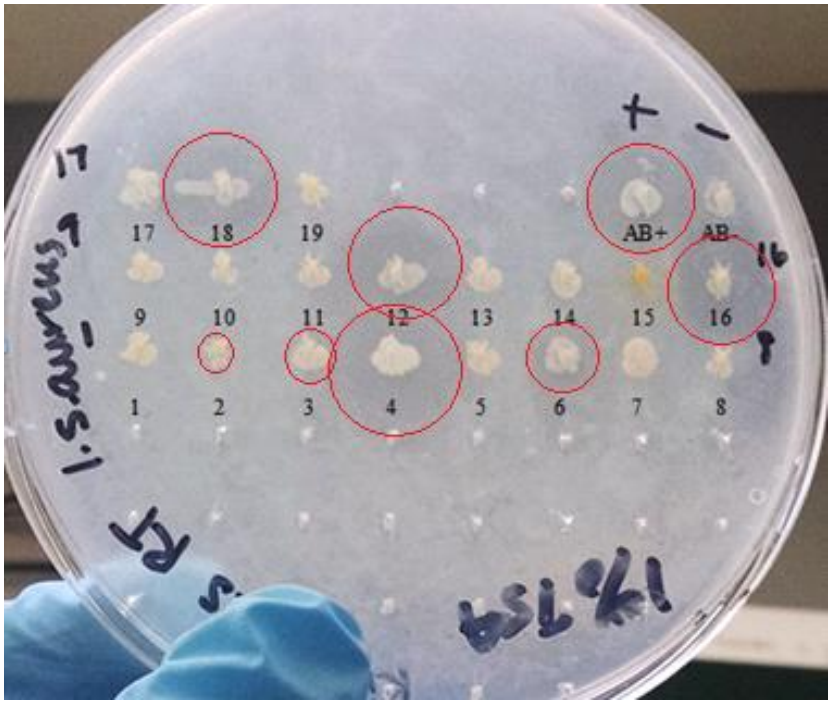


Figure 6. Nineteen independent culture samples of unknown soil bacteria strains numbered 1-19 with antibiotic positive (AB+) and negative (AB-) controls.

The independent-culture antibiotic producers are eliminated as options for co-culture assays. After over 1500 possible co-culture combinations were tested, no combination had reproducible antibiotic activity against *S. aureus*. Therefore, independent producers were chosen for further analysis. Knowing large zones of inhibition may produce more potent antibiotics, independent producers such as the *Pseudomonas* strain, SS400, and *Serratia* strain, SS407, both with large zones of inhibition were chosen.

Antibiotic production was optimized in large scale culture of SS400 by time trial shown in Figure 7. *S. aureus* inhibition appears at about 20 hours and increases as culture time is increased. Natural products were extracted from the cultures of SS400 at maximum antibiotic production time of 36.5 hours.

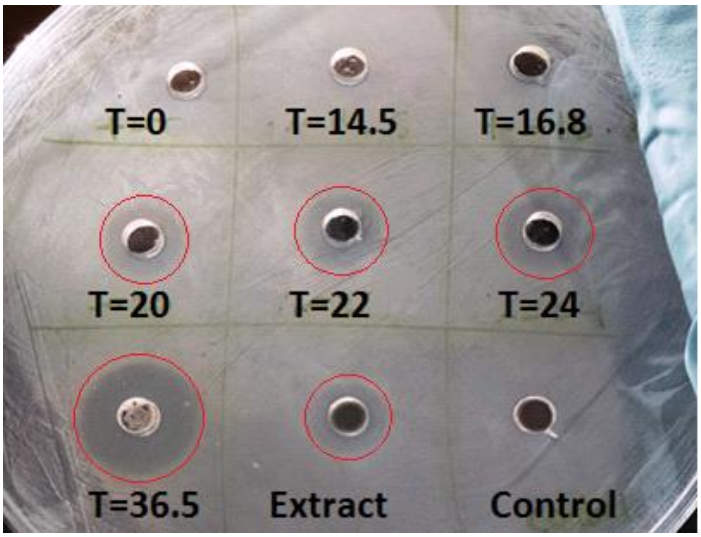


Figure 7. Time trial for antibiotic production in SS400 culture (time in hours). SS400 antibiotic positive purified extract also included.

The SS400 extract was further fractionated by Prep-TLC using 60% ethyl acetate and 40% hexane yielding five visually distinct compounds. Each compound was tested for antibiotic activity against *S. aureus* shown in Figure 8.

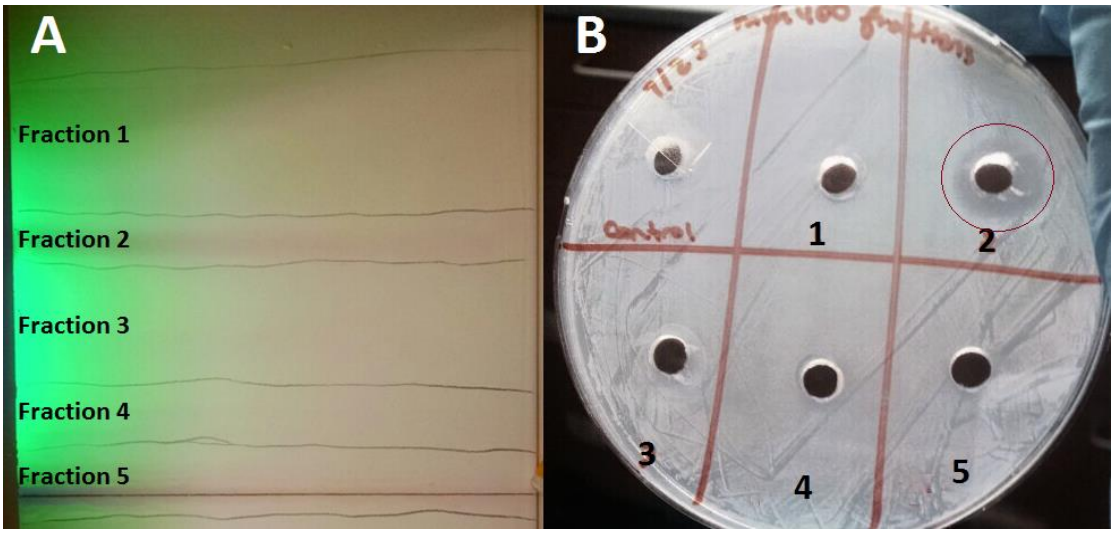


Figure 8. (A) Preparative thin layer chromatography of SS400 extract in 60% hexane, 40% ethyl acetate separated into fractions 1-5. (B) Well method to determine antibiotic activity against *S. aureus* for each fraction (1-5).

Fraction 2 was seen to have a zone of inhibition outlined by a red circle in Figure 8B. This fraction was subjected to ^{1}H NMR (supplementary data 1). When compared to an ^{1}H NMR of extracted dTSB media, there were many similar peaks in the three to five ppm areas indicating there are TSB media small molecules that contaminate the antibiotic extract (supplementary data 2). To eliminate peaks unrelated to the antibiotic compound, a minimal media was used with one carbon source, succinic acid. Succinate, the salt formed by neutralizing succinic acid, can be identified with

ease in an ^{1}H NMR spectrum (δ 2.19, s, 2H) (supplementary data 3). Large scale cultures of SS400 were repeated in minimal media and the antibiotic small molecules produced were separated and purified by column chromotography yielding a pale yellow solid (3.2 mg) ^{1}H NMR ($CDCl_3$-d_1, 400 MHz) δ 5.864 (s, 3H), 5.717 (s, 1H), 2.430 (m, 15H), 2.322 (m, 3H), 1.656 (br, 17H), 1.509 (br, 8H) , 1.291 (br, 97H) (Figure 9). ^{13}C NMR ($CDCl_3$-d_1, 100 MHz) δ 164.24, 103.32, 99.36, 33.80, 31.96, 31.87, 31.47, 29.93, 29.52, 29.15, 29.05, 28.20, 26.93, 23.41, 22.83, 14.31, 1.25 (Figure 10).

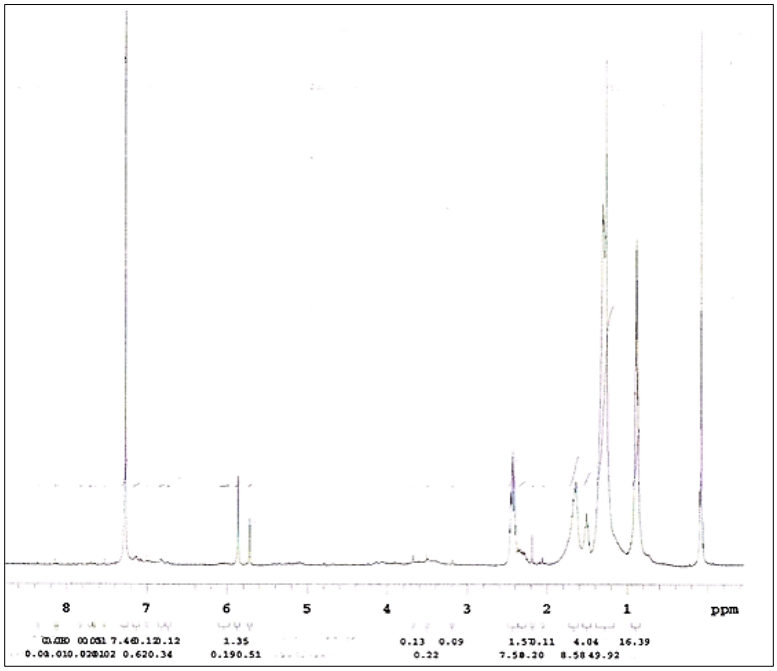


Figure 9. ^{1}H NMR spectrum of SS400 isolated antibiotic.

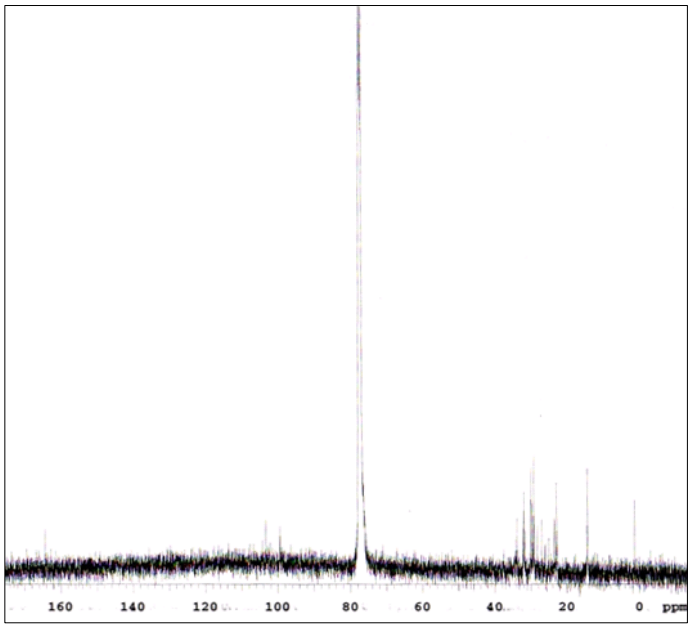


Figure 10. ^{13}C NMR spectrum of SS400 isolated antibiotic.

A 2D COSY ^{1}H NMR was performed yielding cross signals between peaks A and B, and between C and D (see Figure 11). These cross signals indicate there are local interactions of the represented nuclei pairs that are two or three bonds apart in the antibiotic compound. This data provides environmental information between protons in the molecule aiding in characterization of the compound. The ^{1}H NMR and 2D COSY spectra support that the antibiotic compound is aliphatic and may contain multiple stereoisomers as indicated by the various carbon environments in the ^{13}C NMR spectrum.

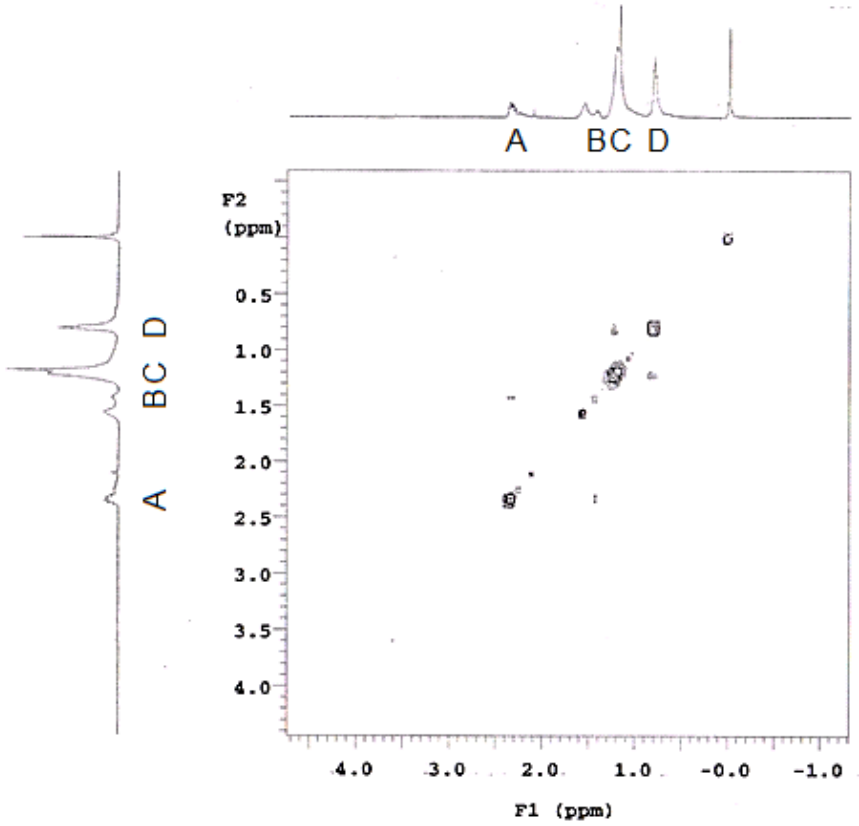


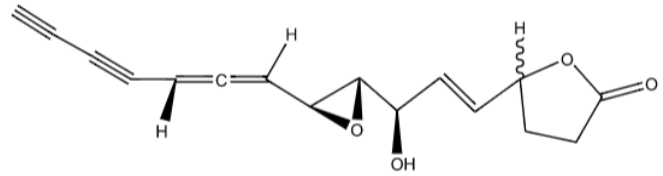
Figure 11. 2-D COSY ^{1}H NMR of SS400 isolated antibiotic compound

Large scale, 1 liter culture of SS407 *Serratia* was grown and compounds were extracted after 36 hours. Prep-TLC was performed with 40% hexane, 60% ethyl acetate yielding 6 fractions. One fraction presented with antibiotic activity against *S. aureus.* Further purification and characterization of the compound is ongoing.

4. Conclusions

New avenues for drug discovery are in demand due to increasing amounts of drug-resistant bacterial strains. Natural product antibiotics are especially desired due to their ability to co-evolve with the resistant strains. Natural products can stem from independent cultures or arise as a secondary metabolites induced by co-culturing, a relatively new method of mimicking competitive behavior *in vitro*. To date, various independent bacteria and co-cultured pairs have been screened for antibacterial activity against *S. aureus* proliferation. Similar research methods by Tyc et al. stated that 33% of bacteria screened had antimicrobial activity found in solo bacteria cultures and 6% had co-culture mediated antibiotic production. This is comparable to the 23% independent producers found in this study, however any yield of co-culture mediated antibiotic production has yet to be seen in this research. Instead, focus was turned to the independently producing strains *Pseudomonas* SS400 and *Serratia* SS407. Currently SS400 isolate characterization is ongoing with evidence supporting the compound is aliphatic with multiple isomers. Other aliphatic antibiotics known to be produced by a *Pseudomonas* are cepacin A and cepacin B (Figure 12).[13]

1)



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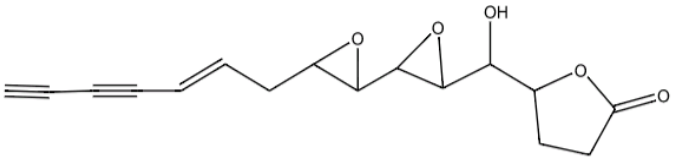

Figure 12. Cepacin A (1) and Cepacin B (2).

The peak multiplicity in the ^{1}H NMR spectrum of cepacin A suggest a mixture of diastereomers. When cepacin A spectral data was compared to the SS400 *Pseudomonas* isolate, both had protons found at a relatively high field (2.74 - 3.47 ppm) indicating that the SS400 isolate may contain epoxide protons.[13] Similarly to cepacin antibiotics, the SS400 isolate has NMR peaks that support presence of an ester (^{1}H NMR peaks 5.864 and 5.717 ppm, ^{13}C NMR 164.24 ppm) and a triple bond (^{1}H NMR peaks 2.430 and 2.322 ppm, ^{13}C NMR 103.32 and 99.37 ppm). Increasing the yield of isolated antibiotics may improve NMR spectra and IR data for further comparison of the antibiotics' structure. *Serratia* SS407 antibiotic optimization time trial, antibiotic purification, and characterization is ongoing. Screening for antibiotic-producing co-culture pairs is ongoing, prepared by Dr. Sarah Seaton's research group.

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