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Isolation of Novel Antibiotic Compounds and Other Approaches to Combat the Rise in Antibiotic Resistant Bacteria

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Abstract

As reported by the CDC in 2019, 35,000 Americans die each year from antibiotic resistant infections. There are on average 2.8 million drug-resistant infections in the US annually, and it is projected that by 2050, 10 million lives each year will be at risk of fatal infections from resistant microbes if the current trends continue. In addition to the rise of drug resistant bacteria, there has also been a steady decline in novel antibiotics developed to treat these increasingly resistant bacteria. Two main approaches to this issue are the isolation of antibiotic compounds with novel mechanisms of action against infectious bacteria and the identification of adjuvant compounds that can decrease the efficiency of resistance mechanisms. Many of the currently marketed antibiotics were discovered through isolation of easily cultured single-culture bacteria. However, since the environment of the laboratory does not reflect the natural environment for most bacteria, thousands of biosynthetic genes that may lead to antibiotics remain silent when cultured under these conditions. By creating a more natural competitive environment representative of the diversity of bacteria in soil, co-cultures of two or more bacteria can stimulate these biosynthetically silent genes and yield antibiotic production from bacteria. This work details the isolation of potentially novel antibiotics produced by cocultured rhizosphere soil bacteria. With two different co-cultures, the potentially novel antibiotic compounds produced by these bacteria have been extracted and isolated using liquid-liquid extractions, solid-phase extractions, and flash chromatography. The compound from one of the co-cultures began characterization using ^{1}H- Nuclear Magnetic Resonance (NMR) and ^{13}C-NMR data. To address the second approach to combating antibiotic resistant bacteria, several compounds in the Wolfe Laboratory's compound library were tested for general adjuvant activity with a panel of bacteria and antibiotics. Additionally, several compounds were tested in a biofilm assay against *Pseudomonas aeruginosa*.

1. Introduction

Infectious diseases are a worldwide, increasingly impactful human health concern. As of 2019, 35,000 Americans die each year from resistant infections with 2.8 million infections in the US annually.[1] Despite wide antibiotic availability, these fatal infections are on the rise due to increasing microbial resistance and, by 2050, 10 million lives a year will be at risk from resistant microbes.[2] Antimicrobial resistance occurs when a microbe survives exposure to an antibiotic with the aid of an advantageous mutation and is able to then proliferate and potentially spread resistance to neighboring microbes of different species through mechanisms of horizontal gene transfer. This resistance can be facilitated by modes of tolerance, such as reducing cell division in the presence of an antibiotic like penicillin, which targets cell wall synthesis.[3] Some of the most common resistant microbes that pose the greatest risk in hospital environments are the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*).[4] To combat this increase in resistant infections,

either new antibiotics need to be developed or alternative methods researched, such as co-dosing antibiotics with adjuvants to restore antibiotic activity by circumventing resistance mechanisms.

Despite the growing concern over increasing antibiotic resistance, large pharmaceutical companies have reduced their focus in discovery and development of novel antibiotics due to the high re-discovery rate and associated costs of natural product isolation. From 1990 to 1999, there were 20 new FDA approved antibiotics but only nine new FDA approved antibiotics from 2000 to 2014.[2,4] While there are currently antibiotics in late stage clinical trials that are near approval, there is a serious lack of early stage candidates entering the pipeline.[5] This decline in novel antibiotics alongside the increase in antibiotic resistant bacteria stresses the need for development of additional novel antibiotics. It is with this goal in mind that this paper investigates the potential isolation of novel antibiotics from soil bacteria as well as methods to increase the activity of known antibiotics through co-dosing as either adjuvants or biofilm disrupters.

Natural products (NP) are secondary metabolites created by organisms and exuded into their environment and compose a large portion of the antibiotics currently in use. Due to the evolutionary advantage gained from producing a unique antibiotic, NPs remain a valuable source of novel antibiotic compounds. NP antibiotics are generally discovered through screening for reduced bacteria growth when exposed to extracts derived from mono-cultures and co-cultures of bacteria, fungi, or other organisms.[6-8] However, NPs can also be discovered through genome mining of biosynthetic pathways and targeting of silent genes.[9-11] Hidden biosynthetic pathways and silent genes may code for the enzymes needed to synthesize active antibiotic secondary metabolites but are not activated in the traditional culture environment of the lab.

The natural environment of the soil is highly diverse with populations varying greatly by abiotic factors such as pH.[12-15] The potential of this diversity has barely been tapped due to difficulties associated with culturing bacteria in the laboratory. As most easily culturable bacteria have already been tested for activity in monoculture antibiotic activity, methods to access previously unculturable bacteria are of high interest. Their secondary metabolites are a source for potentially novel antibiotic compounds. One method for isolation and culturing of previously unculturable bacteria uses *iChip* technology. An *iChip* is placed in the soil and captures single cells in small chambers with thin barriers that allow for diffusion into chambers of environmental growth components. The captured organisms still have access to their natural environment and can be slowly transitioned to *in vitro* growth medias with a 10-15% domestication rate. Results from this method involve the cultivation of entirely different subsets of microorganisms when compared to the traditional petri-dish methods.[16] Through this method, the first novel class of antibiotics in thirty years was discovered from a previously uncultivatable bacterium. This antibiotic class, called Teixobactin, is a promising treatment for multi-drug resistant Gram-positive bacteria and no resistance has been observed *in vitro* against the lead Teixobactin compound.[17,18] Further exploration is needed to fully screen these difficult-to-culture bacteria for antibiotic activity and potentially discover additional novel antibiotics.

Another largely unexplored aspect of the natural diversity of the soil that could yield novel antibiotic compounds involves inter-species competition. This major form of interaction is missing from traditional *in vitro* mono-cultures. Many current antibiotics on the market were isolated from easily culturable mono-culture bacteria.[4,6,8,19] There remain numerous unexpressed and cryptic gene clusters which may code for secondary metabolites that have not been identified, suggesting the *in vitro* mono-culture environment may not be suitable for expression of these genes. By culturing two or more bacteria together in a co- or multi-culture, production of previously unexpressed active compounds increases by as much as 6% and may present a source of novel antibiotic compounds.[20]

The Wolfe Laboratory at the University of North Carolina Asheville (UNC Asheville), has a library over 400 bacterial samples from pitcher plant water and rhizosphere (root level) soil collected in the Rhoades Garden on UNC Asheville's campus and from an undisclosed location in South Western United States. These bacteria have been isolated, purified, and screened for antibiotic production against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*.[6,7] Within this bacterial library, 115 strains have been identified as antibacterial producers in monoculture against *S. aureus,* and 90 against *E. coli*, with genera ranging from *Pseudomonas*[6] and *Serratia*[21], known producers, to *Herbasprillum* and *Aquitalea*, which have not previously been identified as antibiotic natural product producers. All monoculture non-producers are currently undergoing high-throughput screening of activity under co-culture conditions.

Once activity of a co-culture is identified, the active compound is extracted from culturing media and isolated for characterization. The current liquid-liquid extraction and isolation procedure described below often yields only minute amounts of crude extract with not enough pure antibiotic active compound for full characterization. Acquiring suitable quantities of antibiotic compound currently requires numerous scale-ups spanning months, even with optimization of growth time and media. Through implementation of extraction techniques used in Cox et al.'s (2014) work with solid-phase resin beads, extraction yields can potentially be increased and time to characterization decreased since the beads should capture more completely the organic compounds excreted into the supernatant by the bacteria.[22] This method

circumvents the liquid-liquid extraction method which can display high incidences of emulsion, making separation of the aqueous and solvent layers difficult, and has the potential to collect compounds not previously captured in the liquid-liquid extraction.

An alternative solution to the rise in multi-drug resistant bacteria is implementation of adjuvant compounds. Adjuvants, when co-dosed with an antibiotic of low activity, potentiates the activity of the antibiotic, decreasing the minimum inhibitory concentration (MIC). The most commonly encountered pharmaceutically approved adjuvant is clavulanic acid co-dosed with β-lactam antibiotics such as penicillin and amoxicillin.[23] The mechanisms of actions of adjuvants can be highly varied, from efflux pump inhibition[22,24-26] and biofilm disruption[27,28] to blocking genes[27] and proteins responsible for antibiotic alteration.[23] Potential adjuvant compounds can be screened from natural products and their derivatives and may be a viable way for increasing the longevity of antibiotics as well as allow for reintroduction of phased-out antibiotics back into the market. The Wolfe Laboratory has started screening our library of previously synthesized compounds to test for adjuvant activity against a panel of bacteria, *S. aureus*, *E. coli*, *B. subtilis*, and *P. aeruginosa*, with penicillin, amoxicillin, vancomycin, and erythromycin.

Finally, in addition to developing new small molecule antibiotic and adjuvant therapies to treat resistant bacteria, it is also necessary to overcome virulence factors, such as biofilm formation, that prevent small molecule therapies from reaching their biological targets. Biofilms are sessile bacterial communities that often include several species of bacteria cohabitating in a matrix of exopolysaccharides, proteins, and nucleic acids.[27] Biofilms represent a major human health concern due to the nature of the biofilm matrix allowing for protection from antibiotics, with up to 1000 times more resistance, and the increased incidence of chronic infection.[29,30] Biofilm based infections are particularly concerning when related to catheters and other inserted medical devices with infection sourcing from the healthcare professionals inserting the device or from the patients themselves.[31] Approximately 12 million Americans are impacted by these biofilm-based and infection with an economic burden of over $6 billion.[32,33] Despite the high rate of incidence, there are no FDA-approved methods for directly targeting biofilm prevention or disruption. By screening against the biofilm forming bacteria, *P. aeruginosa*, biofilm inhibition or disrupting compounds may be identified and potentially used as an adjuvant in conjunction with other antibiotics as a way to combat both biofilm-forming bacteria and drug resistant bacteria. In a similar line of thought, a portion of the Wolfe Laboratory compound library was screened for biofilm disruption activity against *P. aeruginosa*.

This paper describes methods for extracting, isolating, and characterizing novel antibiotic compounds from soil bacteria mono- and co-cultures as well as assays for testing compounds for their adjuvant activity and biofilm disrupting capabilities. Included within are improvements of isolation and purification methods yielding greater quantities of isolated antibiotic compound leading to the eventual complete characterization of the unknown compound as well as some preliminary results from the adjuvant and biofilm assays.

2. Methodology

All bacterial work was done under a flame using sterile conditions. All media was either autoclaved (121 °C) or filtered through a 0.2 μM Polyethersulfone (PES) filter. All bacteria were stored in 50% glycerol stock at -80 °C and streaked out onto 10% Tryptic Soy Agar (TSA) (3 g Tryptic Soy Broth and 20 g Agar per 1 L deionized (DI) H_2O) plates for laboratory use. Pathogenic bacteria used included *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), *B. subtilis* (ATCC 11774), and *P. aeruginosa* (ATCC 9027).

2.1 Culture Scale-up

2.1.1 mono-culture tryptic soy broth scale-up (1 - 9L)

Initially, $1/100^{th}$ the desired 10% Tryptic Soy Broth (dTSB) (3 g per L DI H_2O) volume (1 – 9L) was inoculated by a singular bacterial colony and shaken (130 RPM on an Excella E25 Incubator Shaker) to allow oxygen exchange for 24 hours at 26 °C. The samples were transferred to a dTSB solution of $1/10^{th}$ the desired volume and shaken for 24 hours at 26 °C. The samples were transferred to a dTSB solution of the desired volume and shaken for 96 hours at 26 °C.

2.1.2 co-culture tryptic soy broth scale-up (1 - 9L)

After scaling up to the full desired volume as described in 2.1.1 and shaking for 24 hours at 26 °C, half of each final volume solution was mixed with half of the corresponding co-culturing bacteria's solution and shaken for 48-96 hours at 26 °C.

2.2 Antibiotic Extraction

2.2.1 liquid-liquid extraction

After scaling up, bacterial solutions were centrifuged at 4,000 rpm and 26 °C for 20 minutes, and the supernatant was collected. The supernatant was then extracted using increasingly polar solvents: hexane, diethyl ether, and ethyl acetate. After collection, the organic solvents were subjected to a brine (saturated NaCl) wash and dried over anhydrous sodium sulfate. Extracts were concentrated under pressure, and the final crude product was weighed.

2.2.2 cell death assay

Crude product from each organic solvent was diluted with 10 μL of Dimethyl sulfoxide (DMSO) per 1 mg of crude. One μL of the dissolved product was added to a 96-well plate in quadruplicate and diluted with 89 μL of Full-Strength Tryptic Soy Broth (FS TSB) (30 g in 1 L) and 10 μL of FS TSB overnight cultures containing either *S. aureus* or *E. coli.* Chloramphenicol and DMSO only columns were used as positive and negative controls. The plate was shaken for 24 hours at 36 °C. Antibiotic activity for each organic solvent was determined using a Biotek plate reader (OD_{590}) after 8 hours and 24 hours of incubation as a measurement of the density of cells within the liquid culture.[7] An active compound would inhibit growth and kill cells resulting in lower absorbance values. All following extractions used only the optimized organic extraction solvent with scale-ups of 3-9 L.

2.2.3 solid-phase extraction

Diaion HP20 resin beads (2% w/v) were rewetted in excess CH_3OH (methanol (MeOH)) and allowed to soak for approximately 15 minutes before decanting off the excess MeOH. Following centrifugation of the bacterial solution, the beads were poured into the scale-up supernatant and agitated for an hour up to 24 hours. The beads were loaded onto a column and six fractions were extracted with DI H_2O (1 L), 20% MeOH (1 L), 40% MeOH (1 L), 60% MeOH (1 L), 80% MeOH (1 L), and 100% MeOH (1 L). Fractions were concentrated under pressure and the final crude product was weighed. Active fractions were identified with the previously described cell death assay in 2.2.2.

2.3 Antibiotic Isolation and Characterization

2.3.1 isolation

Collected crude product, if greater than 25 mg, was loaded onto a normal phase gradient column chromatography (SiO_2, 0%-100% ethyl acetate/hexane). Collection vials were monitored with Thin Layer Chromatography (TLC) and visualized with both UV-vis (254 nm) and potassium permanganate ($KMnO_4$) stain and heat. Collection vials containing related fractions were combined, concentrated under reduced pressure, and weighed. Each fraction was tested on the previously described cell death assay for antibiotic activity.

2.3.2 preparative thin-layer chromatography (PTLC) purification

For finer-scale purification (< 25 mg crude product or extract) active fractions were purified using SiO_2 preparative TLC (PTLC, 5-7 mg per plate) and separated using 50:50 ethyl acetate/hexane. Compounds were visualized with UV-vis (254 nm) and scraped off the plate with a razor blade. Compounds were recovered by collecting silica in a pipette and washing with ethyl acetate. Collection vials were combined, concentrated, and weighed. Each compound was tested on the previously described cell death assay for antibiotic activity.

2.3.3 antibiotic characterization

Characterization began upon obtaining a significant quantity (approximately 20 mg) of antibiotic extract. ^{1}H-NMR, ^{13}C-NMR data was obtained for use in determining the structure of the antibiotic compound.

2.3.4 415/565 extraction and purification conditions

The active compound eluted in hexane with the liquid-liquid extraction method. Gradient column chromatography yielded optimum separation (1%, 5%, 10%, 20%, 50% ethyl acetate). With the solid-phase extraction, active compounds eluted in the 0% MeOH fraction and 60%, 80%, and 100% MeOH fractions. The compound that elutes in the 60%, 80%, and 100% MeOH fractions was fluorescent under short-wave UV light and stains with $KMnO_4$.

2.3.4 582/593 extraction and purification conditions

The active compound eluted in hexane with the liquid-liquid extraction method. Gradient column chromatography yielded optimum separation (1%, 5%, 10%, 20%, 50% ethyl acetate). With the solid-phase extraction, active compounds eluted in the 20%, 80%, and 100% MeOH fraction. The compound that elutes in the 80% and 100% MeOH fractions stains with $KMnO_4$ and does not fluoresce under UV light.

2.4 Microbial Characterization

2.4.1 genomic purification

Pellets of cells (2x 10^9) were harvested via centrifugation of overnight cultures grown in 3 mL dTSB. Using the PureLink™ Genomic DNA Mini Kit and protocol, bacterial DNA was extracted, and Gram-negative procedures were followed for lysing all bacteria. Purified bacterial DNA concentration was measured using the NanoDrop and stored at -20 °C.

2.4.2 PCR procedure

To identify bacteria at the genus level, a 16S rRNA fragment was amplified and sequenced using the universal 16S rRNA primers, 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT). The polymerase chain reactions (PCR) vials contained 5x Phusion Green HF Buffer (10 μL), 10mM dNTP (1 μL), Phusion DNA Polymerase (0.5 μL), 10 μM Universal Primers (2.5 μL), 100ng/mL template, and nuclease-free water up to 50μL. When conducting colony PCR, 2.4.1 was bypassed and instead of adding purified template DNA, a single colony was stirred into the PCR reaction mixture with a pipette tip. A touchdown thermocycler method was used. Once loaded into the thermocycler, the DNA was denatured at 98 °C (5 min), then repeatedly copied at the 16S site for 24 cycles of 98 °C (10 sec), 72 °C (30 sec) (ΔT= -0.5°/cycle), and 72 °C (60 sec), then 12 cycles of 98 °C (10 sec), 60 °C (30 sec), and 72 °C (60 sec)The PCR ended with a final extension period at 72 °C (2 min) before being held at 12 °C continuously until retrieval.

To determine success of the PCR procedure, each PCR product was injected into a 1% agarose gel composed of 0.5 μg/mL ethidium bromide in 1x TAE for size comparison to Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb). After running the gel at 100 mV for approximately 20 minutes for suitable separation, PCR fragments were visualized using UV-vis. Successful PCR products were purified using QiaQuick PCR Purification kit (Qiagen) and procedure and DNA concentration was measured using the NanoDrop and stored at -20 °C.

In the case of multiple bands, a gel extraction was used to purify the PCR product. A Monarch® PCR and DNA clean up kit was used, and the resulting DNA concentration was measured using the NanoDrop and stored at -20 °C.

Preparation of purified PCR products for send-off required dilution of products to 4 ng/μL with 25 pmol 27F primer, and nuclease-free water for a total volume of 15 μL. Samples were shipped to GeneWiz (Cambridge, MA) for sequencing. Genus level identification was generated from input of sequence results into National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) with results acceptable in the >96% range of genetic match.

2.5 Biofilm Inhibition Assay

2.5.1 biofilm inhibition screening assay

Overnight cultures were prepared of *P. aeruginosa* in FS TSB. In columns 1-9 in a 96 well plate, 5 μL *P. aeruginosa*, 94.5 μL FS TSB and 0.5 μL of test compounds (n=6) were added to each well. Columns 10-12 contained ciprofloxacin controls, DMSO controls, and TSB-only controls respectively. Plates were sealed with parafilm and incubated for 24 h at 37 °C, shaking slowly. Plates were then washed two times with 120 μL DIH_2O with the "tap and flick" removal technique, and then dried upside down (with lid on) in an incubator for 45 min-1 h. Then 100 μL of crystal violet (0.05%) was added and allowed to sit for 30 min before excess crystal violet was removed. The plates were washed gently with 120 μL DIH_2O repeatedly until clear and then dried upside down (with lid on) in incubator for 30 min. To dissolve biofilm-bound crystal violet, 100 μL 95% ethanol was added and allowed to sit for 30 min. Biofilm growth was measured with a Biotek plate reader (OD_{570}).

To determine the minimum concentration needed for biofilm disrupting activity, a serial dilution was used. A serial dilution plate was prepared with a ten-fold dilution in DMSO of the stock (100 mg/mL) down each row for an end total of 9 μL in each well. The ciprofloxacin control was also serially diluted to check for proper dilution. Overnight cultures were prepared of *P. aeruginosa* in FS TSB. In columns 1-9 in a 96 well plate, 5 μL *P. aeruginosa*, 94 μL FS TSB and 1 μL of compound (n=3-4) was added to each well based on the dilution scheme of the master plate. Columns 10-12 contained Ciprofloxacin controls, DMSO controls, and TSB-only controls. The rest of the procedure is the same as above.

2.6 Adjuvant Assay

2.6.1 antibiotic master plate

Vancomycin, erythromycin, penicillin, and amoxicillin stocks (1 mL) were made in DMSO (or sterile water for vancomycin) at a concentration of 25600 μg/mL and vortexed to solubilize. Dilution concentrations (25600 μg/mL, 12800 μg/mL, 6400 μg/mL, 3200 μg/mL, 1600 μg/mL, 800 μg/mL, 400 μg/mL, 200 μg/mL, 100 μg/mL) were prepared in vials with DMSO (sterile water for vancomycin) from the stock prior to plating. In a 96-well plate, 200 μL of DMSO was added to columns 1, 11, and 12 and 200 μL of antibiotic was added in the increasing concentrations from column 2 through 10 (Figure 1). The antibiotic master plate was then parafilmed and stored at -20 °C.

2.6.2 compound master plate

The Wolfe Lab's library of synthesized compounds was prepared into 10000 μM and 4000 μM 0.5 mL DMSO stocks. According to the finished plate layout (Figure 1), 14 μL of compound was added to each well of a 96 well plate with both a 10000 μM plate and a 4000 μM plate. The compound master plates were then covered with parafilm and stored at -20 °C.

2.6.3 adjuvant assay

Overnight cultures were prepared in FS TSB and incubated at 37 °C for 12-18 h (12 h for *S. aureus* and *E. coli*, 18 hours for *P. aeruginosa* and *B. subtilis*). Added to a 96 well plate according to the plate layout (Figure 1) in column 1-11 was 178 μL FS TSB, 20 μL bacteria, 1 μL from compound master plate, and 1 μL from antibiotic master plate. In column 12 198 μL FS TSB, 1 μL from compound master plate, and 1 μL from antibiotic master plate was added. Plates were sealed with Glad Press 'n Seal and shaken while incubated and shaken for 18-24 h at 37 °C. Adjuvant activity was measured with a Biotek plate reader (OD_{590}) with adjuvant activity being determined by a reduction in the MIC for the paired antibiotic.

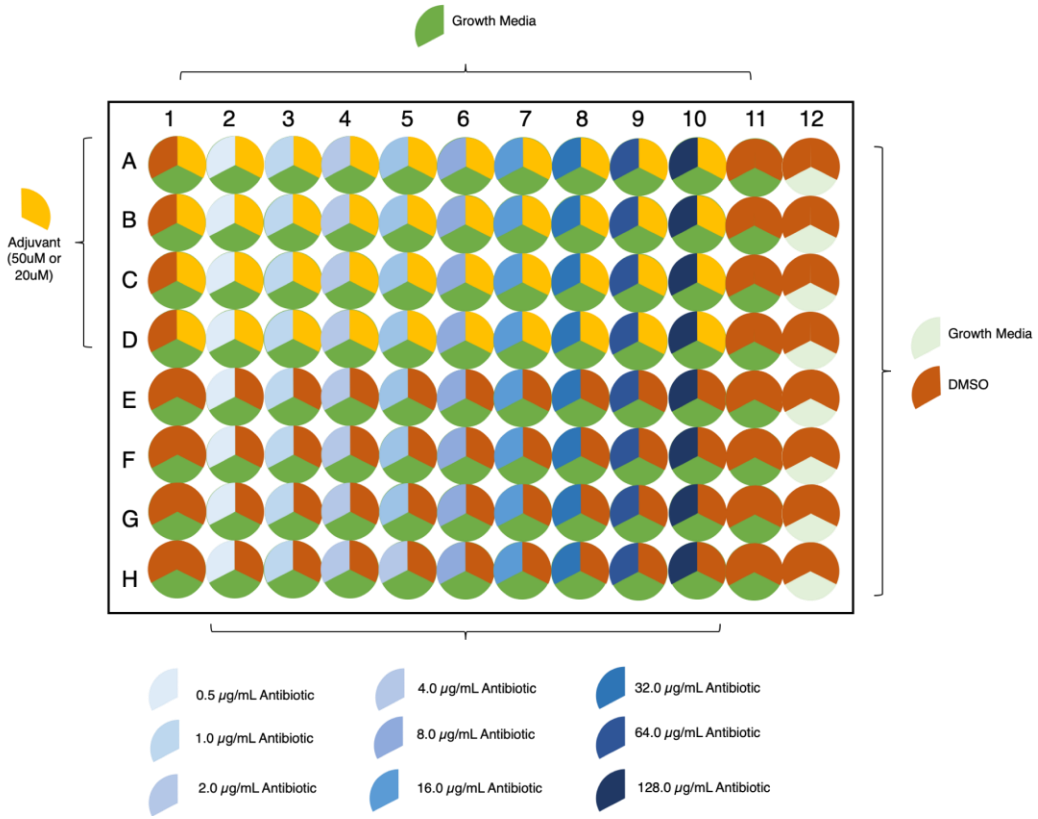


Figure 1. Adjuvant assay plate layout

3. Results and Discussion

3.1 Antibiotic Isolation

Extraction and isolation of antibiotic compounds from one monoculture and two co-cultures has been undertaken (Table 1). Details of each culture are outlined below. Initial extraction methods using liquid-liquid extraction proved to be highly inefficient, yielding a fraction of a milligram of pure compound for each liter of scale-up culture. However, with the implementation of a solid phase extraction, yields have greatly increased, likely due to the increased ability of the resin beads to capture organic compounds. With the improvement of extraction methods, the overall projects have moved forward towards characterizing the potentially novel antibiotic compounds.

Table 1. Antibiotic active fractions from mono- and co-cultures.

	614	*415/565*		*582/593*
	Fraction B	Fraction B	Fraction D	Fraction E
Active Against	*S. aureus* and *E. coli*	*S. aureus*	*S. aureus*	*S. aureus*
Rf Value (% Ethyl Acetate)	0.306 (20%)	0.547 (20%)	0.579 (50%)	0.314 (50%)
Total from Liquid-Liquid Extraction *(Pure Compound per Liter of Scale-up)*			3.1 mg (0.08 mg/L)	4.4 mg (0.12 mg/L)
Total from Solid Phase Extraction *(Pure Compound per Liter of Scale-up)*			24.5 mg (0.68 mg/L)	3.1 mg (0.52 mg/L)

3.1.1 SS 614 monoculture

The monocultured *Herbaspirillum* bacteria, SS 614, was found by previous researchers in the Wolfe laboratory to have strong antibiotic activity against Gram negative *E. coli* and moderate activity against Gram positive *S. aureus* in citrate minimal media after 96 hours of growth (Table 1). The responsible compound was isolated from large liquid culture scale-ups and partially characterized with NMR, IR, and Mass spectroscopy data (not included). Due to the complexity or impurity of this potentially novel antibiotic compound, the full structure was not readily deduced from these data and additional samples were difficult to acquire due to inconsistent yields and inability to fully purify. With improvements in isolation techniques, this monoculture could potentially be revisited as a source of a novel compound active against both Gram-negative and Gram-positive bacteria.

3.1.2 SS 415/ SS 565 co-culture

Random screening of non-producing mono-cultured bacteria in co-cultures with a high-throughput screening methodology showed strong and moderate activity of the SS 415/ SS 565 (415/565) coculture against *S. aureus* and *E. coli* respectively with neither bacterium having antibiotic activity in monoculture.[7] When scaled up, only activity was found against *S. aureus.* SS 415 has not yet been identified but stains as a gram-negative rod (Figure 2). This bacterium grows in a filamentous manner in dTSB and requires 48 h at the 30 mL scale to reach exponential growth.

SS 565 stains as a gram-negative rod (Figure 2) and was identified through 16S rRNA sequencing as a bacterium from the genus *Flavobacterium*, which can fix nitrogen, are nearly ubiquitous in soil and water, promote plant health, and some of which are pathogenic to fish.[34-36] While antimicrobial properties of some *Flavobacterium* have been noted against *E. coli*, *S. aureus*, and *B. subtilis*, only a single compound, Flavocin, has been identified from one strain.[34,37] This genus was noted to show antibiotic activity three times more often in co-cultures than in mono-cultures.[20] Additional reports of antimicrobial activity did not report the responsible active compounds, leaving a large opportunity for discovery of novel compounds produced by this genus. [20,21,34-38] This bacterium grows in a cloudy manner in dTSB and requires 24 h at the 30 mL stage to reach exponential growth.

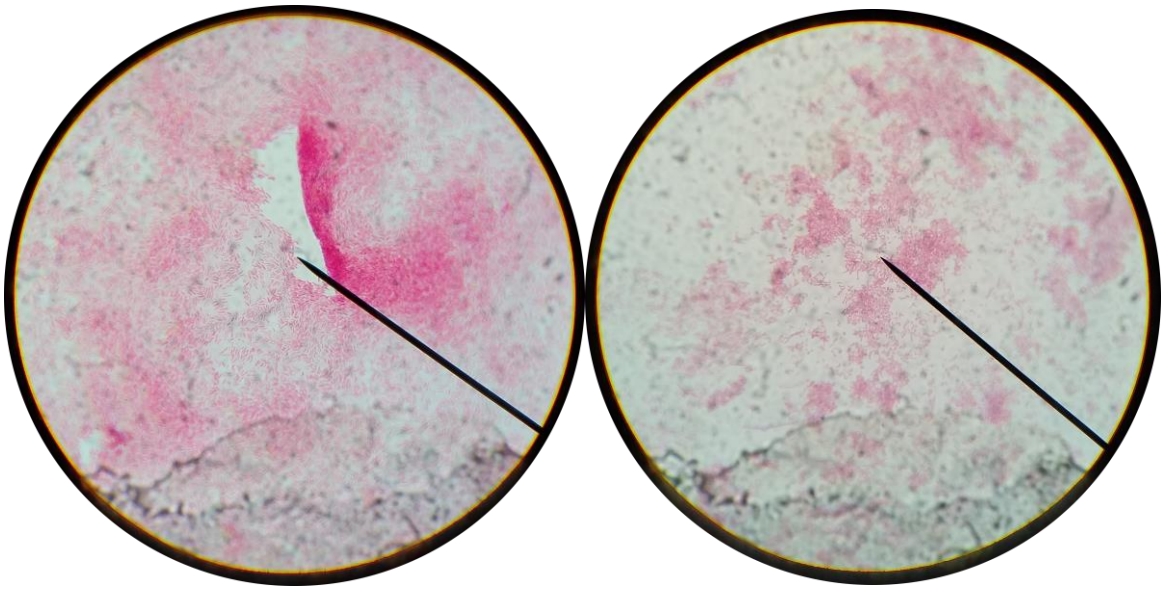


Figure 2. Gram stain images of SS 415 (left) and SS 565 (right) indicating both as gram negative.

The 415/565 co-culture active compounds extract in hexane and produce a crude yield of approximately 7.71 mg/L with liquid-liquid extraction methods. A cell death assay revealed moderate activity against *S. aureus* by Fraction B (R_f= 0.547 in 20% ethyl acetate; average 0.46 mg/L) and activity stronger than the chloramphenicol control against *S.*

aureus by Fraction D (R_f= 0.652 in 50% ethyl acetate; average 0.79 mg/L) (Figure 3a, Table 1). Through PTLC of Fraction D, the active compound, Compound c (R_f=0.580 in 50% ethyl acetate; average 0.08 mg/L) was isolated.

This co-culture displays high rates of emulsion during the extraction phase, prompting lower scale-up volumes to reduce total process time. Yields were still relatively low, so solid-phase extraction methods were implemented to increase yield, narrow fractions of interest, and reduce overall extraction time. With the solid-phase extraction, active compounds against *S. aureus* eluted in the 0% MeOH fraction and 80% and 100% MeOH fractions, corresponding to Fraction D (Figure 3b). In Figure 3b there also appears to be a small amount activity against *E. coli* in the 0% and 40-100% fractions. The previously isolated active compound from Fraction D was identified via TLC plate to be in the 60%, 80%, and 100% MeOH fractions was purified with PTLC and yielded approximated 1.3 mg/L of pure compound (R_f= 0.579 in 50% ethyl acetate) with a 1 hour mix time of the beads in the supernatant and 1.28 mg/L when the resin was allowed to mix with the supernatant overnight (Table 1). There did not appear to be a significant increase in yield with the longer mix time with the beads indicating either a possible upper limit reached in regard to concentrations of compounds held by the beads or that one hour of mixing is all that is needed for collection of all produced antibiotic compound. This demonstrates a large improvement in yield with reduced time and effort and, with further optimization in the resin extraction, should increase the speed of discovery in the Wolfe Laboratory.

After purification through PTLC, the characterization stage of the pure Fraction D compound began. Two H^1-NMRs were generated from new (Figure 4) and old (Figure 5) samples of the compound. What was referred to as pure Fraction D appeared to still be a mix of at least two compounds as evidenced by the likely unrelated multitude of peaks visible on the H^1-NMR. However, through virtue of how compound concentration affects peak area, it was possible to compare the peak areas of the new and old samples to develop an idea of which peaks were related and derived from one compound or another. It is unlikely that the same concentrations of compounds were collected in both samples. Ratios were calculated using the peak area of the new sample over the peak area of the old sample. Peak area ratios that were 1 ± .50 are indicated by red boxes (Figure 4). Based on these peaks, the compound appears to have several aromatic hydrogens as well as carbohydrate chains. The ^{13}C-NMR cannot be as easily separated by compound since there are no peak areas (Figure 6). Future work focusing on obtaining the mass of the compound with Mass Spectrometry would aid in determining which peaks are relevant to the compound of interest.

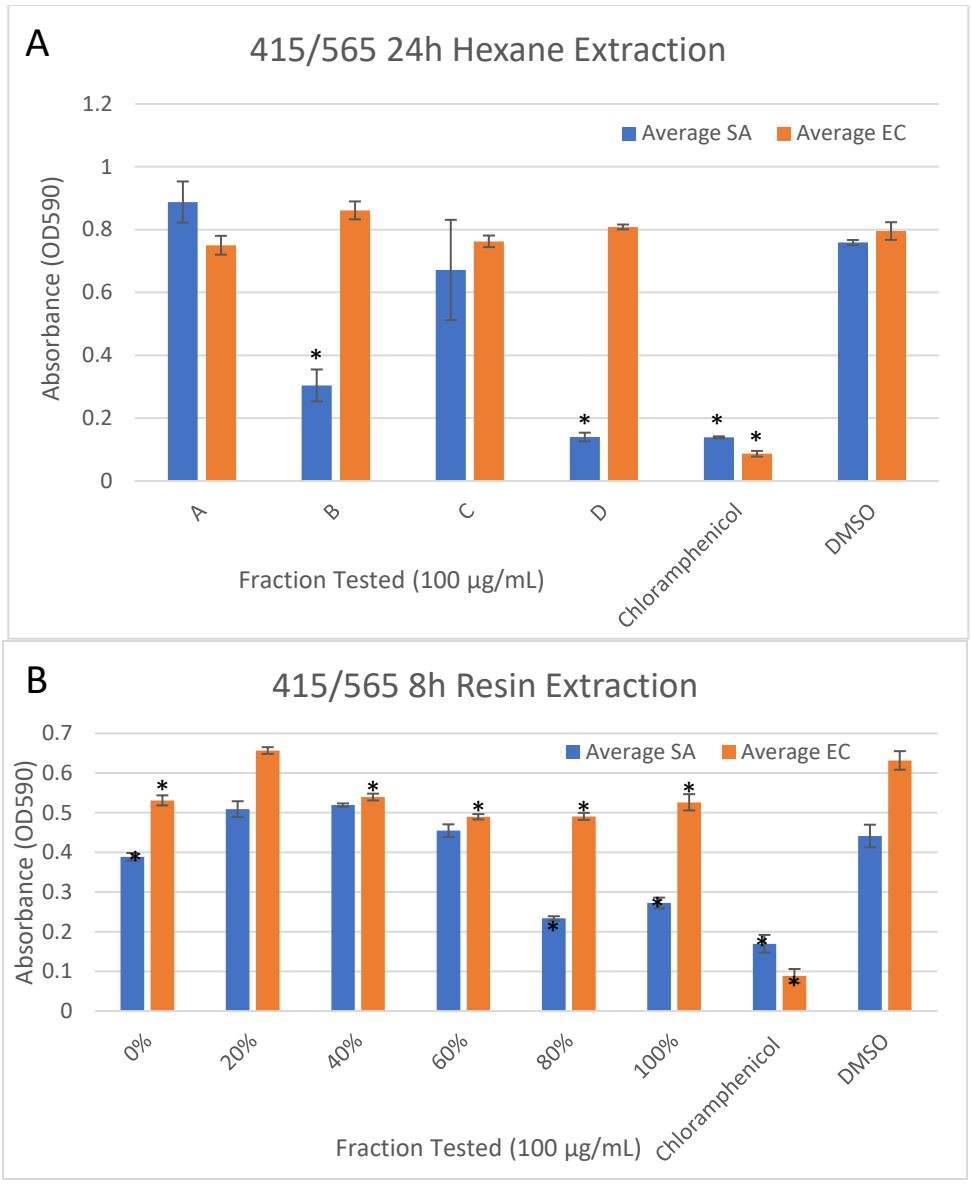


Figure 3. Average absorbance for the cell death assay results for 415/565 liquid-liquid extraction (A) and solid-phase extraction methods (B). Higher absorbance generally indicates more cells in solution and thus less antibiotic activity. Error bars show 95% confidence.

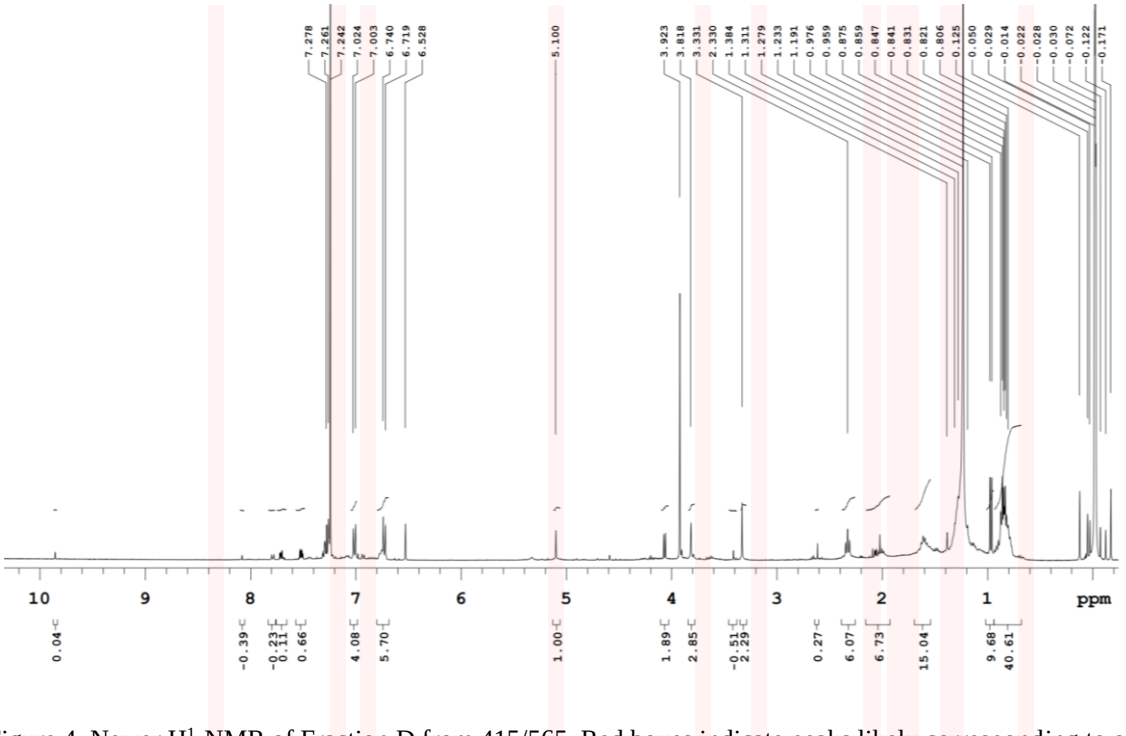


Figure 4. Newer H[1]-NMR of Fraction D from 415/565. Red boxes indicate peaks likely corresponding to one compound.

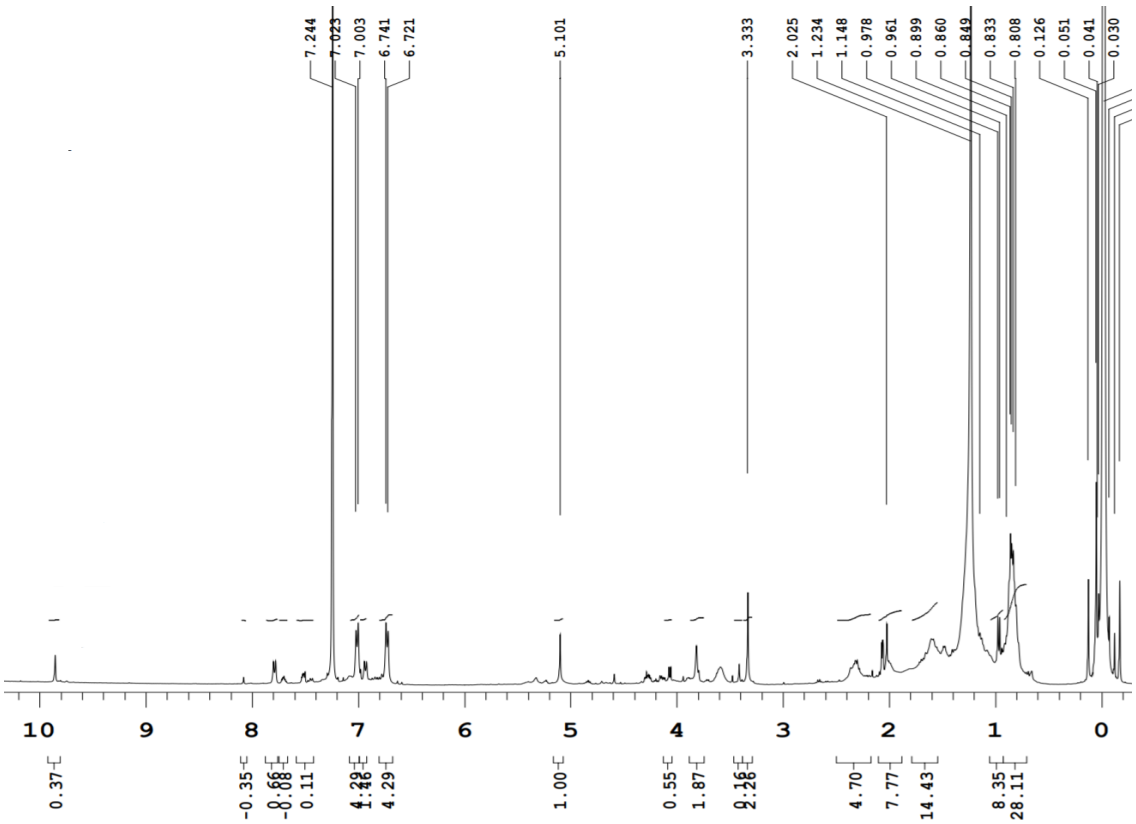


Figure 5. Older H[1]-NMR of Fraction D from 415/565.

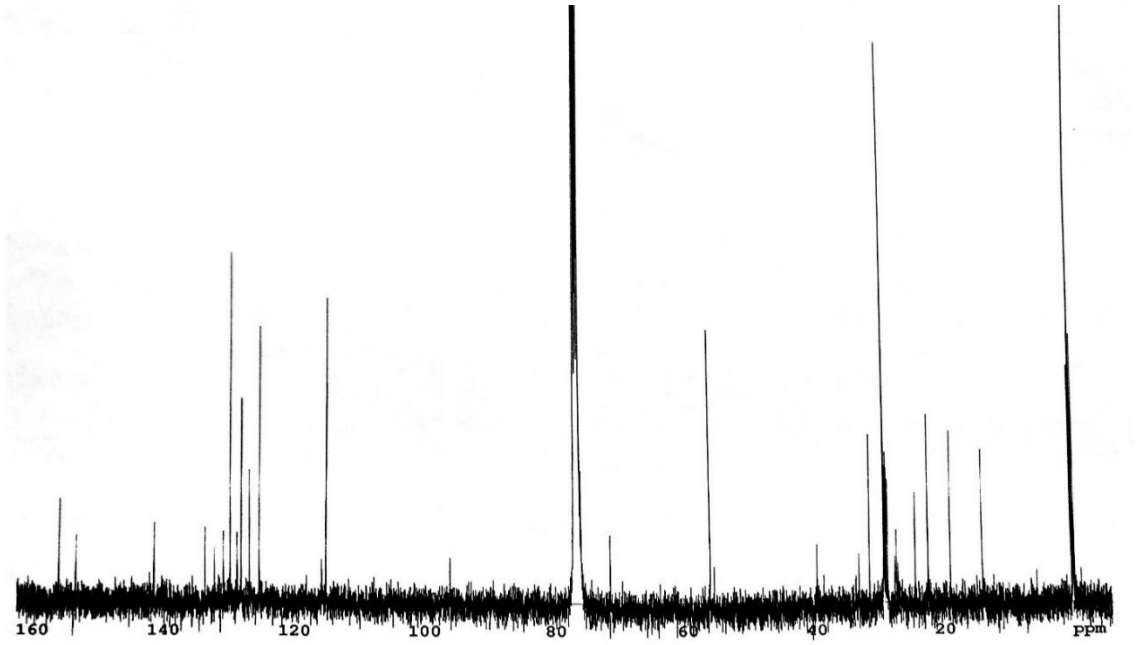


Figure 6. C^{13}-NMR spectra of Fraction D from 415/565.

3.1.3 SS 582/SS 593 co-culture

Screening of co-cultures with a high-throughput screening methodology showed strong and moderate activity of the SS 582/SS 593 (582/593) coculture against *S. aureus* and *E. coli* respectively with neither bacterium having antibiotic activity in monoculture. When scaled up, only activity was found against *S. aureus.* SS 582 has not yet been identified but stains as a gram-positive filament (Figure 7). This bacterium grows in small balls and often turns the media an apple-juice color. SS 593 has not yet been identified but stains as a gram-positive filament (Figure 7). This bacterium grows in small balls and often turns the media an orange-juice color.

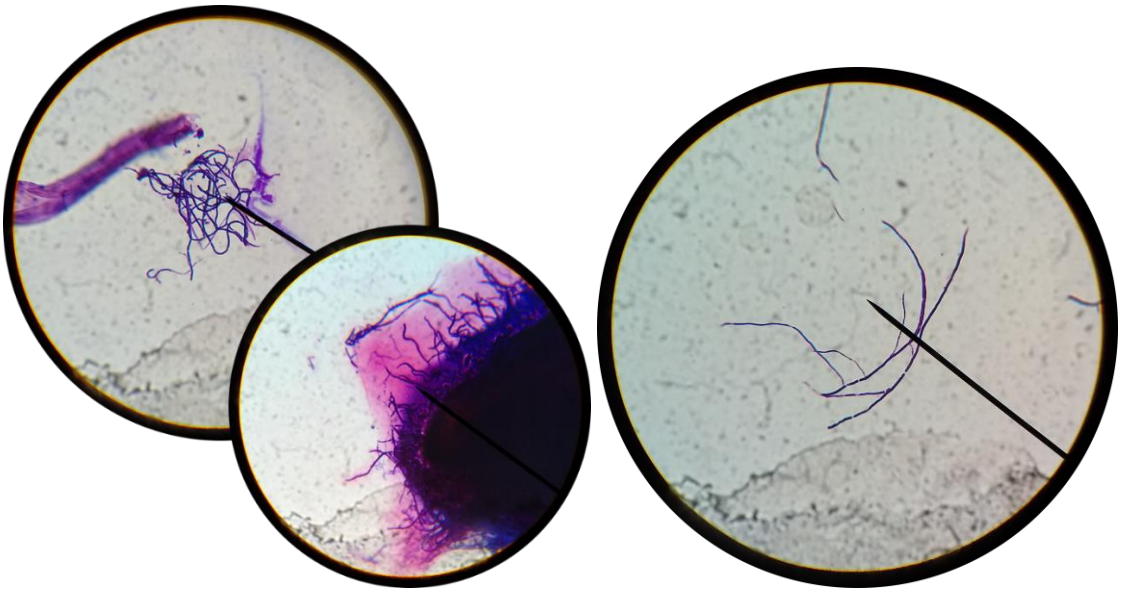
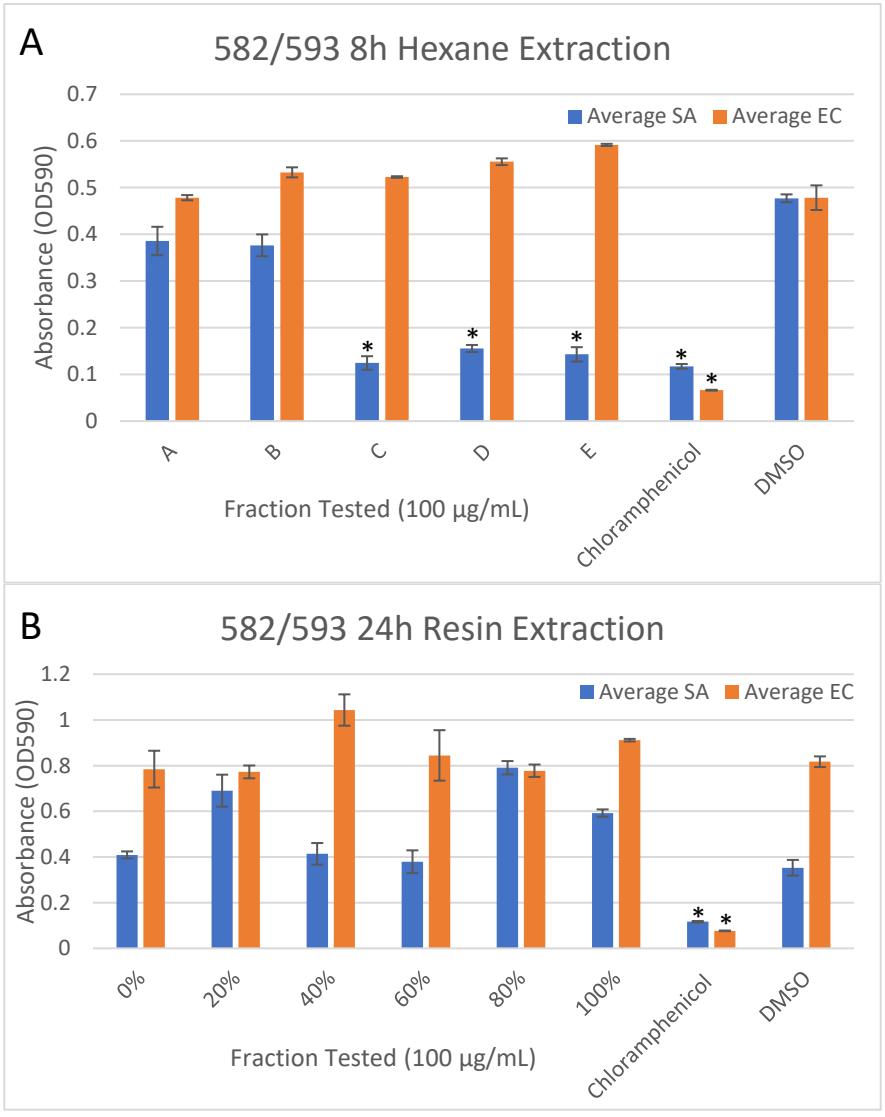


Figure 7. Gram stain images of SS 582 (left) and SS 593 (right) indicating both as gram positive.

The 593/582 co-culture active compounds extract in Hexane and produce a crude yield of approximately 7.5 mg/L. A liquid inhibition assay revealed strong activity against *S. aureus* by Fractions C-E (Figure 8a). This activity was determined to originate from a single compound found in all three fractions hereon referred to as Fraction E (R_f= 0.314 in 50% ethyl acetate; average 3.4 mg/L) (Table 1). Through PTLC of Fraction E, the active compound (R_f=0.567 in 50% ethyl acetate; average 0.12 mg/L) was isolated.

Yields were still relatively low even with optimization of co-culture length, so solid-phase extraction methods were implemented to increase yield, narrow fractions of interest, and reduce overall extraction time. With the solid-phase extraction, active compounds eluted in the 20, 80, and 100% MeOH fraction (Figure 8b). In Figure 8b, the active fractions appear as a much higher absorbance when compared to the negative DMSO control. This result comes from altered growth patterns of the pathogenic bacteria. Further purification of the Fraction E compound indicates successful isolation through this method (Figure 8c).

The 80 and 100% fractions were purified and yielded 0.52 mg/L (R_f= 0.314 in 50 % ethyl acetate) when the resin was mixed with the supernatant overnight (Table 1). Furthermore, after purification with a PTLC and an additional assay, the active compound showed strong activity not only against *S. aureus* but also moderate activity against *E. coli* (Figure 8c). This marks the first instance where the activity seen during the initial screening against *E. coli* has been recovered and documented with either co-culture. This may likely be due to higher purity from the resin extraction and PTLC methods allowing for more accurate measurement of the antibiotic activity in the cell death assay.



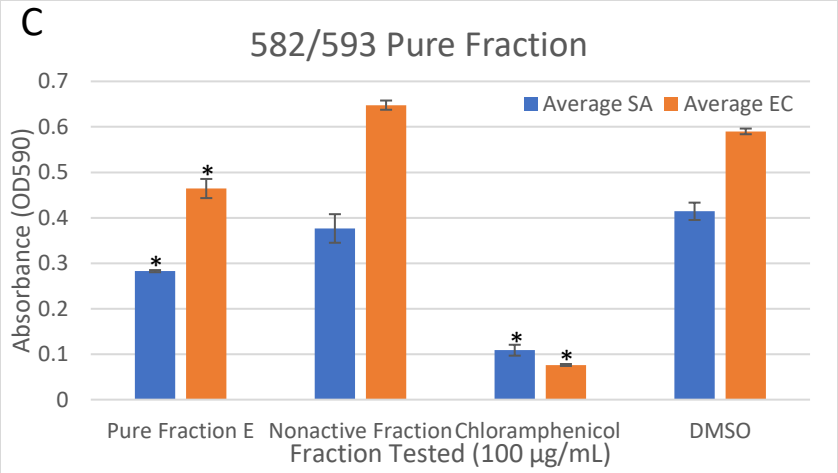


Figure 8. Average absorbance for the cell death assay results for 582/593 liquid-liquid extraction (A), solid-phase extraction (B), and the purified compound. Higher absorbance generally indicates more cells in solution and thus less antibiotic activity. Error bars show 95% confidence.

The compound in the 20% active fraction was not previously identified with the liquid-liquid extraction and shows that the resin method can capture a wider variety of compounds at assumedly higher concentrations. This fraction was not investigated in the scope of this paper. These results further emphasize the improvements associated with the resin solid-phase extraction and will be used in further scale ups for collection of the active compound before proceeding into characterization.

3.2 Assays

3.2.1 biofilm assay

Biofilms present a difficult barrier for antibiotics to penetrate and are a serious health concern due to the reduced treatability of infections. Therefore, biofilm disrupting compounds are of great interest to extend the abilities of antibiotics. Once the base-level biofilm assay had been developed with reliable controls and consistent results, an initial screen of several compounds was undertaken. As used by Murray et. al and Cutrona et al., instead of determining the average absorbance, the ratio of the absorbance sample and the negative control was calculated and plotted.[7,27] With this method, the compounds were determined to be nonactive at any of the tested concentrations.

3.2.2 adjuvant assay

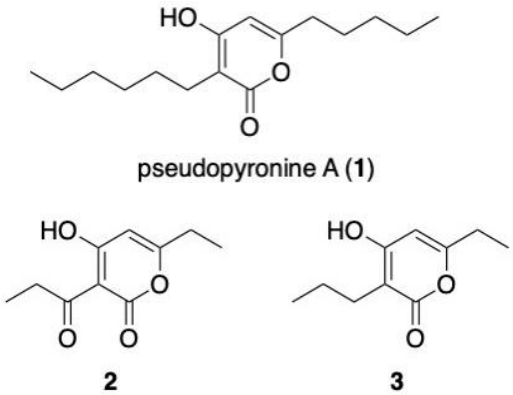


Figure 9. Pseudopyronine A (1) and short chain derivatives (2 and 3).[6]

Another approach to extend the activity of antibiotics is to co-dose with an adjuvant compound that disrupts mechanisms of resistance. From a panel screening against a set of known antibiotics, two compounds synthesized previously in the Wolfe Laboratory[6] (figure 9) which were not individually antibiotic active, LB52 (adjuvant 2) and LB54 (adjuvant 3), showed adjuvant activity with penicillin G against *P. aeruginosa* (Table 2). This indicates that the presence of the adjuvant compounds increased the antibiotic ability of penicillin G against *P. aeruginousa* with as much as a 256-fold increase. As the first discovered antibiotic, penicillin has remained in use as an antibiotic treatment through the implementation of adjuvants such as clavulanic acid.[23] Further work has been done to identify the mechanism of action of this adjuvant activity with the biofilm assay being one such method highlighted in this paper.

Table 2. Adjuvant activity of Adjuvant 2 and 3 at 50 μM with initial antibiotic MIC and, in brackets, fold change in MIC.

	***P. aeruginosa* Adjuvant MIC (μg/mL)**		
Antibiotic	No Adjuvant	Adjuvant 1 (50 μM)	Adjuvant 2 (50 μM)
Amoxicillin	2	2 [1]	2 [1]
Erythromycin	0.5	0.5 [1]	0.5 [1]
Penicillin G	>128	0.5 [>256]	128 [>1]
Vancomycin	1	1 [1]	1 [1]

4. Conclusions

Development of novel antibiotics remains crucial for the continued health of humankind in the battle against multi-drug resistant bacteria. However, additional strategies, such as targeting biofilm formation and co-dosing with adjuvant compounds, may provide the answer to the problem of continued resistance.[22,27] The methods implemented to screen and isolate active antibiotic compounds produced from the Wolfe Laboratory bacterial library could easily be converted to a high-throughput assay screening for biofilm inhibition or adjuvant activity.[7] It is with these new approaches that novel ways to combat antibiotic resistant bacteria may be elucidated.

Antibiotic discovery and isolation from soil bacteria co-cultures is a long process with ideal purification of active compounds requiring creative solutions to retain yield and ultimately successfully characterize. With the introduction of solid-phase extraction methods, yield of antibiotic compound was increased by as much as 11-fold which greatly progressed the project towards the characterization stage. Furthermore, this method led to two unexpected results: a previously missed active antibiotic fraction was collected from the 582/593 coculture and the original active fraction's activity was expanded to include moderate antibiotic activity against *E. coli.* These results support the use of the solid-phase extraction as a viable way to not only increase yield and purity but also capture a wider spectrum of compounds. There remains a need for better purification methods, such as with High Performance Liquid Chromatography (HPLC), for the purpose of more successfully isolating the pure active compound for easier characterization.

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6. References

1. CDC. Antibiotic Resistance Threats in the United States, 2019. *U.S. Department of Health and Human Services, CDC*; **2019.**
2. O'Neill, J. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. *Review on Antimicrobial Resistance* **2016**, 1-7.

3. Levin-Reisman, I.; Brauner, A.; Ronin, I.; Balaban, N. Q. Epistasis between Antibiotic Tolerance, Persistence, and Resistance Mutations. *PNAS* **2019**, *116* (29), 14734–14739.
4. Bologa, C.G.; Ursu, O.; Oprea, T.I.; Melançon, C.E.; Tegos, G.P. Emerging Trends in the Discovery of Natural Product Antibacterials. *Current Opinion in Pharmacology* **2013**, *13*, 678−687.
5. Butler, M.S.; Blaskovich, M.A.; Cooper, M.A. Antibiotics in the Clinical Pipeline at the End of 2015. *Journal of Antibiotics* **2017**, *70(1)*, 3-24
6. Bouthillette, L. M.; Darcey, C. A.; Handy, T. E.; Seaton, S. C.; Wolfe, A. L. Isolation of the Antibiotic Pseudopyronine B and SAR Evaluation of C3/C6 Alkyl Analogs. *Bioorganic & Medicinal Chemistry Letters* **2017**, *27*, 2762-2765.
7. 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.
8. Cueto, M.; Jensen, P. R.; Kauffman, C.; Fenical, W.; Lobkovsky, E.; Clardy, J. Pestalone, a New Antibiotic Produced by a Marine Fungus in Response to Bacterial Challenge. *Journal of Natural Products* **2001**, *64*, 144-1446.
9. Fedorova, F.D.; Moktali, V.; Medema, M.H. Bioinformatics Approaches and Software for Detection of Secondary Metabolic Gene Clusters. *Methods in Molecular Biology* **2012**, *944*, 23-45.
10. Weber, T. In Silico Tools for the Analysis of Antibiotic Biosynthetic Pathways. *International Journal of Medical Microbiology* **2014**, *304*, 230-235.
11. Khater, S.; Anand, A.; Mohanty, D. In Silico Methods for Linking Genes and Secondary Metabolites: The Way Forward. *Synthetic and Systems Biotechnology* **2016**, *1*, 80–88.
12. Schloss, P.D.; Handelsman, J. Status of the microbial census. *Microbiol. Mol. Biol. Rev.* **2004,** *68*(4), 686-691.
13. Schloss, P.D.; Girard, R.A.; Martin, T.; Edwards, J.; Thrash, J.C. Status of the Archaeal and Bacterial Census: an Update. *MBio*. **2016**, *7*(3), 201-216.
14. Fierer, N.; Jackson, R. B. The Diversity and Biogeography of Soil Bacterial Communities. *Proceedings of the National Academy of Sciences* **2006**, *103* (3), 626–631.
15. Dunbar, J.; Barns, S. M.; Ticknor, L. O.; Kuske, C. R. Empirical and Theoretical Bacterial Diversity in Four Arizona Soils. *Appl. Environ. Microbiol.* **2002**, *68* (6), 3035–3045.
16. Nichols, D.; Cahoon, N.; Trakhtenberg, E.M.; Pham, L.; Mehta, A.; Belanger, A.; Kanigan, T.; Lewis, K.; Epstein, S.S. Use of Ichip for high-throughpup *in situ* cultivation of "uncultivatable" microbial species. *American Society for Microbiology* **2010,** *76*(8), 2445-2450.
17. Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schäberle, T. F.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V. A.; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.; Zullo, A. M.; Chen, C.; Lewis, K. A New Antibiotic Kills Pathogens without Detectable Resistance. *Nature* **2015**, *517* (7535), 455–459.
18. Karas, J. A.; Chen, F.; Schneider-Futschik, E. K.; Kang, Z.; Hussein, M.; Swarbrick, J.; Hoyer, D.; Giltrap, A. M.; Payne, R. J.; Li, J.; Velkov, T. Synthesis and Structure−activity Relationships of Teixobactin. *Annals of the New York Academy of Sciences* **2020**, *1459* (1), 86–105.
19. Hover, B.M.; Kim, S.; Katz, M.; Charlop-Powers, Z.; Owen, J.G.; Ternei, M.A.; Maniko, J.; Estrela, A.B.; Molina, H.; Park, S.; Perlin, D.S.; Bradey, S.F. Culture-Independent Discovery of the Malacidins as Calcium-Dependent Antibiotics with Activity against Multidrug-Resistant Gram-Positive Pathogens. *Nature Microbiology* **2018**, *3*, 415–422.
20. Tyc, O.; van den Berg, M.; Gerards, S.; van Veen, J.A.; Raaijmakers, J.M.; de Boer, W.; Garbeva, P. Impact of interspecific interactions on antimicrobial activity among soil bacteria. *Frontiers in Microbiology* **2014,** *5*, 1-10.
21. Jones, M.B.; Nierman, W.C.; Shan, Y.; Frank, B.C.; Spoering, A.; Ling, L.; Peoples, A.; Zullo, A.; Lewis, K.; Nelson, K.E. Reducing the Bottleneck in Discovery of Novel Antibiotics. *Microbial Ecology* **2017,** *73(3)*,658-667.
22. Cox, G.; Koteva, K.; Wright, G.D. An unusual class of anthracyclines potentiate gram-positive antibiotics in intrinsically resistant Gram-negative bacteria. *J. Antimicrob. Chemother.* **2014**, *69*, 1844-1855.
23. Cox, G.; Sieron, A.; King, A.; De Pascale, G.; Pawloski, A.; Koteva, K.; Wright, G.D. A Common Platform for Antibiotic Dereplication and Adjuvant Discovery. *Cell Chemical Biology*. **2017**, *24*, 98-109.
24. Sjuts, H.; Vargiu, A.V.; Kwasny, S.M.; Nguyen, S.T.; Kim, H.S.; Ding, X.; Ornik, A.R.; Ruggerone, P.; Bowlin, T.L.; Nikaido, H.; Pos, K.M.; Opperman, T.J. Molecular basis for inhibition of AcrB multidrug efflux pump by novel and powerful pyranopyridine derivatives. *Proc Natl Acad Sci U S A*. **2016,** *113*(13), 3509-3514.

25. Ayhan, D.H.; Tamer, Y.T.; Akbar, M.; Bailey, S.M.; Wong, M.; Daly, S.M.; Greenberg, D.E.; Toprak, E. Sequence-Specific Targeting of Bacterial Resistance Genes Increases Antibiotic Efficacy. *PLOS Biology* **2016,** *14*(9).
26. Taylor, P.L.; Rossi, L.; De Pascale, G.; Wright, G.D. A Forward Chemical Screen Identifies Antibiotic Adjuvants in Escherichia coli. *ACS Chemical Biology* **2012,** *7* (9), 1547-1555.
27. Cutrona, N.; Gillard, K.; Ulrich, R.; Seemann, M.; Miller, H.B.; Blackledge, M.S. From Antihistamine to Anti-infective: Loratadine Inhibition of Regulatory PASTA Kinases in *Staphylococci* Reduces Biofilm Formation and Potentiates β-Lactam Antibiotics and Vancomycin in Resistant Strains of *Staphylococcus aureus*. *ACS Infectious Diseases* **2019**, *5* (8), 1397-1410
28. Maiden, M.M.; Hunt, A.; Zachos, M.P.; Gibson, J.A.; Hurwitz, M.E.; Mulks, M.H.; Waters, C.M. Triclosan Is an Aminoglycoside Adjuvant for Eradication of *Pseudomonas aeruginosa* Biofilms. *Antimicrobial agents and chemotherapy*, **2016,** *62*(6).
29. Oliveira, W.F.; Silva, P.M.S.; Silva, R.C.S.; Silva, G.M.M.; Machado, G.; Coelho, L.; Correia, M.T.S. *Staphylococcus aureus* and *Staphylococcus epidermidis* infections on implants. *J. Hosp. Infect.* **2018,** *98*(2), 111−117.
30. Blackledge, M.S.; Worthington, R.J.; Melander, C. Biologically inspired strategies for combating bacterial biofilms. *Curr. Opin. Pharmacol.* **2013,** *13* (5), 699−706.
31. Zheng, Y.; He, L.; Asiamah, T.K.; Otto, M. Colonization of medical devices by staphylococci. *Environ. Microbiol.* **2018,** *20* (9), 3141−3153.
32. O'Toole, G.A. Microbiology: a resistance switch. *Nature* **2002,** *416* (6882), 695−696.
33. Wolcott, R.D.; Ehrlich, G.D. Biofilms and chronic infections. *JAMA* **2008,** *299* (22), 2682−2684.
34. Shenin, Y.D.; Kruglikova, L.F.; Vassyuk, L.F.; Kozhemyakov, A.P.; Popova, T.A.; Tchebotar, V.K. New metanolite with fungistatic activity produced by associative nitrogen-fixing bacteria belonging to genus *Flavobacterium*. *Nitrogen Fixation: Fundamentals and Applications* **1995**, 777.
35. Abdul-Mutalib, N.A.; Amin, N.S.; Malina, O.; Muhaimin, R. A.; Natsumi, I.; Kenji, S.; Yukihiro, T.; Kosuke, T.; Toshinari, M.; Yoshihito, S. The prevalence of foodborne pathogenic bacteria on cutting boards and their ecological correlation with background biota. *AIMS Microbiology* **2016**, *2*, 138-151.
36. Einsoglu-Atalay, V.; Atasever-Arsian, B.; Yaman, B.; Cebecioglu, R.; Ozilhan, S.; Ozen, F.; Catal, T. Chemical and molecular characterization of metabolites from *Flavobacterium sp. PLoS One* **2018**, *13*, 1-17.
37. Leon, J.; Liza, L.; Soto, I.; Torres, M.; Orosco, A. Marine bacteria producing antibacterial compounds isolated from inter-tidal invertebrates. *Peruvian Journal of Experimental Medicine and Public Health* **2010**, *27*, 215-221.
38. Grossart, H.P.; Schlingloff, A.; Bernhard, M.; Simon, M.; Brinkhoff, T. Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. *FEMS Microbiology Ecology* **2004**, *47*, 387-396.