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Co-culture Screens for the Isolation of Antibiotics from *Sarracenia* Pitcher Plant Bacteria

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Abstract

New antibiotics must be continually developed to combat multidrug resistant bacteria. Bacterial co-culture has been previously described as a method to induce antibiotic production in microorganisms. Using a bacterial library of isolates from *Sarracenia* pitcher plants in Western North Carolina, a high throughput screening method was developed and optimized to identify bacteria that produce antibiotic compounds in either single culture or co-culture. Three assay methods were designed and tested: the circular well assay, the agar pour-over assay, and the replicator assay, with the replicator assay showing the least ambiguous results. For antibiotic producers, the culture was scaled up to liter volumes and the antibiotics were isolated using liquid-liquid extraction followed by chromatography. Work is ongoing to increase the yield of each compound, and to determine their structures using ^{1}H NMR, ^{13}C NMR, and IR spectroscopy.

Keywords: Co-culture, antibiotics, bacteria

1. Introduction

The emergence of multidrug resistant bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a significant threat to human health. These drug resistant bacteria have increased in prevalence due in part to inappropriate and excessive use of antibiotics in the fields of both medicine and agriculture.[1] Most large pharmaceutical companies have stopped developing new antibiotics, and most new antibiotics are being discovered by smaller research groups.[1] Natural products, organic molecules that are isolated from living organisms, continue to be the most abundant source of new antibiotics, constituting two-thirds of the new antibiotic therapies approved in the United States in the past 30 years.[1] Clinically useful natural products are typically secondary metabolites, ones that are not required for survival and only produced under specific conditions. Some genomic studies suggest that a significant portion of the genome of some bacteria are related to biosynthesis pathways that are not expressed under any currently known conditions.[2] One method that is frequently found in the literature is the use of bacterial co-culture as a means of stimulating the production of antibiotic secondary metabolites. Co-culture is a way of emulating natural competition between bacteria, and has been shown as a way to stimulate antibiotic production.[3]

The use of bacterial co-culture for antibiotic production is well documented in the literature. A screen of *Streptomyces tenjimariensis* against fifty-three other marine bacteria found twelve co-culture interactions that as much as doubled production of the antibiotic istamycin.[4] A screen of *Streptomyces lividans* against many different mycolic acid containing bacteria found that the antibiotic alchivemycin A was only produced when cells were in direct contact, and not when they were separated by a dialysis membrane.[5] It was found that biphenomycin C was produced by *Streptomyces griseorubiginosus*, and the compound was converted to the active antibiotic compound Biphenomycin by *Pseudomonas maltophila* in co-culture.[6] The method used by Seyedsayamdost et al. is particularly useful for this

study, because the mixed culture and antibiotic screening were combined into a single step. Seyedsayamdost et al. screened twenty actinomycete bacteria in all possible co-culture combinations on an agar plate, and a second layer of agar containing *S. aureus* culture was poured on top of the actinomycete mixed cultures and monitored for signs of inhibition in the growth of the *S. aureus* lawn.[7] An example of this type of assay is shown in Figure 1.

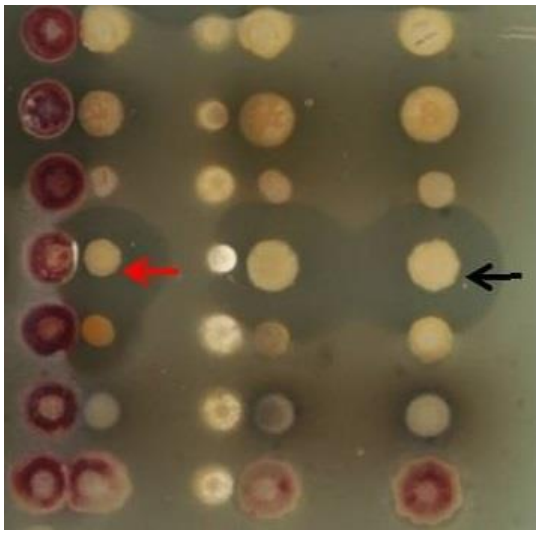

Figure 1. Mixed culture agar pour-over assay from with marked zones of *Staphylococcus aureus* inhibition.[7]

The purpose of this study was to modify the screening method used by Seyedsayamdost et al. to screen all possible binary interactions between a library of bacteria isolated from *Sarracenia* pitcher plants in western North Carolina. This method was used to quickly screen for and identify any single cultures or binary interactions that produced antibiotic compounds that were active against pathogenic bacteria such as *S. aureus*. The cultures that produced antibiotics were scaled up to liquid culture at liter volumes. The antibiotics were extracted using centrifugation and liquid-liquid extraction followed by preparative thin layer chromatography (PTLC) and column chromatography. Once purified, spectroscopic methods including ^{1}H NMR, ^{13}C NMR, and IR spectroscopy can be used to fully characterize these compounds, and tests can be done to quantify their effectiveness against pathogenic bacteria.

2. Methods

All agar and media solutions were sterilized by autoclave. All procedures were carried out under sterile conditions, and all media and tools were autoclaved or flame sterilized before use.

2.1 Acquisition And Maintenance Of Specimens

Cultures of *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were purchased from Microbiologics Inc. and maintained on Mueller Hinton agar plates at 4º C. The *Sarracenia* isolates were obtained from Dr. Sarah C. Seaton of the UNC Asheville Department of Biology. These bacterial cultures were isolated from liquid samples of *Sarracenia* pitcher plants from two sampling locations in Western North Carolina. These 96 isolates were divided into 5 sets and maintained on 10% tryptic soy agar (TSA, 3g tryptic soy broth powder and 20g agar per 1L ultra-pure water) at 23ºC. Also obtained from Dr. Seaton were two strains of *Pseudomonas fluorescens*, one with a single active antibiotic gene (Ab+) and one with the antibiotic producing gene deleted (Ab-). These were used as positive and negative controls in co-culture assays, and were maintained on TSA plates at room temperature.

2.2 Preparation Of Co-Culture Antibiotic Screens

Liquid cultures of *S. aureus* and *E. coli* that were used as overlays for antibiotic screening assays were prepared by inoculating a single colony from the agar plate into a culture tube containing 10 mL of sterile Tryptic Soy Broth (TSB)

and incubated in a shaker at 37ºC for 2 days. Liquid cultures of *Sarracenia* isolates were prepared similarly in well plates containing 1 mL of 10% TSB and agitated in the VWR S-500 orbital shaker at 150 rpm and 23ºC for two days. Three different antibiotic screens were used – the circular well assay, the agar pour-over assay, and the pin replicator assay. For the circular well assay, 50 µL of each isolate was pipetted into wells of a 96 well plate, such that each combination of two bacteria in the set was prepared. Separate wells containing each organism by itself were also prepared to exclude any single producers. 50 µL of liquid culture of *S. aureus* or *E. coli* was spread on top of a 10% TSA plate and allowed to dry. A Pasteur pipet was used to drill circular holes into the agar, and each mixed culture was pipetted into a well on this plate. The plate was incubated for two days at 37ºC and monitored for zones of inhibition. An example is shown in Figure 2.

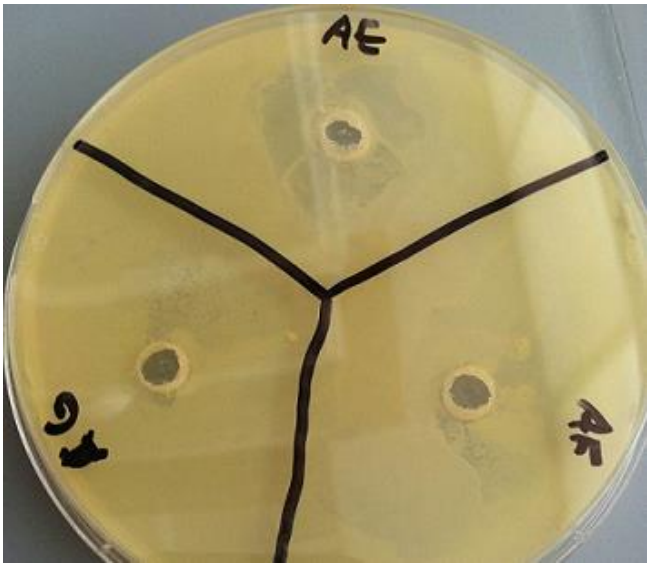

Figure 2. Circular well assay pairing specimen A against specimens E, F, and G. Culture A shows some inhibition against culture AE with an irregular zone of inhibition in the growth of *S. aureus*.

For the pour-over assay, the mixed bacterial cultures were prepared as described previously. 1 µL of each mixed culture was pipetted directly onto the surface of a 10% TSA plate and allowed to grow. Then, 3.0 mL of *S. aureus* or *E. coli* was added to 300 mL of sterilized 10% TSA that had been cooled to 40ºC, and this was carefully poured in a thin layer to cover the whole surface of the plate. The cultures were allowed to grow for two days at room temperature and observed for zones of inhibition. A schematic diagram of the assay setup is shown in Figure 3. An example plate is shown in Figure 4.

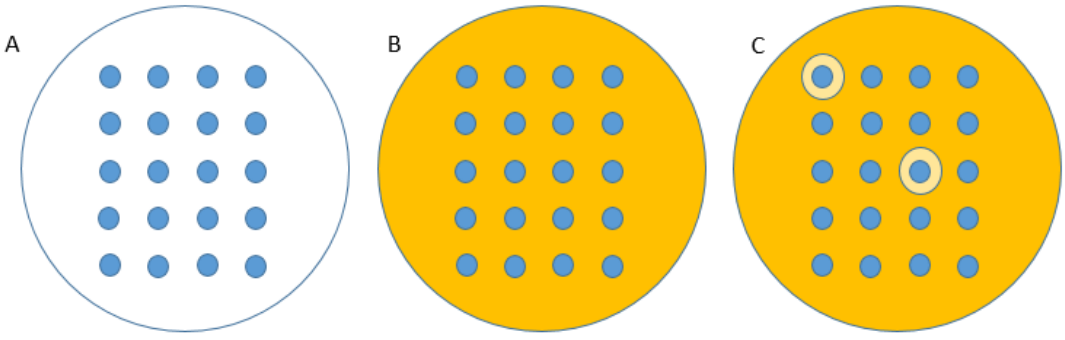

Figure 3. A schematic diagram of the pour-over assay. In A, the mixed cultures of bacteria are pipetted directly onto the agar plate. In B, a layer of agar inoculated with *S. aureus* or *E. coli* is poured on top of the mixed cultures. Zones of inhibition are seen in C, in the top left and middle of the plate.

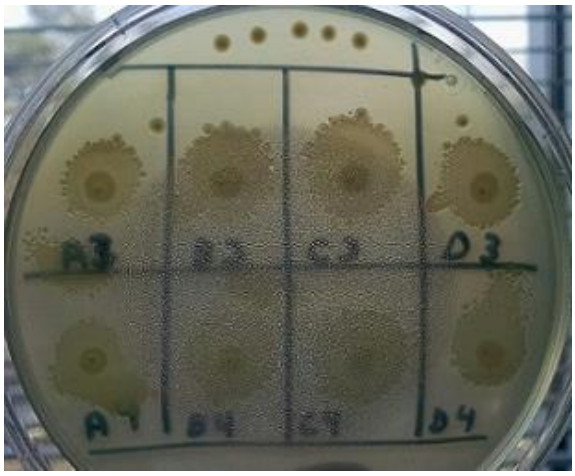

Figure 4. A *S. aureus* agar pour-over plate. Each organism shows overgrowth into the agar overlay and at least one organism shows high mobility in the agar layer.

For the replicator assay, 50 µL of *S. aureus* or *E. coli* was spread evenly on top of a 10% TSA plate and allowed to dry. Unknown mixed cultures were prepared in a 96 well plate as described before, but using 10 µL of each organism. A 96 pin replicator was used to transfer these mixed cultures and pressed into the agar plate which was labeled for orientation. The plate was allowed to grow for two days at room temperature and observed for zones of inhibition. An example of this plate is shown in Figure 5.

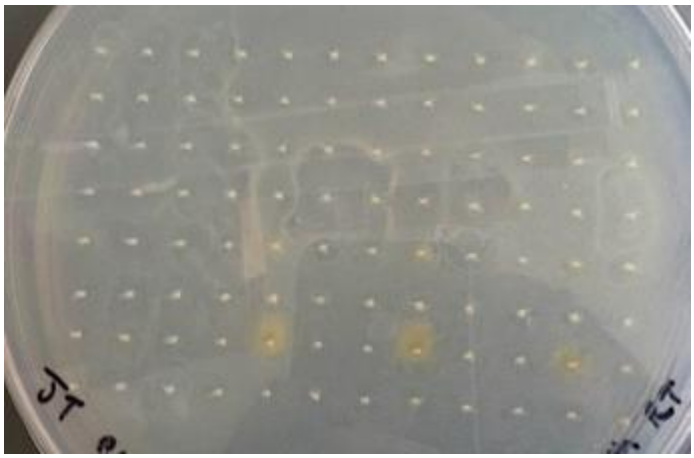

Figure 5. Replicator assay with *S. aureus* overlay. Zones of inhibition can be seen, but shape is still irregular.

2.3 Large Scale Culture And Time Trial

Three single producer hits were scaled up to 1 L volumes – SS401 (*Pseudomonas*), SS405 (*Chromobacterium*), and SS408 (*Streptomyce*). SS401 and SS408 were grown in 10% TSB, and SS405 was grown in a minimal media composed of 12.5 mM Glucose, 35 mM K_2HPO_4, 22 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, and 1.2 mM $MgSO_4$. Specimens were inoculated into 10 mL of growth media and allowed to grow at 23 ºC for one day, then transferred into 1 L of growth media and incubated at room temperature in the New Brunswick Excella E25 incubator shaker at 150 rpm and allowed to grow for 3-7 days, or when cell density began to decrease. Cell density was monitored by measuring absorbance at 600 nm. SS405 was later scaled up to a volume of 4 L, and in this trial absorbance values and antibiotic activity were tested in quadruplicate at 4, 18, 28, 48, 56, and 75 hours, and the flasks were taken out at 75 hours.

2.4 Extraction And Separation Of Extracts

After the growth period, the contents of each flask was poured into centrifuge tubes and cells were spun down at 5000 rpm for 10 minutes. The supernatant was collected, and a small amount was passed through a syringe filter and pipetted into wells in a *S. aureus* overlay plate to test for antibiotic activity. The rest of the supernatant was extracted in a separatory funnel with ethyl acetate (3x). The organic layers were then combined, washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated under reduced pressure. Each crude extract was tested for antibiotic activity by dissolving in minimal dimethyl sulfoxide, diluting 1 µL of this to 100 µL using 10% TSB, and pipetting into wells of a *S. aureus* overlay plate. The extracts were further separated using preparative thin layer chromatography (PTLC) on TLC Silica gel 60 F_{254} 20x20 cm plates for SS405 and SS408, or by normal phase SiO_2 column chromatography for SS405. Separated fractions were tested for antibiotic activity in the same way as the total extract.

For the large scale SS405 culture, an additional step was added to attempt to remove the glucose before separation. First, the extract was divided into 2 fractions. The first fraction was extracted as described above and labeled "A." The second fraction was dissolved in ethyl acetate and washed three times with 1 M NaOH in a separatory funnel. The organic fraction was then dried over Na_2SO_4, concentrated under reduced pressure, and labeled "B." The aqueous NaOH washes were combined and acidified to pH < 4 with 1M HCl. The acidified aqueous solution was then re-extracted with ethyl acetate. The resulting organic layer, labeled "C," was dried over Na2SO4 and concentrated under reduced pressure. Fractions A, B, and C were all tested in the same way as the total extract, and this plate is shown in Figure 4. Afterwards, the washing process was repeated with fraction A.

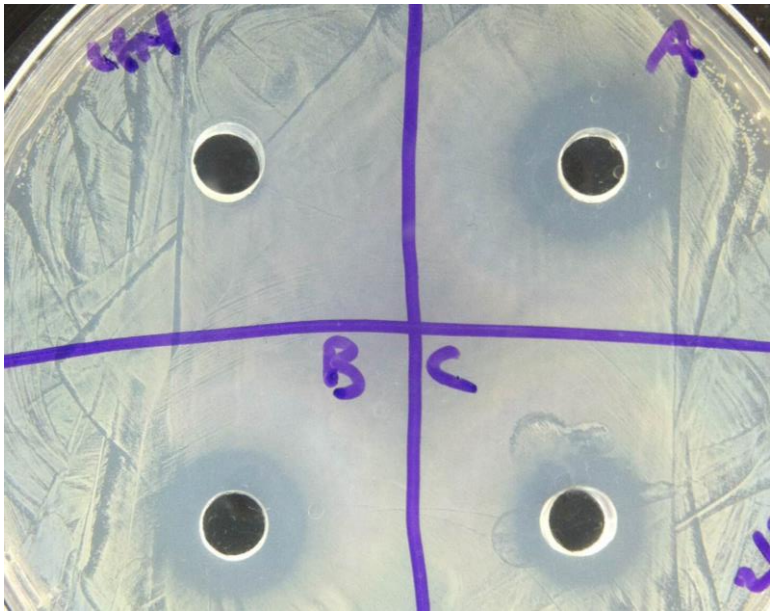

Figure 5. Antibiotic assay for base wash procedure. Fraction B has significantly more antibiotic activity than fraction C, though both show less activity than the unwashed fraction A.

3. Results

Of the three assay types shown in Figures 1, 2, and 3, the pin replicator assay gave the least ambiguous results. This method appears to be an effective large scale screen for antibiotic activity against *S. aureus*. So far, the assays for *E. coli* have not been as successful, as it was never possible to get an even lawn of *E. coli* using this method. Alternative methodology needs to be developed so that specimens can be quickly screened for activity against both Gram-positive and Gram-negative bacteria.

Out of 96 isolates that were tested, 22 were found to be single producers. Frequently, even when all single producers were removed from a set, additional single producers would be found. No interactions were found to produce antibiotics exclusively in co-culture that could be replicated. This was unexpected given the prevalence of co-culture

antibiotic production that is seen in the literature. This might indicate that some changes should be made in culture conditions in order to stimulate competition mediated antibiotic production.

In general, large scale cultures had very low yields. 1 L cultures typically produced less than 10 mg of total extract, and the antibiotic fraction was a small portion of this amount. To combat this, the SS405 culture was grown to 4 L, which had the highest yield of 62.7 g of total extract. Data from the time trial analysis of the 4 L SS405 culture is shown in Table 2. The culture reached maximum average absorbance of 0.95 ± 0.02 at 75 hours, with a zone of inhibition of 5.5 ± 0.6 mm. No zones of inhibition were seen until 48 hours. If possible, it would be beneficial to further increase the culture volume to obtain more active compound. This should make the NMR data easier to interpret.

Table 1. Summary of time trial data.

Sampling Time (hours)	Average Absorbance	Average Zone of Inhibition (mm)
4	0	0
19	0.018 ± 0.004	0
28	0.31 ± 0.03	0
48	0.8 ± 0.1	4 ± 1
56	0.76 ± 0.05	5 ± 1
75	0.95 ± 0.02	5.5 ± 0.6

Separation of extracts via chromatography was very poor. A summary of all four separations that were done on SS401, SS405, and SS408 and solvent conditions is shown in Table 2. Wide bands were seen on the PTLC plate, and often one compound would separate into multiple bands. This might have been due to overloading of the plate, which caused uneven separation. Subsequent attempts to separate by column chromatography were not much more successful. Two things were done to improve this. The first was the use of a minimal glucose media for growing SS405, which greatly reduced the number of fractions in the extract. This is particularly useful because many of the components of TSB are unknown. However, glucose had poor solubility in all organic solvents used, which led to the presence of glucose in every fraction after column chromatography. To alleviate this, the previously described base wash step was added. As shown in Figure 4, the majority of the active component remained in the organic layer B, while most of the glucose was thought to have washed out with the basic layer. A significant portion of the antibiotic compound was found in fraction C, suggesting that the active compound is mildly acidic. The column that was run on the washed extract showed clear separation of at least two components, but glucose was still present in almost every fraction. This suggests that additional methods need to be developed to remove the glucose before separating in a column, or alternative separation methods need to be explored. For characterization to proceed, it is important for pure fractions to be obtained, and currently the amount of glucose in each fraction is overwhelming the active compound.

Table 2. Summary of the separation of single producer trials. PTLC was run on all extracts except the SS405 in glucose medium, which was run on a column. Fractions B3-5 were a single compound in 3 bands on the PTLC plate.

Strain	Growth Media	PTLC/Column Solvent System	Active Fraction(s)
SS401 (*Pseudomonas)*	10% TSB	50/50 Ethyl Acetate/Hexanes	None
SS408 (*Streptomyces)*	10% TSB	80/20 Ethyl Acetate/Hexanes	1&2
SS405 (*Chromobacterium*)	10% TSB	Ethyl Acetate	B3-5 & B10
SS405	Minimal Glucose	50/50 Ethyl Acetate/Hexanes →100% Ethyl Acetate	Unknown

4. Conclusion

The pin replicator assay has been shown as an effective way to quickly screen many bacteria at once for antibiotic activity against *S. aureus*. However, no antibiotic activity exclusive to co-culture was observed using this methodology. Experimenting with different growing conditions may be worthwhile to stimulate antibiotic production in co-culture. Additionally, a different methodology should be developed that allows for the screening of *E. coli* or other Gram-negative bacteria in addition to *S. aureus*. To aid in the separation of antibiotics extracted from single producers, additional minimal media should be developed. Additionally, new methods need to be developed that will allow for the separation of active fractions from the carbon source so that pure antibiotic compounds can be isolated and characterized. Once pure samples of the antibiotic compound are obtained, it can be characterized by ^{1}H NMR, ^{13}C NMR, and IR spectroscopy. Once characterized, the effectiveness of these compounds will be measured by obtaining minimum inhibitor concentration (MIC) values for each active compound. If the antibiotic compounds can be successfully isolated and identified, they may eventually serve as drug therapies that can counteract drug resistant bacterial strains.

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6. References

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