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Antibiotic Activity of Natural Products Produced by Bacteria Isolated in North Carolina and Utah

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Abstract

In order to treat the growing number of antibiotic resistant pathogens, the isolation and production of new antibiotics is necessary. The need to discover novel antibiotics is increased by the lack of interest of large pharmaceutical companies. The three bacterial strains being studied herein were isolated from the phytotelma of *Serracenia* pitcher plants found in Western North Carolina, or from soils collected on public lands in Utah, U.S. *Pseudomonas* CM/CP G1 was grown in a 25 mM sodium succinate minimal media, *Streptomyces* SS568 was grown in both glucose and acetate minimal medias (12.5mM), and SS827 was grown in 12.5 mM citrate minimal media. Antibiotic activity of crude extracts were tested against Gram-positive *Staphylococcus aureus* (grown on 10% Triptic Soy Agarose plates). The presence of antibiotic compounds has been confirmed for all strains. Compounds isolated via column chromatography from SS568 and SS827 were probed for antibiotic activity against *S. aureus*. Structural characterization of active compounds using 1D and 2D NMR, IR, and LC-MSMS are ongoing.

1. Introduction

Since the discovery of penicillin in 1929[1], humans and bacteria have been in a race for survival. Bacterial selection has resulted in multidrug resistant bacteria, and humans are working to both modify old antibiotics and isolate new antibiotics to combat these resistant strains. The ability of bacteria to selectively evolve resistance to known antibacterial compounds has been attributed to several factors, including the excessive use of antibiotics in livestock and agriculture industries, as well as the over prescription and unnecessary prescription in clinical industries.[1] Specifically in the case of the Gram-positive bacteria *Staphylococcus aureus*, it has been noted that the development of resistant strains may occur during the treatment of other infections.[2] When a patient is being treated for a different bacterial infection, antibiotics may be present in lower levels at the site of the *Staphylococcus aureus* in relation to the levels at the site of the bacteria which is being treated. It is expected that *Staphylococcus aureus* selects for resistance at this low concentration of antibiotics, and is then resistant to this type of antibiotics at higher concentrations, rendering this antibiotic ineffective in the treatment of this strain of bacteria.[2]

The growing populations of drug resistant strains of *Staphylococci* bacteria are of particular concern for their high mortality rates, and their tendency to affect at-risk populations such as hospital patients, the elderly, and children, in addition to the growing effect on lower-risk populations.[2] When a strain of bacteria becomes resistant to a certain drug, it also becomes more easily resistant to similar drugs. Gram-negative bacteria, in particular, have a variety of mechanisms for developing resistance to antibiotics,[3] and can become resistant to entire classes of antibiotics.[4] The growth of drug-resistant bacteria calls for more aggressive discovery and development of antibiotic compounds, including a combination of genomic research with high through-put screening.[2] In the past, the re-isolation of known antibiotics stymied advances in new drug development, and most new antibiotics released to market were modifications of known antibiotics.[1,5,6]

One promising avenue for the isolation of novel antibiotic compounds is microbial co-culture.[5] Microbial co-culture, or the growth of multiple strains of bacteria in the same culture, attempts to mimic natural environmental conditions in which bacteria compete with one another for limited resources.[5] While in competition with one another, bacteria produce natural compounds, including antibiotic compounds.[7] This production of secondary metabolites (metabolites produced that are not necessary to the bacteria to survive) once speculated to be both competitive and cooperative, has been found to be more competitive in nature.[8] Burgess *et al.* demonstrated this competitive nature by exposing bacterial cultures to cell free supernatants and observing, through a methicillin resistant *Staphylococcus aureus* (MRSA) inhibition assay, the production of antibacterial natural products. This concludes that interactions between microorganisms in culture, while not well understood, do induce an increase in antibiotics, which could be due to competition.[9] Abrudan *et al.* screened 13 bacteria with one another for suppression and induction of antibiotic production, and concluded that the interactions between the bacteria were competitive.[8]

The culture of bacteria in standard laboratory conditions has resulted in rediscovery of natural compounds.[10] The importance of mimicking natural environments by growing microbes in co-culture has been emphasized.[11] There were initially concerns that mixed fermentation, while being effective at producing novel compounds, could lack reproducibility.[10] However, several effective strategies for consistent production of natural products via microbial co-culture have been seen, refuting the concern of irreproducibility.[10] [MT1] One Strain Many Active Compounds (OSMAC), is the term used to describe the method of modifying the genome of a strain of fungus or the culture conditions in which it is grown to better exploit its secondary metabolic pathways.[11] Abrudan *et al.* describe two methods for growth of bacteria, social and asocial. For asocial growth, one strain is grown across an entire plate, called the target strain, and another bacterial strain, called the focal strain, is grown in a colony over the target strain. If the focal strain produces secondary metabolites with antibiotic activity, it can kill the target strain, producing a zone of inhibition, or ring of dead target strain. In social growth, a third bacteria, a modifier, can be grown alongside the focal strain over the target strain. This modifying strain allows for the induction or suppression of the antibiotic activity of the focal strain.[8] The Wolfe group uses a similar method to search for antibiotic production in social and asocial conditions.[12,13]

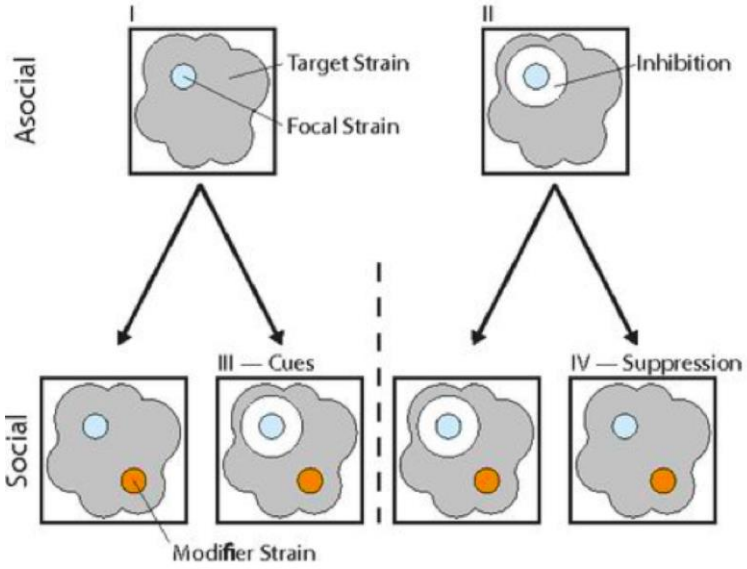

Figure 1. From Abrudan *et al*. Figure 1 (A), Schematic of Social and Asocial inhibition assays.

Gomez-Escribano and Bibb modified *Streptomyces coelicolor* M145, suppressing expression of four production pathways of secondary metabolites, to reduce the number of pathways in which carbon and nitrogen would be used. Additional point mutations were also made. These modifications resulted in higher production of secondary metabolites with a simpler metabolic profile. The modified strains are ideal candidates for heterologous expression of genes which may induce production of novel or difficult to produce secondary metabolites. The simplification of the metabolite profile may lend these strains as gene carriers for promising antibiotic compounds produced only in low levels in nature. [14]

Manteca *et al.* examined the liquid culture of *Streptomyces coelicolor* for cell differentiation and antibiotic production. The growth of *S. coelicolor* in 20 ml liquid culture was observed in several growth stages, a first mycelium, death stage, and second mycelium. It was after the death stage and in the second mycelium stage at which production of antibiotics by *S. coelicolor* was optimized. Liquid cultures more densely inoculated began production of antibiotics before the second mycelium, but production was still optimized at the later mycelium. Additionally, the denser cultures reached the second mycelium growth phase after shorter culture time than the less densely inoculated cultures. [15]

Dr. Amanda Wolfe, of the Chemistry Department, and Dr. Sarah Seaton, formerly of the Biology Department, at the University of North Carolina Asheville, have worked in collaboration to isolate >400 strains of bacteria from both soil and aquatic environments in western North Carolina. These have been grown in a variety of methods in order to induce or optimize the production of antibiotic secondary metabolites. Their groups co-cultured many of the bacteria in groups of two, but also found many bacteria strains which produce antibiotic compounds when grown in mono-culture (called single producers).[12] This experiment attempted to exploit the production of antibiotic secondary metabolites by bacteria which are single producers, and to isolate and identify antibiotic compounds.

2. Experimental Methods

2.1. Sterile Technique

The workplace was first sterilized by washing with ethanol. The laboratory work was completed under a flame from a propane torch, unsterilized metal or glass was first sterilized by flame, and lids/ coverings for sterile containers were flamed before replacement. Plastics were purchased sterile or were sterilized by autoclave (121 °C, 20 minutes). The methods were carried out under sterile conditions unless otherwise specified.

2.2. Preparation of Culture Media

2.2.1. liquid media

Triptic Soy Broth (TSB) was made using Bacto™ Tryptic Soy Broth Soybean-Casein Digest Medium, 30 g/L for full strength (FS TSB), 3 g/L for 10% TSB. Minimal media carbon sources were made with one of succinate, acetate, citrate, glucose, and salicylate (each 12.5 mM, except for succinate which was used at a concentration of 25 mM) in a mix of salts ($K_2HPO_4 \cdot 3H_2O$ 0.035M, KH_2PO_4 0.022M, $(NH_4)_2SO_4$ 0.008M), and 0.0012M $MgSO_4$. The solid additives were dissolved in deionized water for all types of liquid media, and these were autoclaved (121 °C, 20 minutes).

2.2.1. solid media

Agar plates were made using Difco™ Triptic Soy Agar Soybean-Casein Digest Agar. Full strength and 10% mixtures of Triptic Soy broth were used (as prepared above), adding 20g of the agarose powder to the mixture before adding deionized water. These were heated to aid in solution, and autoclaved (121 °C, 20 min).

2.3. Culture of Bacteria

2.3.1. small scale (3-30 ml)

Under sterile technique, 3 ml (up to 30 ml) of each carbon source was inoculated with a colony of the bacteria, and the bacteria was allowed to grow for 2 days in a shaker (eppendorf New Brunswick Excella 25 Inc Shaker) at varying rotations per minute (above 90 rpm), and at room temperature (23-26 °C).

2.3.2. large scale (increments of 1 Liter)

Each respective liter of liquid media was prepared, in a 2 liter Erlenmeyer flask, then autoclaved. A small scale culture was first prepared, then mixed into the liter of media, in a 1% ratio of starter culture to liquid media (10 ml starter culture to 1 L culture) This was grown loosely covered (to preserve sterile conditions) for some length of time in the shaker. For growth of more than 1 liter, multiple flasks were used.

2.4. Storage of Bacteria

2.4.1. long-term

Bacterial strains preserved in 50% glycerol stocks at –80 °C.

2.4.2. regular use

Bacteria were stored on solid media plates. They were streaked from a purchased source, glycerol stock, another solid media plate, or liquid culture. Bacteria were grown for approximately 2 days (depending on the speed of growth) at 23 °C (for terrestrial and aquatic bacteria) or 37 °C (for pathogenic bacteria like *S. aureus*). Once single colonies were present, the plates were parafilmed and could be kept refrigerated for up to two-weeks. To inoculate a media or streak a new plate, a single colony was removed and used to streak or inoculate.

2.5. Well Assay on TSA

S. aureus was grown in 3 ml of FS TSB (cultured for 2 days, 37 °C), then was spread evenly over a 10% TSA plate. Excess liquid culture was removed, and the remaining culture allowed to dry. The end of a glass pipette was sterilized and used to cut wells into the plate. Test compounds in solution (1 % DMSO in minimal media or sterile DI H_2O) were pipetted into each well (50 µl).

2.6. Well Assay in Liquid Culture

Dilution assays (10-fold) were prepared using 96-well plates for master plates and test plates. Master plates were made using 9 µl DMSO in each of the 96 wells. The top row contained 1 µl of a) a compound of interest dissolved in DMSO; b) pure DMSO (negative activity control); or c) the antibiotic chloramphenicol (100mg/ml) (positive activity control). The dilution was performed taking 1 µl from the top well, and mixing it in the well below, down each column. Test plates each contained 10% overnight culture of *S. aureus* in FSTSB (10% culture to fresh FSTSB) and 1% of the test compound in DMSO. Compounds were either transferred from a pre-prepared master plate, or added directly to each well. Absorbance was read for each well on a BioTek plate-reader at 590nm using a Standard S. Aureus Assay Protocol_590nm_blanks.prt, analyzed using Gen5 2.09 software data package. Antibiotic activity was assessed visually and by absorbance values. No established cut-offs for activity had been established, activity was considered antibiotic if less bacteria growth was present than the negative control (DMSO).

2.7. Agar Bacterial Inhibition Assay

200 µl FS TSB inoculated with *S. Aureus* (cultured for two days, 37 °C) was spread over a 10% TSA plate, and dried. 3 µl of cells cultured in media (small scale culture, described above) was pipetted onto the plate (4 per plate, each in own quadrant for small plate, 6-9 cell cultures per larger plate). These bacteria on the plates was left to grow at 23 °C for 1-3 days. The zone of inhibition (if present) was measured to asses the potency of antibiotic activity.

2.8. Extraction of Secondary Metabolites: *CM/CP G1*

Each liter culture of bacteria was centrifuged at 8000 rpm for 12 minutes, and the supernatant was collected. The supernatant was treated with 50 ml saturated aqueous NaCl (brine) per 1 liter of solution. This was extracted three times with ethyl acetate. The organic layers were combined, washed with brine, dried over anhydrous sodium sulfate (Na_2SO_4), and concentrated under reduced pressure.

2.9. Extraction of Secondary Metabolites: *SS568*

The cell free supernatant of the SS568 cultures was saturated with sodium chloride (approximately 100 ml by volume dry NaCl per 400 ml supernatant), then extracted with hexanes, diethyl ether, and ethyl acetate respectively. Each layer was dried over anhydrous sodium sulfate (Na_2SO_4) and concentrated under reduced pressure.

2.10. Purification of Secondary Metabolites

Organic compounds extracted and concentrated from bacterial culture were purified using normal phase (SiO_2, XX-XX% EA/Hex) flash or gradient column chromatography. Ethyl acetate to hexane ratios were determined using thin layer chromatography (TLC). TLC was used to consolidate fractions from the column.

2.11. Time Trial

The production of secondary metabolites was optimized by time. The growth of cells is checked over a period of 72 hours, in exponential increments. Growth was monitored by optical density at 600 nm. The production of antibiotic compounds was checked by a small sample of culture at each of these time points. A 3 ml fraction of the liquid media was removed from the culture flask, filtered through a 0.22 µm syringe filter to remove cells, and concentrated by evaporation or increased airflow. These concentrated cell free fractions (dissolved in minimal media, not DMSO) were tested by well assay for antibiotic activity against *S. aureus.*

3. Results and Discussion

3.1. *Pseudomonas* species, *CM/CP G1*

The *Pseudomonas* species CM/CP G1 was seen to be a single producer against *S. aureus,* while grown in mono culture in succinate minimal media. The growth of CM/CP G1 in various minimal media carbon sources was optimized, and the bacteria showed the greatest absorbance, indicating cell growth or production of secondary metabolites, in succinate minimal media.

Table 1. The growth of the bacteria CM/CP G1 in various minimal media carbon sources for optimization of growth and antibiotic production.

Media Source	Absorbance (at 600 nm)	Antibiotic Activity (Y/N)
TSB	0.223	Y
Acetate	0.013	NA
Citrate	0.304	NA
Glucose	0.140	NA
Salicylate	0.004	NA
Succinate	0.458	Y

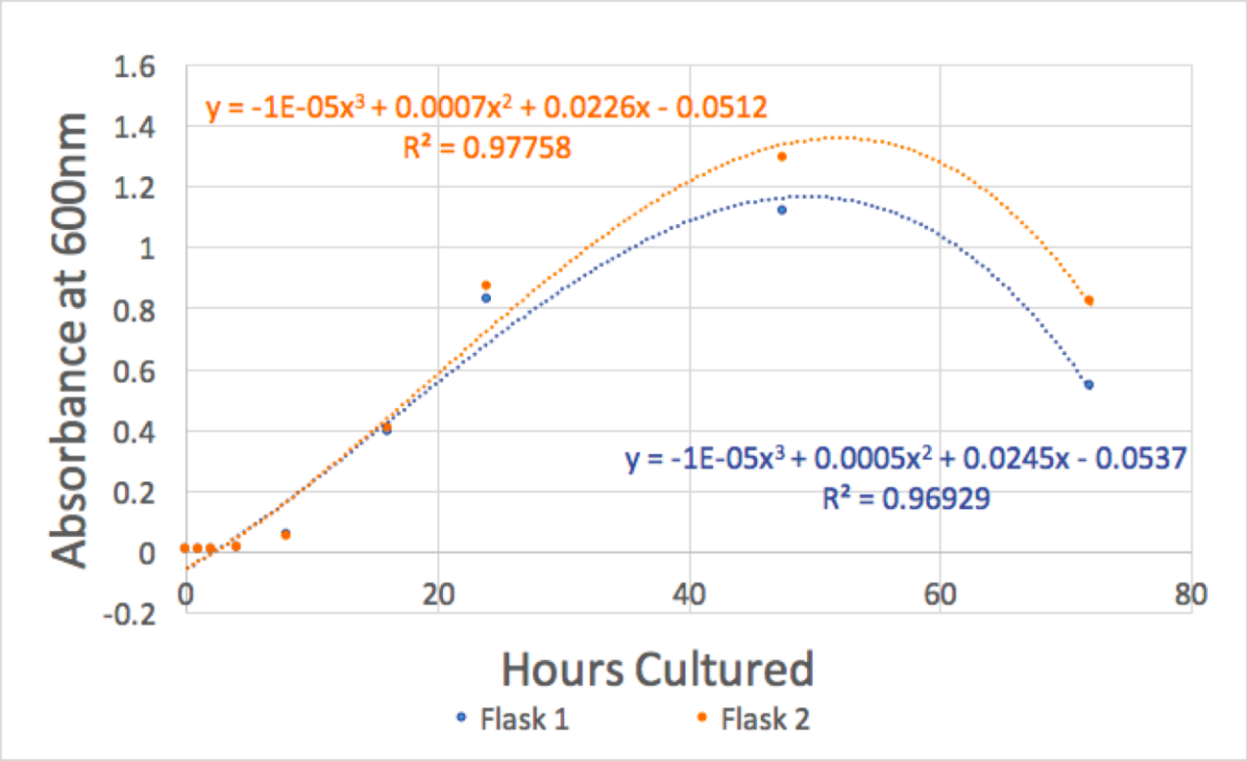

Figure 2. The growth of CM/CP G1 in succinate minimal media over 72 hours.

A time trial was conducted over 72 hours for the growth of CM/CP G1 in succinate minimal media (Figure 2). The method of checking bacteria growth of CM/CP G1 by absorbance values was seen to be effective, because the peak in absorbance between 40 and 60 culture hours (figure 2) correlates with the beginning of antibiotic production. Antibiotic production was demonstrated through a test of each time point during the trial in a well assay, testing against *S. aureus*. Samples from 24, 48, and 72 hours produce zones of inhibition measuring 1.65 cm after 24 hours in the assay (figure 3), and these increased minimally (0.1 cm) after an additional 24 hours in the assay.

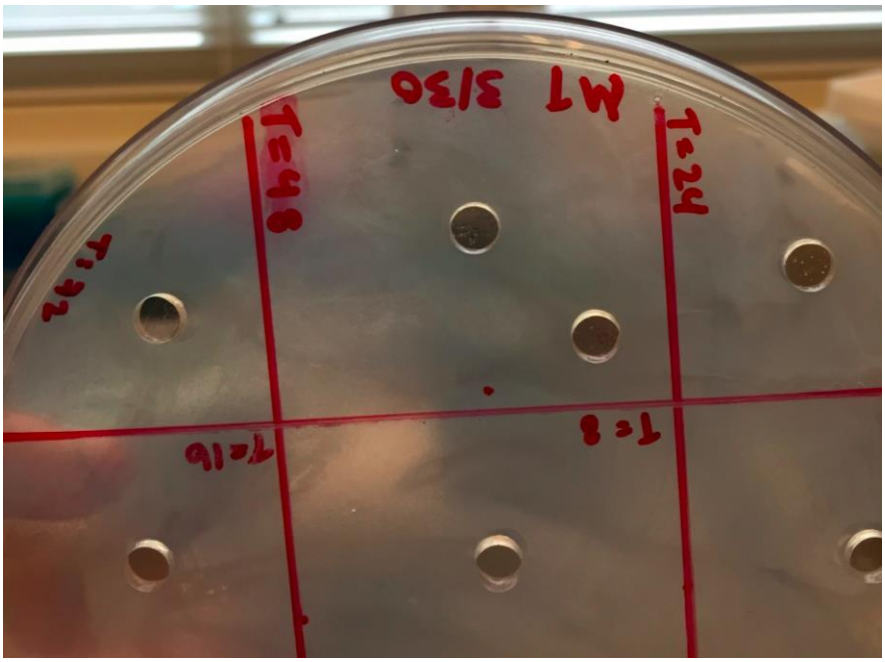

Figure 3. Well assay illustrating production of antibiotic activity, indicated by the darker ring around wells, in time trials from 24, 48, and 72 hours after inoculation.

CM/CP G1 cultured in 3 liters of succinate minimal media produced 64.9 mg of crude product after culture for 3 days (74 hours). Crude product was dissolved in 1:9 ethyl acetate: hexane solution with a few drops of dichloromethane (DCM). This solution was purified by column chromatography (20% ethyl acetate 80% hexanes), and tested by well assay. No antibiotic activity was observed. Likely this lack of antibiotic activity was due to the very low concentration of product.

The bacteria CM/CP G1 was later grown in several incubations of 3-6 liters of succinate minimal media, but the antibiotic active compound could not be successfully and consistently isolated. One isolated compound showed a zone of antibiotic activity against *S. aureus* in one well assay, it is pictured outlined in blue ink, but this result could not be replicated (Figure 4).

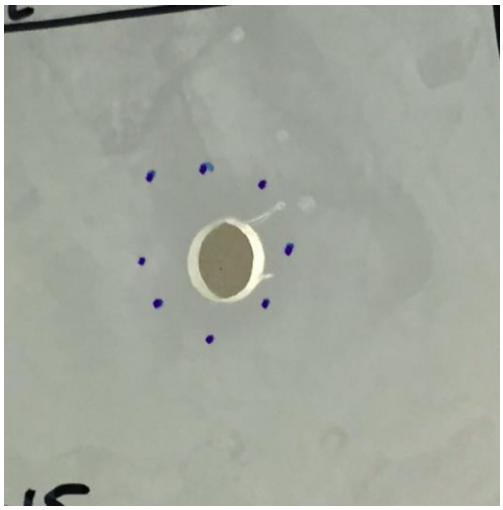

Figure 4. Compound produced by CM/CP G1 with possible antibiotic activity, results not reproducible.

3.2. *Streptomyces* species, *SS568*

The *Streptomyces* bacteria, SS568, was seen to produce antibiotic compounds when grown in glucose minimal media after 3-5 days (at 3 days, 1.1cm zone was just below the activity of the control, at 7 days surpassed the activity of the control, with a lighter zone of 1.7 cm). In acetate minimal media, antibiotic activity was observed after 11 days, exhibiting a 1.1 cm and 1.45 cm zone of inhibition, and a 2 cm zone after 16 days (Figure 5).

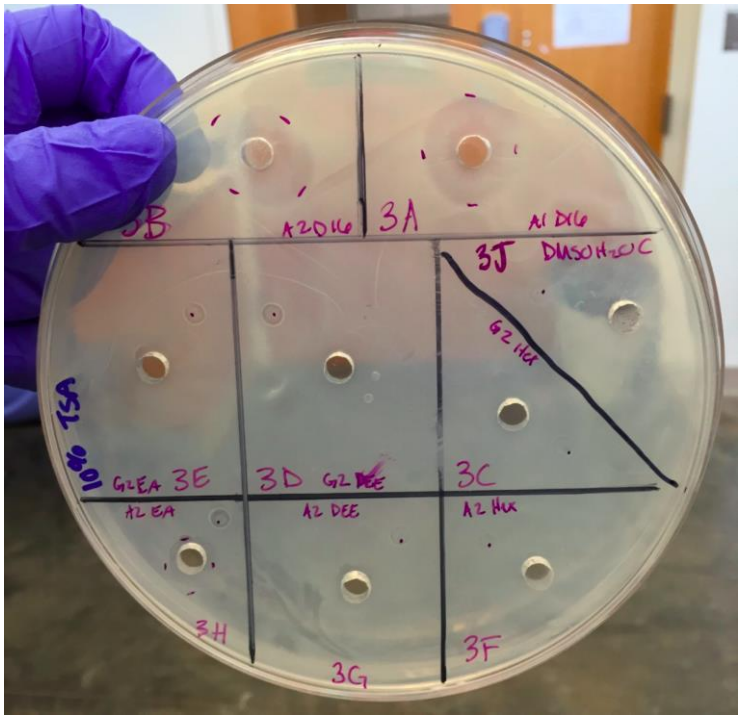

Figure 5. The antibiotic activity against *S. aureus* as indicated by a zone of inhibition.

Figure 5 Antibiotic activity present in 3A and 3B resulted from the 16 day culture of SS568 in acetate minimal media. Wells 3C-3J represent respectively hexane (3C), diethyl ether (3D), ethyl acetate (3E) extractions from *SS568* in glucose minimal media; hexane (3F), diethyl ether (3G), and ethyl acetate (3H) extractions from *SS568* in acetate MM; and a control 1% DMSO in sterile DI H_2O solution.

The 3 liter cultures of SS568 grown in acetate and glucose minimal medias were extracted using a varied solvent technique, and each extraction layer was tested for antibiotic activity. The activity of fractions remained relatively the same across the two medias, ethyl acetate most active, diethyl ether less active, and hexanes not active, with the more antibiotic active fractions in the more polar solvents (figure 5; 3C-3J). Activity was shown using the bacterial assay technique described in the methods section (2.7), in which 3 µl of each crude product in 1% DMSO/sterile DI H_2O solution was added to the plate which was covered in *S. Aureus*. The location of the compound in solution was indicated by a purple dot, and activity was indicated visually by a zone of inhibition.

The diethyl ether (DEE) and ethyl acetate (EA) extractions were found to have 10-11 and 6 different compounds visualized on silica TLC plates using potassium permanganate stain ($KMnO_4$), long wave ultraviolet radiation (UV), or short wave UV. These were isolated from the crude product via column chromatography (2-10% and 2-15% graduated solvent system of Ethyl Acetate to Hexanes in silica gel, both separations of compounds in EA crude extracts). The baseline fraction produced a zone of inhibition when tested against *S. aureus* (Figure 6).

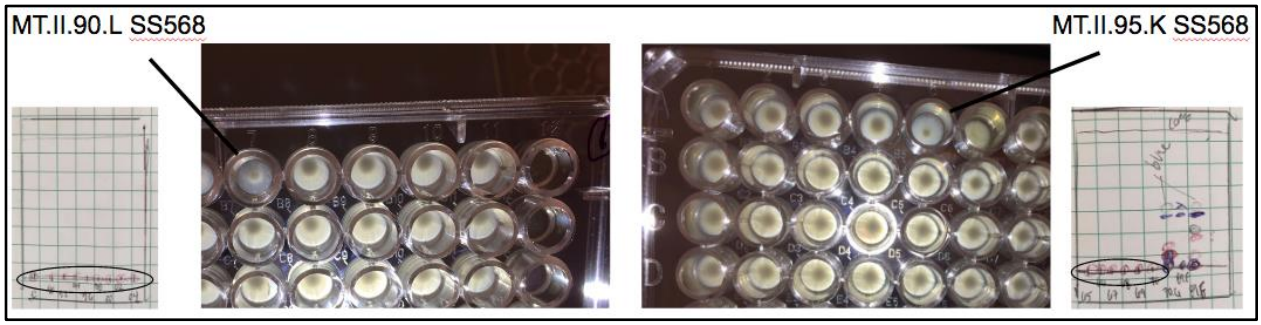

Figure 6. Baseline fraction of *SS568* visually shows antibiotic activity against *S. aureus*.

Figure 6 The vials showing antibiotic activity against *S. aureus* contain a compound visualized at the bottom of a thin layer chromatography plate, indicating relatively high polarity of compound. Antibiotic activity is indicated by the decrease in pellet size at the bottom of the well, indicating an inhibition or termination of cell growth by the compound in solution.

3.3. *Pseudomonas* species, *SS827*

The bacteria SS827 was cultured using a 10% inoculation (small culture grown for one day, used for a 300 ml culture, grown for one day, used in 3-L culture) for three days. Growth time and carbon source optimized according to growth assays by Elizabeth Murray. The 6-L culture was centrifuged (12 minutes, 4000 rpm), extracted (sat. NaCl, extracted 3x with ethyl acetate, dried with anhydrous Na_2SO_4), producing 113.0 mg of an oily, yellow crude product. This crude product produced a 1.5cm zone of inhibition against *S. aureus* in a well assay (1% DMSO in FSTSB) as compared to the positive activity control, chloramphenicol (Z.O.I. = 2.7 cm) and negative activity control, 1%DMSO in FSTSB (Z.O.I. = 0.0 cm) (Figure 7). The compounds in the crude extract were isolated using 10-40% EA/Hex gradient column chromatography.

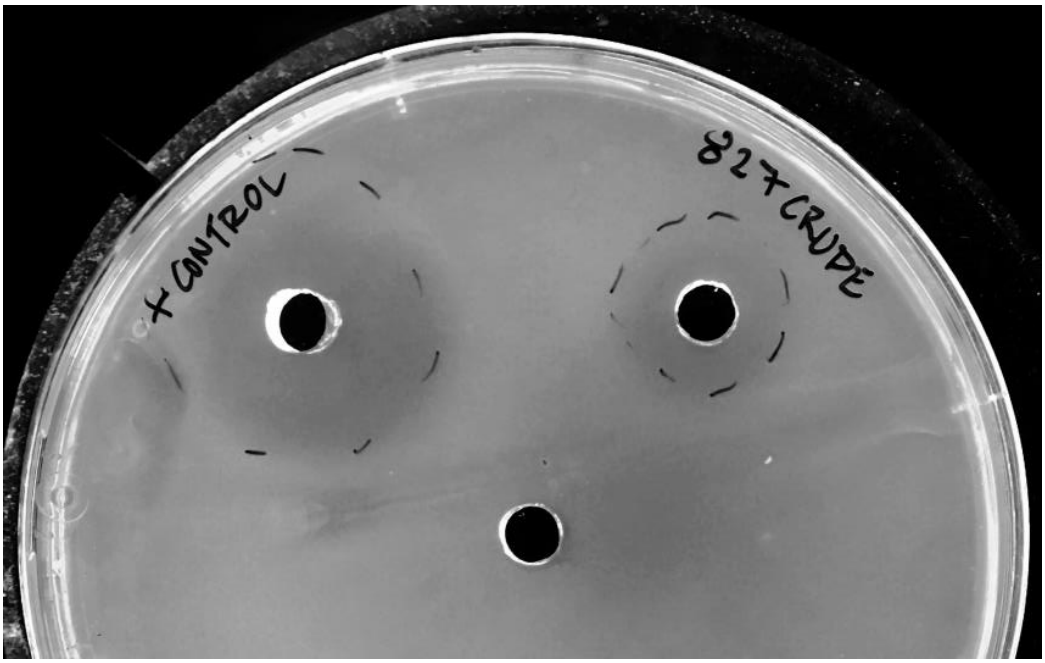

Figure 7. The antibiotic activity of SS827 crude extract against *S. aureus*.

Figure 7. The zone of inhibition of the crude extract as compared to the positive (chloramphenicol) and negative (DMSO) controls. Z.O.I.s are outlined, and the center well is the negative control. Black and white used to emphasize contrast between growth and death of *S. aureus*.

The fractions isolated via column chromatography were tested in a well assay in liquid culture (2.6) as follows. Fractions were collected and combined based on visualization with $KMnO_4$, long wave ultraviolet (UV) and short wave UV, then dried. To each vial, 5 µl of DMSO was added. From this, 1 µl was removed, and diluted to 100 µl with DMSO. This DMSO was blown down, then a new 5 µl of DMSO was added to the vial (next day). The compounds. in DMSO, from either the vial or the diluted vial (stored in freezer), were added to each well, 1% by volume. Absorbance was read at 16 and 28 hours of growth (supplemental). The crude extract still showed antibiotic activity after the column (not all of the crude extract was transferred, some remained in the vial TBH.I.2). Antibiotic activity against *S. aureus* was seen in both the crude extract and the 100-fold dilution of the crude extract, as well as for the fractions collected in vials TBH.I.5.13 *SS827*, TBH.I.5.12 *SS827*, and TBH.I.5.4 *SS827*, activity was compared to a chloramphenicol positive control, 100 mg/ ml (1% solution), and to the negative DMSO control (1% solution). Compounds were considered to show antibiotic activity when the pellet appeared smaller than in the negative control, indicating that cell growth was decreased or inhibited by the test compounds after 16 hours of growth (figure 8). Some errors must be noted while performing the well assay: 1) the researcher stopped in the middle of the assay for a brief period (~30 minutes) and the test compound was added to rows 2, 4, 6, 8, 10, 12, and the bottom six wells in row 9 after this brief period. The hourly time of growth was measured from 4 pm, because it was the middle point of the start and finish of the well. 2) It is possible that the test compound for vial TBH.I.5.5 *SS827* (100-fold dilution) was added to the well for TBH.I.5.4 *SS827* (1% in FSTSB), so the activity of TBH.I.5.4 *SS827* seemingly indicated by the assay may be a false result caused by excess DMSO in solution.

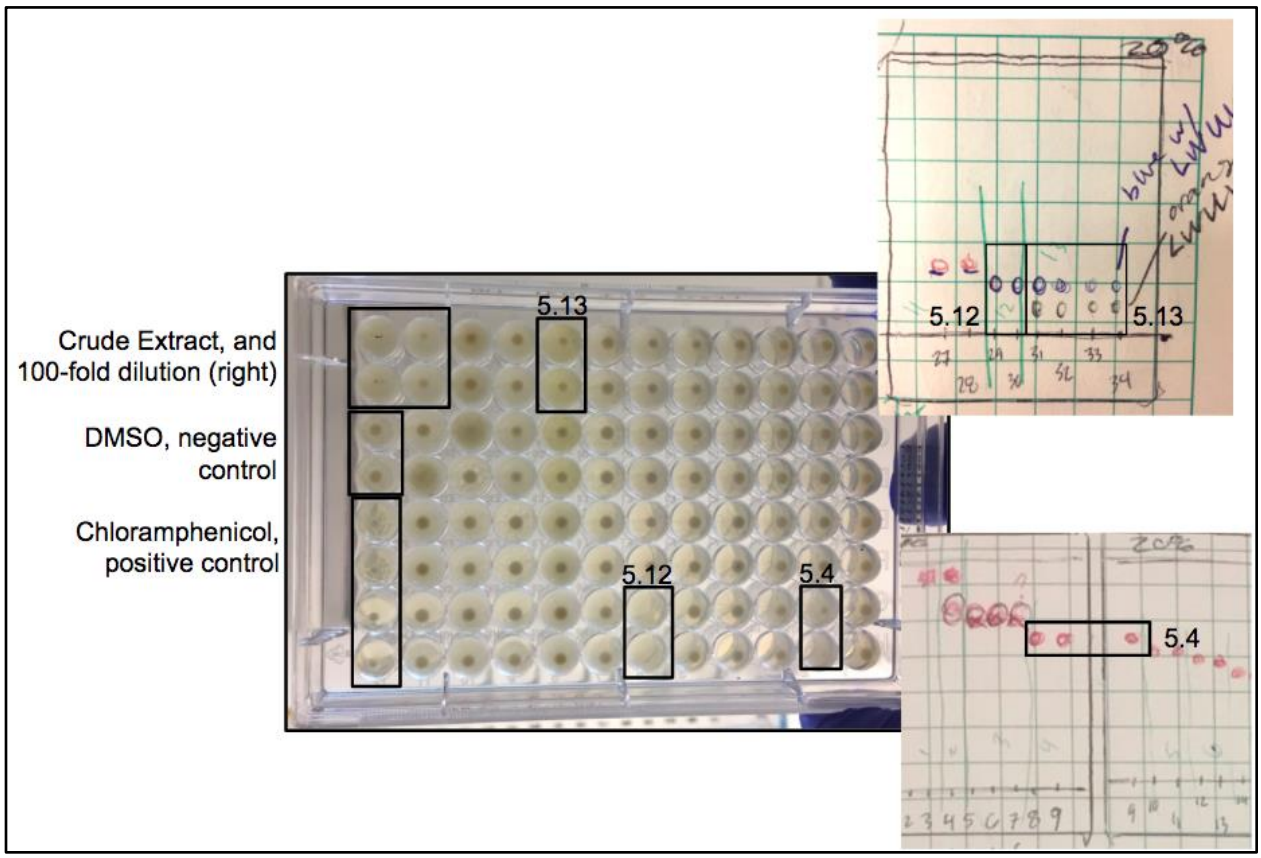

Figure 8. The antibiotic activity of compounds isolated from the secondary metabolites of bacteria *SS827*, grown in citrate minimal media.

Figure 8. Antibiotic activity, against *S. aureus*, of fractions visualized via column chromatography was assessed using a 96 well liquid culture assay, using chloramphenicol (100 mg/ml, diluted to 1% in FSTSB) as a positive activity control, and DMSO (1% in FSTSB) as a negative control. TLC plates are shown which were used to combine fractions after column chromatography. The activity of vial 5.4 is questionable due to an error in protocol.

4. Conclusions

Antibiotic activity against *S. aureus* has been confirmed and isolated from the secondary metabolic profile of the bacterial strains *SS568* (a *Streptomyces*) and *SS827*, and antibiotic activity was visualized, but not reproducibly, from the bacteria strain *CM/CP G1* (a *Pseudomonas*).

5. Acknowledgements

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7. Supplemental

Supplemental figure 1. Fractions from the column of *SS827* were arranged as so when tested for antibiotic activity against *S aureus*.

	1	2	3	4	5	6	7	8	9	10	11	12
A	5.1	5.1	5.5	5.5	5.9	5.9	5.13	5.13	5.17	5.17	TBH 2	THB 2
B	5.1	5.1	5.5	5.5	5.9	5.9	5.13	5.13	5.17	5.17	TBH 2	TBH 2
C	5.2	5.2	5.6	5.6	5.1	5.1	5.14	5.14	5.18	5.18	***	DMSO
D	5.2	5.2	5.6	5.6	5.1	5.1	5.14	5.14	5.18	5.18*	5.18?	DMSO
E	5.3	5.3	5.7	5.7	5.11	5.11	5.15	5.15	5.19	5.19*	5.19?	Chloram
F	5.3	5.3	5.7	5.7	5.11	5.11	5.15	5.15	5.19	5.19**	5.19	Chloram
G	5.4	5.4	5.8	5.8	5.12	5.12	5.16	5.16	5.2	5.2	****	Chloram
H	5.4	5.4	5.8	5.8	5.12	5.12	5.16	5.16	5.2	5.2	****	Chloram

Supplemental figure 2. Absorbance data after 16 hours of growth.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.439	0.518	0.493	0.539	0.504	0.597	0.528	0.519	0.664	1.212	0.839	0.324
B	0.567	0.601	0.655	0.575	0.555	0.755	0.608	0.548	0.726	1.266	0.84	0.429
C	0.583	0.59	0.506	0.616	0.68	0.722	0.525	0.759	0.881	1.279	0.604	0.574
D	0.632	0.573	0.5	0.663	0.692	0.723	0.539	0.753	0.852	0.551	1.342	0.603
E	0.649	0.616	0.588	0.564	0.595	0.488	0.68	0.958	0.552	0.626	0.847	0.203
F	0.546	0.608	0.568	0.545	0.594	0.531	0.65	0.978	0.552	0.592	0.827	0.194
G	0.531	0.618	0.617	0.58	0.506	0.414	0.602	0.977	0.541	0.755	0.538	0.206
H	0.491	0.606	0.634	0.592	0.537	0.394	0.608	1.045	0.54	0.768	0.57	0.242

Supplemental figure 3. Absorbance data after 28 hours of growth.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.414	0.494	0.441	0.49	0.456	0.616	0.469	0.428	0.511	1.199	0.671	0.318
B	0.54	0.625	0.621	0.583	0.533	0.765	0.615	0.458	0.686	1.193	0.808	0.328
C	0.549	0.619	0.547	0.592	0.641	0.713	0.577	0.759	0.891	1.312	0.685	0.562
D	0.564	0.61	0.577	0.644	0.672	0.719	0.579	0.725	0.826	0.622	1.289	0.581
E	0.569	0.626	0.564	0.578	0.627	0.463	0.637	0.823	0.605	0.616	0.804	0.198
F	0.516	0.623	0.551	0.569	0.616	0.483	0.619	0.847	0.603	0.595	0.782	0.195
G	0.489	0.545	0.564	0.595	0.554	0.515	0.592	0.899	0.611	0.696	0.597	0.202
H	0.449	0.54	0.535	0.573	0.53	0.459	0.546	0.839	0.563	0.626	0.599	0.301