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Isolation, Characterization, and Antibiotic Extraction of Bacterial Strains

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Abstract

Multidrug resistant bacteria pose a threat to human health due to overprescription and misuse of antibiotics, an industry of agriculture reliance, and the decline of novel antibiotic discovery. According to the CDC, at least two million people in the United States each year are infected with multidrug resistant bacteria and more than 23,000 succumb to those infections. Natural product (NP) isolation remains a robust source of novel antibiotics even though rediscovery is an ongoing problem. This research reports the isolation of 101 bacteria from soil samples collected in the Southwestern United States and subsequent antibiotic screening of those bacteria. Bacteria from a library of isolates found to be strong antibiotic producers were then identified by 16S rDNA analysis and subjected to scale up and extraction to isolate the produced antibiotic. Currently, antibiotics produced by three bacteria, a *Bacillus* strain (SS729), a *Microbacterium* strain (SS452B) and a *Pseudomonas* strain (SS827B) are being characterized. SS452B was found to have antibiotic activity against Gram-positive *Staphylococcus aureus* while the other two, SS729 and SS827B were found to have antibiotic activity against both Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* in liquid inhibition assays.

1. Introduction

The World Health Organization has recognized multidrug resistant bacterial infections as one of the top human health problems. The most common multidrug resistant bacteria, often referred to by the acronym "ESKAPE" pathogens, include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter species*.[1] The Center for Disease Control estimate at least two million people in the United States each year are infected with multidrug resistant bacteria and more than 23,000 succumb to those infections.[2] Conservatively by 2050, it is estimated that 10 million people will be at risk for a multidrug resistant bacterial infection with a financial burden of 100 trillion dollars.[3] The discovery of novel antimicrobial compounds has been declining rapidly from 20 new FDA approved antibiotics (1990-1999) to only 9 new FDA approved antibiotics (2000-2014).[2,4] With a decline in novel antibiotic discovery coupled with the increasing threat of multidrug resistant bacteria to human health, development of new antibiotics is urgent.

Natural products (NP) remain a robust source of novel antibiotics.[4] There has been a decline in pharmaceutical investment in NP isolation due to the high costs and the ongoing problem of rediscovery. Large pharmaceutical industries are turning their heads to quicker and more financially benefiting projects. Many of the available antibiotics today are alternative forms of NPs synthesized from bacteria. Bacteria have developed a vast array of mechanisms and secondary metabolites for survival due to the rate of advantageous mutations through exponential cell division and genetic exchange. Over generations, these organisms have evolved to produce NPs as a means of communication or successful competition between other microbes.[5] In the past 12 years, microbial diversity census estimates have flourished. It is estimated that bacterial diversity on earth is between 10^7 and 10^9.[6] Before 2005, only 78,000 species had been characterized while by 2016 over one million species had been characterized.[6,7]

Researchers are now concluding that they may be approaching an accurate microbe diversity due to the decline of discovery.[7] However, we still may be far from understanding the microbe diversity fully. Specifically, soil microbes are challenging to isolate. It is estimated that 99% of bacteria isolated from the environment cannot be grown in conventional laboratory settings and are therefore unculturable.[8] Environmental survival conditions are often extremely hard to replicate, and therefore observing NP production is challenging. With the growing understanding of microbes and NP's medical potential, many cutting edge researchers are seeking ways to access the reservoir of unculturable bacteria.

Although there has been a decline in large pharmaceutical interest in NP isolation, recently NP isolation research has been revisited due to improvements in culturing and isolation methods. In February 2018, a laboratory at Rockefeller University in New York published the findings of a novel antibiotic class isolated from soil in New York City with activity against MRSA and no found resistance. This new class of antibiotics, Malacidins (Figure 1A), were found from biosynthetic gene clusters using sequencing, bioinformatics analysis and heterologous expression to screen soil for calcium-dependent antibiotics, and a bacterial host was used to produce the active compound.[9] These compounds are currently in preliminary testing. In addition, Teixobactin (Figure 1B), which is a new class of antibiotic, was discovered using unconventional isolation methods.[10] The ichip is one of the new technologies being employed to access environmental bacteria previously unculturable under conventional laboratory conditions. Use of the ichip allows capture of individual bacterial samples in their natural environment. Once grown, they are moved into the conventional laboratory, *in vitro.*[11] Their discovery of activity also led to the discovery of a new bacterial specie now named *Eleftheria terrae,* a Gram-negative bacterium which produces the unusual depsipeptide.[10]

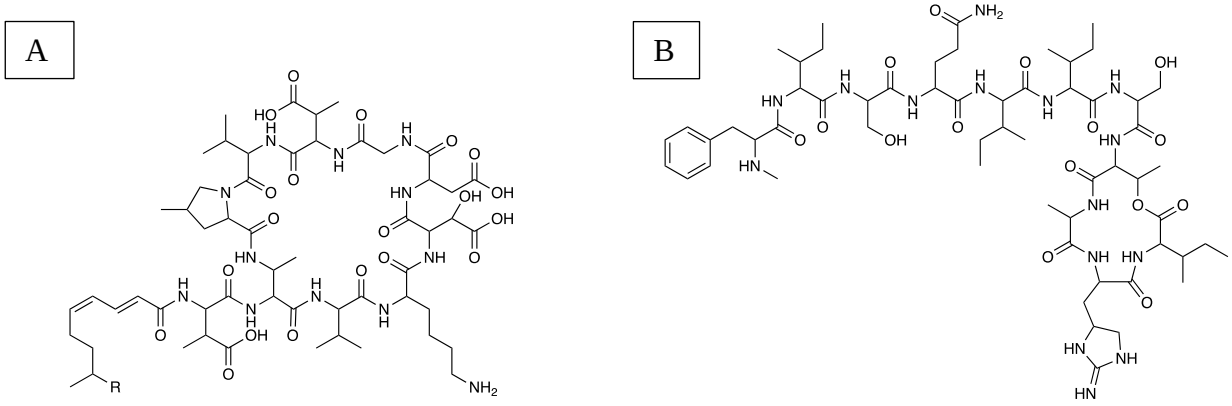

Figure 1. Recent Antibiotic Discoveries: a) Malacidins (A; R = Me, B; R = Et), calcium-dependent antibiotic, isolated from soil in 2017.[9] b) Teixobactin, isolated with ichip technology from soil in 2015.[10,11]

Often times microbes isolated in conventional laboratory settings, are not exposed to typical environmental stressors or microbial interactions. In particular, soil microbes have been found to hold "cryptic" gene clusters not expressed when grown *in vitro,* which suggests there is a potential to induce the expression of a novel antibiotic.[5,12] Bacteria-bacteria competition as a means to induce novel antibiotic expression may lead to alternative antibiotic discoveries.[5] One study found that 6% of previously non-antibiotic producing microbes show induction of antibiotic activity when in bacterial-bacteria competition.[13] New methods to observe secondary metabolites produced from bacteria in conventional laboratory conditions may also take the stage with antibiotic discovery and give insight to bacteria-bacteria interactions. A review of imaging mass spectrometry (IMS) in 2016 shows how these technologies may further novel NP discovery.[14] The use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) IMS allows research to simultaneously visualize dozens of metabolites with biological phenotypes of interest from an agar plated bacterium. This method allows for the observation of secondary metabolites previously undetected in monoculture and bacteria-bacteria interactions.[14]

Since the beginning of antibiotic discovery, NPs remain to be the most abundant and promising source of antimicrobial compounds.[4] Although the rediscovery of antibiotics and isolation methods continue to be an issue, there is still more information to be gathered from classic isolation methods. The Wolfe research laboratory at UNC Asheville discovered a previously isolated antibiotic, pseudopyronine B from a soil microbe in Western North Carolina. Although this antibiotic compound had previously been isolated, they were able to generate structure antibiotic activity relationship profile along with expanding knowledge of antibiotic production in *Pseudomonas*

species.[15] The Wolfe Laboratory is in the early stages of finding unique bacteria and optimizing bacterial conditions with the ultimate goal of finding antibiotics through bacteria-bacteria interactions. Possible research will expand in collaboration with the Shank research laboratory at UNC at Chapel Hill with IMS technologies. This collaboration along with the future development of bacteria-bacteria interaction methods would help expand the success of novel antibiotic isolation.

This work specifically aims to discover and isolate novel NPs with antibiotic activity through: 1) screening of bacteria from under-explored environments, and 2) through the use of bacteria-bacteria competition to induce production of cryptic NPs. Following isolation, this work also specifically examines three strains of bacteria which have been found to produce NPs with antimicrobial activity. Well-known antibiotic producers isolated from the Southwestern United States, *Bacillus* (SS729) and *Pseudomonas* strain's (SS827B) NP(s) are being characterized.[15,16] Antibiotic activity of a *Microbacterium* strain (SS452B), not been previously recognized to produce antibiotics,[17] is also being characterized.

2. Methodology

All methodology was carried out under aseptic conditions, with all solutions either autoclaved (121 °C) or filtered through 0.2μM PES filter.

2.1. Microbial Isolation and Antibiotic Screening

Four soil samples were aseptically collected from public land in the southwestern United States. (Sample 1: Unknown, Sample 2: 38°1'19.943"N 111°57'41.69"W, Sample 3: 38°22'26.264"N 111°34'37.309"W, Sample 4 37°22'38.594"N 108°25'27.171"W). Samples were taken on 20 May 2016 and 24 May 2016. The top centimeter of soil was discarded with a sterilized spatula. Approximately 0.5 g of soil beneath was collected in a sterile eppendorf and processed within two weeks of collection.

Samples were hydrated with phosphate buffer, vortexed, and allowed to settle. Four, 10% supernatant dilutions of 50μL and 100μL volumes were spread onto dilute Tryptic Soy Agar (dTSA; 10% Tryptic Soy Broth, 1.5% Bacto agar) plates and *Actinomycete Isolation Agar* (AIA). Plates were incubated at 22 °C until colonies appeared. Colonies were selected based on differences in form, elevation, margin, surface, opacity, and pigmentation. Each colony was then streak purified three times to ensure purity. Pure bacterial cultures were stored as glycerol stocks (20% glycerol, −80 °C) for use in subsequent experiments.

2.1.1. agar spread plate method:

Target pathogen was agitated in Luria Broth overnight at 26 °C. A bacterial lawn was created by spreading 70μL of target pathogen onto a dTSA plate. 1.7μL of desired cell suspension grown in dilute Tryptic Soy Broth (dTSB; 10% Tryptic Soy Broth) was spotted onto dried pathogen lawn plates. Plates were monitored up to a week at room temperature for zones of inhibition against target pathogen.

2.1.2. agar overlay method:

dTSA was melted via microwave and cooled to 50 °C in a hot water bath. 100mL of target pathogen overnight suspension in Luria Broth was added to 4mL of dTSA, gently inverted and poured onto pre-warmed, 37 °C, dTSA plates. Plates were slightly rocked for even coverage and left to dry. 1.7μL of desired overnight cell suspension in dTSB was spotted onto dried overlay plates and monitored at room temperature up to a week for zones of inhibition against target pathogen.

2.2. Interaction-mediated Antibiotic Production

Each well of a 96-well master plate was filled with 150μl of dTSB and inoculated with multiple, unique isolates determined not to produce antibiotics in previous monoculture screening via agar spread plate/agar overlay methodology. Control wells include a known antibiotic producer (SS 400) as a positive control and un-inoculated

dTSB as a negative control. Each isolate was also grown in dTSB overnight and diluted 10-fold into a monoculture 96-well plate with control wells. Master plates were incubated at 26 °C for 1 days, and subsequently used to generate co-culture mixtures of all pair-wise combinations (50µl of each of two organisms) (Figure 2).

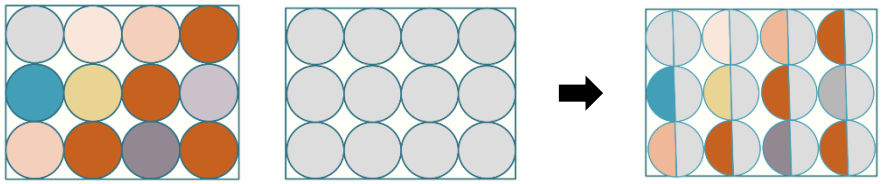

Figure 2. Screen for Co-culture Antibiotic Production. Demonstration of a master plate (far left) containing many different isolates and a homogenous isolate plate (middle). These two plates are combined to generate the co-culture plate (right). This co-culture plate would then be pin replicated onto a dTSA plate.

Co-culture mixtures were then pin-replicated onto dTSA plates previously spread or overlaid with target pathogen of either *E. coli* or *S. aureus* and incubated (22 °C, 2 days) to establish colonies. Plates were monitored over the course of a week for zones of inhibition.

The 96-well generated co-culture mixture plate was then used to make quadculture 96-well plates. Co-culture 96-well plates were randomly paired. One pairing was rotated 180°. These were combined in a further plate in a one to one ratio (50ul of each organism pairs, each 50µL contained two isolates). These were pin replicated and followed the protocol for co-culture growth and monitoring.

2.2. Microbial Characterization

2.2.1. gram-staining

Samples were placed onto a microscope slide by pipetting 10µL of sterile deionized water and smearing a colony. The slide was allowed to dry completely and then subjected to gentile heat fixation via flame. The smear was subjected to crystal violet (1 minute), Iodine (1 minute), decolorizer (5 seconds), safranin (45 seconds). Between each step, the smear was washed thoroughly with deionized water. Bacterial cells were visualized under bright-field microscopy at 10,000x magnification.

2.2.2. genomic purification

Bacterial DNA was acquired using PureLink™ Genomic DNA Mini Kit and protocol. Samples were grown in 4mL dTSB overnight and cells (2x 10^9) were harvested via centrifugation. Protocol for bacterial cell lysate was followed with respect to Gram-staining results. All samples were subject to purifying genomic DNA using a spin column-based procedure. DNA elution from spin column for each sample was completed twice with 50µL PureLink® Genomic Elution Buffer to a total volume on 100µL. Purified bacterial DNA was kept at -18 °C.

2.2.3. pcr and identification

Genus-level identification was determined using16S rRNA amplification and DNA sequence analysis. Universal 16S rRNA primers, 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT). Polymerase chain reactions (PCR) were completed for 50µL reactions containing 5x Phusion Green HF Buffer (10µL), 10mM dNTP (1µL), Phusion DNA Polymerase (0.5µL), 10µM Universal Primers (2.5µL), 100ng/mL template, and nuclease-free water. Thermocycler was set for initial denaturing at 98 °C (3 minutes), followed by 29 cycles of 98 °C (30 seconds), 48 °C (30 seconds), and 72 °C (5 minutes). After 29 cycles were complete, sample underwent a final extension at 72 °C (5 minutes).

PCR products were visualized for size separation on 1% Agarose gel containing 0.5 µg/mL ethidium bromide in 1x TAE. A Quick-Load® Purple 2-Log DNA Ladder (0.1 - 10.0 kb) was used for size reference. Products (approximately

1460 bp) were visualized under UV light and purified using QiaQuick PCR Purification kit (Qiagen). Purified PCR product send-off was prepared in a total volume of 15 μL for sequencing at 4 ng/μL products, 25pmol 27F primer, and nuclease-free water. Samples were sent to GeneWiz (Cambridge, MA) for Sanger sequencing. Sanger sequencing results were cross checked with the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) for genus level identification.

2.3. Antibiotic Extraction

2.3.1. minimal media optimization

Bacteria shown to produce an antibiotic in the screen were further subjected to a minimal media screen (0.35 M K_2HPO_4; 0.22 M KH_2PO_4; 0.08 M $(NH_4)_2SO_4$; 200mM $MgSO_4$) containing a single carbon source: sodium acetate (12.5mM), sodium citrate (12.5mM), D-glucose (12.5mM), or sodium succinate (25mM). Bacteria cultures were first grown in dTSB at 26 °C for 24 hours. Liquid cultures were diluted (10:1) in a round bottom 96-well plate with minimal media to a total volume of 100 μL (n=8). 96-well plates shaken in the BioTek plate reader at 26 °C and optical density (OD_{590}nm) was continuously measured over 96 hours. Growth curves were determined for each minimal media.

2.3.2. antibiotic time-trial

Bacterial samples were grown in dTSB at 26 °C overnight. Liquid culture was transferred to optimal minimal media in a 10 % dilution. After 24, 48, 72, and 96 hours, 1mL samples were removed and filtered through a 0.22μM syringe filter. Samples were concentrated and loaded into a 96-well plate with pathogen (n=2). The plate was agitated up to 18 hours and optimal density was read at 590nM in a BioTek plate reader. Optimal time for antibiotic production was determined based on density of pathogen.

2.3.3. minimal media scale-up (1 – 9L)

dTSB was inoculated with bacterial samples in 1/100th the desired optimal minimal media (1-9 L) volume and agitated for 24 hours at 26 °C (10 mL/1 L). Samples were transferred to $1/10^{th}$ desired volume of optimal minimal media and agitated for 24 hours at 26 °C. (100 mL/1 L). Samples were transferred a third time to a desired volume of optimal media. This volume was agitated at 26 °C for the optimal antibiotic production time previously determined.

2.3.4. antibiotic extraction

Following growth in minimal media, the bacteria were centrifuged at 4,200 rpm for 20 minutes at 26 °C. Supernatant was collected and extracted sequentially in increasingly polar solvents: hexane, diethyl ether, and ethyl acetate. Organic extracts were then collected, washed with saturated aqueous NaCl, dried over sodium sulfate, and concentrated under reduced pressure. Crude product for each organic extract was weighted.

2.3.5. Organic extraction optimization

Each organic extract was dissolved in 10μL of DMSO and 1μL of the dissolved product was added a 96-well plate containing a 10% TSB overnight liquid *S. aureus* culture with a 10% dilution in full strength TSB. Plates were shaken up to 18 hours at 36 °C. Organic extract containing antibiotic was determined from Biotek plate reader (OD_{590}).

2.3.6. column chromatography

The crude organic extract was loaded onto a normal phase gradient column chromatography (SiO_2, 10%-40% ethyl acetate/hexane). Collection tubes were visualized on Thin Layer Chromatography (TLC) with UV-vis (254nm) and potassium permanganate. Retardation factors (R_f) were measured with optimal separation (20%-40% ethyl acetate/hexane).

3. Results and Discussion

101 bacterial samples from the Southwestern United States were isolated and purified using the microbial isolation and antibiotic screening methodology. Sample 1 contained 3.45 x 10^5 colony forming units/gram soil (cfu/g), Sample 2 contained 1.07 x 10^6 cfu/g, Sample 3 was inconclusive, and Sample 4 contained 1.29 x 10^4 cfu/g. These findings give us a glimpse of the vast array of bacteria in each gram of soil. Cfu count is only estimated to represent 1% of the total bacteria in a particular soil sample grown on nutrient agar.[8] Sample three was inconclusive due to a large variety of cfu counts.

These 101 bacterial isolates were added to the Wolfe/Seaton library of previously isolated bacterial samples. Of the 101 bacteria purified, agar overlay protocols were used to determine antibiotic production against both Gram-positive *Staphylococcus aureus* (*S. aureus)* and Gram-negative *Escherichia coli* (*E. coli).* Qualitatively, the agar overlay technique was found to be a more accurate way of visualizing zones of inhibition as opposed to the spread plate methodology due to a more uniform lawn of pathogen. Out of the 101 soil isolates, 72% were recorded to have zones of inhibition in monoculture via this agar overlay methodology. Previous research reported 33% of isolates showed antibiotic production in monoculture via an alternative agar overlay methodology.[13] Our findings show an increase in antibiotic producers which may be due to a purely qualitative screening, adapted methodology along with possible overlap in species isolation.[13] To date, approximately 378 co-cultures and 192 quad-cultures have been screened; no induction of antibiotic production has been noted both involving spread plate and agar overlay techniques. Previous research on bacteria-bacteria interaction of 2798 unique bacterial mixtures showed 6% of isolates with an induced antimicrobial activity in co-culture. Methodology for these findings varied by pouring an agar overlay over OmniTrayTM plates already containing individual samples.[13] Our technique varies distinctly by premaking agar overlays and spotting our target organism overtop. These variable techniques along with the capabilities of screening large quantities of isolates may be reasons for inconsistent data. Alternative methodology is currently being pursued by the Wolfe research laboratory.

Currently, a high-throughput screening methodology is being used to rescreen the Wolfe/Seaton bacterial library, which had originally been screened via agar spread plate and agar overlay method. This quantitative data is being used to find strong antibiotic producers (30% inhibition against pathogen) and antibiotic producers (+2 SD below 100% pathogen) from the Wolfe/Seaton library of over 400 bacterial samples through statistical analysis.[18] This new methodology will ultimately be employed in bacterial interaction assays. All antibiotic producers found via this new methodology have been subjected to 16S rRNA amplification and DNA sequence analysis (Table 1). To date, 69 antibiotic producers have been identified, nine antibiotic producers have been identified with multiple results, and a remaining three are currently being identified. Of those identified, the highest abundant genera were *Pseudomonas (30%)* and *Serratia (*17%). *Aquitalea, Bacillus, Brevibacillus, Brevundimonas, Chromobacterium, Chryseobacterium, Collimonas, Herbaspirillum, Microbacterium, Paenibacillus, Staphylococcus, Streptomyces, Streptrophomonas* encompass the remaining identified isolates. Out of these identified, *Psuedomonas*[15, 19, 20], *Serratia*[21], *Bacillus*[22], *Brevibacillus*[23], *Chromobacterium*[24], *Collimonas*[25], *Paenibacillus*[26], *Staphylococcus*[27], *Streptomyces*[28], and *Streptrophomonas*[29] are all known antibiotic producers. Isolates which have not yet been found to produce NP antibiotic compounds include *Aquitalea, Brevundimonas, Chryseobacterium, Herbaspirillum,* and *Microbacterium.*

Table 1. Sequencing results from microbial characterization protocol of all isolates from the Wolfe/Seaton library with antibiotic production. Sequencing results with multiple results, had equal genes level hits when cross referenced.

SAMPLE NUMBER:	GENUS-LEVEL SEQUENCING RESULTS:
RGRF B10	*Pseudomonas*
SS 400	*Pseudomonas*
SS 422	*Pseudomonas*
SS 439	*Bacillus, Brevibacterium*
SS 440	*Aquitalea, Chromobacterium*
SS 447	*Chromobacterium*
SS 448	*Aquitalea, Chromobacterium*
SS 449	*Chromobacterium*
SS 452B	*Microbacterium*
SS 454	*Chromobacterium*
SS 519	*Brevibacillus*
SS 548	*Paenibacillus*

SS 560	*Pseudomonas*
SS 581	*Collimonas*
SS 609	*Bacillus*
SS 614	*Herbaspirillum*
SS 616	*Pseudomonas*
SS 617	*Pseudomonas*
SS 619	*Pseudomonas*
SS 620	*Pseudomonas*
SS 623	*Pseudomonas*
SS 625	*Pseudomonas*
SS 626	*Pseudomonas*
SS 636	*Pseudomonas*
SS 637	*Pseudomonas*
SS 639	*Pseudomonas*
SS 640	*Pseudomonas*
SS 642	*Aquitalea*
SS 643	*Aquitalea*
SS 650	*Pseudomonas*
SS 651	*Kluyvera, Leclercia, Enterobacter*
SS 654	*Serratia*
SS 655	*Serratia*
SS 656	*Chryseobacterium*
SS 659	*Serratia*
SS 660	*Serratia*
SS 661	*Pseudomonas*
SS 662	*Pseudomonas*
SS 669	*Serratia*
SS 670	*Serratia*
SS 672	*Serratia*
SS 673	*Serratia*
SS 674	*Serratia*
SS 675	*Klugvera, Leclera*
SS 677	*Pseudomonas*
SS 682	*Serratia*
SS 692	*Cedecea, Enterobacter, Raoultella, Lelliottia, Kluyvera*
SS 696	*Serratia*
SS 697	*Bacillus*
SS 701	*Herbaspirillum*
SS 719	*Brevundimonas*
SS 720	*Streptrophomonas*
SS 722	*Microbacterium*
SS 723	*Microbacterium*
SS 724	*Pseudomonas*
SS 729	*Bacillus*
SS 730	*Serratia*
SS 735	*Streptomyces*
SS 739	*Bacillus, Brevibacterium*
SS 746	*Streptomyces*
SS 751	*Streptomyces*
SS 752	*Bacillus*
SS 764	*Bacillus*
SS 779	*Bacillus, Brevibacterium*
SS 783	*Streptomyces*
SS 792	*Streptomyces*
SS 794	*Bacillus, Brevibacterium*
SS 821	*Staphylococcus*

SS 827B	*Pseudomonas*

3.1. Antibiotic Isolation

Antibiotics produced by three bacteria, *Pseudomonas* strain (SS827B), a *Bacillus* strain (SS729), and a *Microbacterium* strain (SS452B) are being characterized (Table 1). Determination of optimal antibiotic production and bacterial growth was determined following protocols for antibiotic production (Table 2).

Table 2. Optimal growing conditions and optimal antibiotic extraction for bacterial samples being explored.

Sample Number	Optimal Minimal Media	Antibiotic Production Optimal Time	Optimal Organic Extraction
SS 827B	Glucose	96 - 120 hours	Ethyl Ether
SS 729	Succinate	72 hours	Ethyl Acetate
SS 452B	Glucose	72 hours	Hexane

3.1.1. SS 827B

SS 827B was determined to strongly inhibit *S. aureus* and *E. coli* with high-throughput screening methodology.[18] Previous work had been completed for SS 827B before optimization had been confirmed. Prior to optimization development, researchers isolated a total of 39 L of SS 827B grown in 12.5mM Citrate ($48 < x < 72$ hours). These scale-ups underwent multiple columns for optimal separation. Results from fractions were inconclusive due to low yield or a loss of active compound.[30] Minimal media growth and antibiotic extraction optimization showed alternative growing conditions (Table 2). SS 827B was grown in 1-L scale up in both 12.5mM Glucose (70 hours) and 25mM Succinate minimal media (72 hours) and extracted following antibiotic extraction protocol. Liquid inhibition assays showed antibiotic production in succinate extracted with ethyl acetate, but optimal antibiotic production in glucose extracted with diethyl ether. Time trial protocol was used to determine optimal time for SS 827B in glucose media at 96 hours. This data was furthermore confirmed through more thorough screening methods.[31] SS 827B was grown and extracted in optimized conditions for 96 hours (crude yield: 53.95 mg/L). Antibiotic activity of crude was confirmed using liquid inhibition assay. Column chromatography was performed twice for optimal compound separation (30% ethyl acetate/hexane). Liquid inhibition assay with *S. aureus* of fractions showed active compound in Fraction A (R_f = 0.96, 0.44, 0.37, 0.25, 0.18; 1.18 mg/L). With additional TLC investigation, the active compound is expected to be R_f values 0.44 or 0.37 when compared to inactive fractions. Due to a low active compound yield, SS 827B was grown under a longer incubation time of 120 hours and extracted in optimized conditions (crude yield: 88.33 mg/L). Column chromatography was performed with optimal TLC compound separation (30% ethyl acetate/hexane). Further purification is currently underway.

Pseudomonas have been well known antibiotic producers with previous antibiotic isolation of phenazines, phloroglucinols, pyoluteorin, pyrrolnitri, cyclic lipopeptides, to volatile hydrogen cyanide.[19, 20] The Wolfe research laboratory at UNC Asheville previously discovered an antibiotic, pseudopyronine B from *Pseudomonas* species in Western North Carolina.[15] Pseudopyronine B was compared via TLC and confirmed not present in active fraction from SS 827B.[30] Further fraction characterization will confirm similar or novel structures to previous isolates referenced above.

3.1.2. SS 729

SS 729 was originally found to be a producer against both *S. aureus* and *E. coli* in agar overlay assays. Confirmation of production via high-throughput screening methodology was completed.[18] SS 729 was determined to inhibit *E. coli* and strongly inhibit *S. aureus.* SS 729 growing conditions were optimized via minimal media optimization, antibiotic time-trial, and organic extraction optimization (Table 2). More thorough screening methods have not been applied but may give further insight into optimal antibiotic isolation methodology. SS 729 was scaled up and extracted under the previously optimized conditions. Crude yield varied from 3.7 mg/ L to 17.85 mg/L. Drastic changes in yield may possibly be due to inconsistent growing conditions. Further scale-ups will lead to higher precision. Column chromatography was performed with optimal compound separation (25% ethyl acetate). Antibiotic activity of fractions was determined in a liquid inhibition assay with *S. aureus* and showed slight antibiotic activity in fraction F (R_f= 0.56;

total 4.5mg). Antibiotic activity did not show visually strong results. The structure of the active compound in fraction F was assessed with ^{1}H NMR analysis and is currently being evaluated.

Bacillus strains have been known to produce a wide variety of antibiotics. Gramicidin, tyrocidine, bacitracin, mycobacillin, surfactin, bacilysin, and subtilin were all isolated before 2001 from a variety of *Bacilli*.[32] More recently, macrolactin, bacillaene, difficidin, amylocyclicin have been isolated.[33] Further scale-up and fraction characterization of SS 729 will confirm similar or novel structures to previous isolates above.

3.1.3. SS 452B

SS 452B was determined to originally inhibit *S. aureus* but not *E. coli* in early stages of high-throughput screening methodology.[18] Optimization was completed through thorough screening methods for minimal media, time trial, and extraction methodology.[34] SS 452B was scaled up and extracted under optimized conditions (crude: 17.27 mg/L). Column chromatography was performed with optimal separation (30% ethyl acetate). Antibiotic activity of fractions was determined in a liquid inhibition assay against *S. aureus* and showed activity in Fraction I (R_f = 0.66) and G (R_f= 0.40). After the fractions were reduced, TLC was completed, and results were inconclusive. R_f values may not be accurate. SS 452B was rescaled up and extracted under optimized conditions (crude: 9.91 mg/L). Column chromatography has yet to be performed. Upon method corrections to the high-throughput screening assay, SS 452B was determined to not inhibit *S. aureus* or *E. coli.*

Although there has been considerable examination of antibiotic resistance mechanisms in *Microbacterium* species,[35] as of the date of this publication, there are no reports of *Microbacterium* producing antibiotic metabolites in the literature. With *Microbacterium* antibiotic production unknown, it would be an immense discovery for the Wolfe research laboratory at UNC Asheville and may be a source of a novel NP with antibiotic properties. Since this is potentially a novel antibiotic producer, reconfirmation of antibiotic activity in high-throughput screening assay or additional scale-ups may help confirm the presence or lack of antibiotic production. If it is found to be a non-producer, SS 452B will be used in future bacterial interaction assays for induced antibiosis.

4. Conclusion

This research has demonstrated the early stages of isolation techniques and optimized characterization techniques for NP antibiotic discovery with the ultimate goal of NP isolation from bacteria-bacteria interactions. 101 soil bacterial isolates were successfully purified from the Southwestern United States along with work with NP isolation from three bacterial species. All three bacterial species undergoing NP isolation are currently in their compound purification steps as well as intended NP characterization in the future.

5. Acknowledgements

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