

Antimicrobial Producing Bacteria as Agents of Microbial Population Dynamics

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Dynamics

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Abstract

The need for new antibiotics has been highlighted recently with the increasing pace of emergence of drug resistant pathogens (MRSA, XDR-TB, etc.) (15). Modification of existing antibiotics with the additions of side chains or other chemical groups and genomics based drug targeting have been the preferred method of drug development at the corporate level in recent years(59). These approaches have yielded few viable antibiotics and natural products are once again becoming an area of interest for drug discovery.

We examined the antimicrobial “Red Soils” of the Hashemite Kingdom of Jordan that have historically been used to prevent infection and cure rashes by the native peoples. Antimicrobial producing bacteria were present in these soils and found to be the reason for their antibiotic activity. After isolation, these bacteria were found to excrete their antimicrobials into the liquid culture media which we could then attempt to isolate for further study. Adsorbent resins were employed to capture the antimicrobial compounds and then elute them in a more concentrated solution.

As part of a drug discovery program, we sought a way to quickly characterize other soils for potential antibiotic producing bacteria. The community level physiologic profile was examined to determine if this approach would allow for a rapid categorizing of soils based on their probability of containing antimicrobial producing microorganisms. This method proved to

have a high level of variability that could not be overcome even after mixing using a commercial blender.

The role of these antimicrobial producing bacteria within their natural microbial community has largely been confined to microbe-plant interactions (10, 89) (32) (77). The role of antimicrobial-producing microorganisms in driving the diversity of their community has not been a focus of considerable study. The potential of an antimicrobial-producing bacterium to act as a driver of diversity was examined using an artificial microbial community based in a sand microcosm. The changes in the microbial assemblage indicate that antimicrobial-producing bacteria may act in an allelopathic manner rather than in a predatory role.

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List of Abbreviations

Deoxyribonucleic acid: DNA

Bisphenol A: BPA

Community level physiologic profile: CLPP

Minimum inhibitory concentration: MIC

Chapter 1: Introduction

Natural Product Drug Discovery

Natural product antimicrobials have been used for centuries by native peoples all around the world, but it wasn't until the late 1800s that people began to search for single compounds that could be used to kill disease causing bacteria. Soil and plants had been used to prevent wounds from becoming infected or to treat rashes as far back as the Roman and Byzantine empires (87). All cultural groups have medicinal folklore that has some level of effectiveness for any number of ailments. Many modern common therapeutic agents have their origins in natural sources, such as: Paclitaxel, also known as Taxol, which was derived from the yew tree (102); Morphine is derived from the opium poppy; Atropine comes from the belladonna leaf; and Aspirin is from white willow bark (48).

In the late 1800s scientists had begun to accept the germ theory of disease which proposed that bacteria and other microbes were responsible for a variety of ailments including many sicknesses as defined in Koch's Postulates (42). The first antibiotic to be used in hospitals appeared in the 1890s in the form of pyocyanase which was derived from microbes by Rudolf Emmerich and Oscar Low (36). In 1928 Sir Alexander Flemming noted that *Staphylococcus aureus* could be readily killed by the fungus *Penicillium notatum*, but it wasn't until 1942 that penicillin was massed produced and turned into a commercial antibiotic by Howard Florey and Ernst Chain. This commercialization of penicillin ushered in an era of drug discovery and many new antimicrobials were discovered over the next half century.

With the discovery of individual antimicrobial compounds from plants, bacteria, and the fungi, people were able to begin to more effectively treat a wider variety of diseases and infections. These natural products have been used extensively over the last seventy years. Such

heavy use has both helped people live longer, healthier lives, and it has also resulted in the selection of many of today's modern "superbugs" that are highly drug-resistant (59). This rise of the super-bug has shown a need for a new round of drug discovery to take place.

Current drug development methods have been slow to produce effective new antibiotics as they have primarily focused on modifying existing classes of antibiotics or using genomics to identify new drug targets (15). Unfortunately this approach is yielding results too slowly to keep up with the rapid pace of emergence of drug resistant bacteria (15). The need for new antibiotics from natural sources, and new classes of antibiotics, was a major driver in the direction of this research. Exploring the possibility of discovering new antimicrobials from bacteria was chosen for this research because bacteria have been at war with each other for survival for approximately 4-5 billion years. This would lead one to think that bacteria might be the best source for finding compounds to kill other bacteria.

Jordan's Red Soil

With this in mind, we decided to take a closer look at medicinal folklore to identify places that we felt would be possible source of novel antimicrobial producing bacteria. After talking with a research colleague, we chose the Hashemite Kingdom of Jordan where a "Red Soil" has been used for thousands of years to prevent infections in wounds as well as to clear rashes on the skin (38). It was hypothesized that this soil's medicinal property would be due to the presence of bacteria that produced possibly novel antimicrobials that could be isolated and studied for possible drug development.

Appearance of bisphenol A

Once we had individual bacterial isolates, we began to grow them in larger batch cultures in an effort to produce enough of the desired antimicrobial that the structure could be purified by chromatography and identified through Nuclear Magnetic Resonance (NMR) imaging and Mass Spectrometry. Each culture was fractionated several times in order to test each fraction for active compounds and to reduce the number of compounds being studied. It was at this point that bisphenol A (BPA) was identified as being present in the culture material. BPA is used widely in industrial settings in the manufacture of plastics as well as a lining for metal cans used to hold food and has recently been gained notoriety as hazardous as it produces estrogenic effects in humans (35). BPA was also linked to a higher incidence of heart disease, diabetes and liver abnormalities in humans (64).

BPA degradation by microorganisms has been observed (71) (93) (95) (94), but BPA synthesis has not been confirmed. We sought an explanation to the appearance of BPA in our microbial cultures as synthesis of BPA by a microorganism would have a significant impact by possibly becoming a low cost option of obtaining BPA for use in industrial applications.

Community Level Physiologic Profiling and the Biolog EcoPlate™ system

Since the list of places to look for novel bacteria and antimicrobials is nearly limitless, we sought a way to reduce the areas we would look at to a more manageable level. We decided we would focus only on soils as soils harbor high microbial diversity. We looked for a way to quickly categorize the soils so over time we could develop a method in which we could quickly decide if a soil should be used in the labor intensive process of characterizing as many of the microbes present as possible. This characterization of a soil would allow us to decide to focus on a particular set of characteristics based on a perceived likelihood of containing antimicrobial-

producing microorganisms. We chose to look at community level physiologic profiling (CLPP) as this method can give a representation of what a microbial community is capable of utilizing (46) (103). This knowledge would allow for the quick categorization of a soil into groups that would tell us the likelihood of having antimicrobial producers within it, after a sufficient number of soils had been characterized to establish a baseline. Once enough soils had been tested, both for their CLPP and for antimicrobial-producers, we might be able to determine that a certain CLPP had a higher likelihood of containing antimicrobial-producers than other CLPPs and we could then decide to focus on soils fitting that CLPP description.

Biolog manufactures 96-well plates containing different substrates linked with a tetrazolium dye for use in microbial species identification that relies on a substrate utilization pattern. Utilization of a substrate will result in the reduction of the tetrazolium dye and the production of color in the well which can then be read on a microplate reader. They also produce a 32-well (repeated 3 times on a 96-well plate) version called an EcoPlate™ that is designed to allow for the categorization of a microbial community's CLPP based on the pattern and intensity of response when read on a microplate reader. This CLPP pattern could be linked with the prevalence of antimicrobial producing bacteria in such a way as to allow us to quickly determine which soils we would examine at greater length.

Keystone Species

Of personal interest was the effect that antimicrobial-producing bacteria have on the microbial community in a soil. Are these producers the driving factor in diversity or do they simply co-exist with the other microorganisms present? In macro organisms, Robert Paine described a Keystone Species as one which played a large role in the population dynamics, such

as diversity, regardless of its own number in the community (84) (85). If bacteria had the ability to function this way in a microbial community has not been explored previously. I did not set out to definitely prove that bacteria do or do not function in this capacity but rather do they have the ability to function as a Keystone type species or if they are functioning in an allelopathic manner by competitively excluding other microorganisms.

Adsorbent Resins

Attempts to increase antimicrobial production by co-cultivating the producer with a sensitive “prey” organism were unsuccessful, as they failed to show any detectable increase in antibiotic activity of the cell-free culture filtrate (Tanner, unpublished data). It is possible that the failure to observe stimulation was due to binding of the extracellular antimicrobial compound(s) by the susceptible target microbial cells. This binding would remove the antimicrobial compound from the media, even though production could have been stimulated. As an alternative, we have employed adsorbent resins as surrogates for target cells.

Adsorbent resins (e.g., XAD) have been shown to increase production of antibiotics by microorganisms (47, 74, 98), perhaps by overcoming feedback inhibition/repression (47) or preventing degradation (98). Adsorbent resins also have the potential to become a method for efficiently extracting an antimicrobial compound from other compounds produced in cell culture. We chose to focus on the ability of adsorbent resins to bind and subsequently release the compounds being produced by the isolated bacteria. Adsorbent resins have the ability to bind compounds through polar/non-polar interactions or through ionic interactions depending on the resin selected. Not all resins have the ability to bind the same compounds so a range of resins

was tested for each isolate to examine which resins bound the antimicrobial compounds most efficiently.

Objectives

- (1) Identify source of bisphenol A (BPA) in the fractions of antimicrobial producing microorganisms. Is the BPA being produced by the microorganisms or is it being leached out of the plastic growth flasks?
- (2) Identify the source and basis for the antimicrobial ability of the Jordanian “Red Soils.” Is the source microbial (biological) or mediated by mineral/metals (abiotic)?
- (3) Develop a method of rapid soil screening for antimicrobial-producing microorganisms using community level physiologic profiling allowing for characterization of a soil based on its likelihood to contain antimicrobial-producing bacteria.
- (4) Develop a method for more rapid antimicrobial isolation than the freeze dry/fractionation method used currently in the laboratory.
- (5) Determine if antimicrobial producing bacteria have the ability to be major drivers of microbial diversity in a manner consistent with the “Keystone Species” concept as described by Robert Paine.

Chapter 2: Microbial-mediated release of bisphenol A from polycarbonate vessels

This work has been published in Letters in Applied and Environmental Microbiology with the following list of authors: Nicholas H. Oberlies, Chen Li, Robert J. McGivney, Feras Q. Alali, Justin R. Tanner and Joseph O. Falkinham, III.

Abstract

Aim: Identify the source of bisphenol A [(2, 2'-bis(4-hydroxyphenyl) propane] in cultures of an antibiotic-producing *Bacillus* sp. strain grown in polycarbonate flasks.

Methods and Results: Although a culture of an antibiotic-producing *Bacillus* sp. strain grown in a new, rinsed polycarbonate flask yielded bisphenol A (BPA), duplicate cultures grown in thoroughly washed polycarbonate flasks did not. Cells of *E. coli* strain C were grown in new polycarbonate flasks rinsed 3-times with 100 ml distilled H₂O. BPA was only recovered from cultures grown in new polycarbonate flasks, but not from the autoclaved medium incubated in parallel.

Conclusions: BPA was present in either *Bacillus* or *E. coli* cultures, probably due to its release from inadequately washed polycarbonate flasks. Standard autoclaving did not result in BPA appearance; microbial growth was required. Polycarbonate vessels for microbial cultures should be thoroughly washed to avoid the appearance of BPA in culture medium.

Significance and Impact of the Study: This study rigorously demonstrates that the presence of bisphenol A (BPA) in culture medium was a consequence of microbial growth or metabolism in inadequately washed polycarbonate flasks. Since BPA exhibits antimicrobial and estrogenic

activity, searches for novel drugs or production of recombinant chemotherapeutic agents could be derailed by the artifactual appearance of BPA.

Introduction

As part of our ongoing research to identify novel antimicrobial agents produced by nonobligate predator bacteria (19, 20), an organic extract of a culture of a strain of *Bacillus* sp. recovered from a soil sample collected on the Jordanian shore of the Dead Sea was found to produce high levels of antimicrobial activity against *Staphylococcus aureus* (MIC = 4 $\mu\text{g ml}^{-1}$). Upon bioactivity-directed fractionation of that organic extract, one of the constituents was found to be bisphenol A [2, 2'-bis(4-hydroxyphenyl) propane; BPA]. The isolated BPA had modest antimicrobial activity (i.e., MIC against *Staphylococcus aureus* 288 $\mu\text{g ml}^{-1}$) in confirmation of data by others (37). In repeated experiments we sought to confirm the production of BPA by the *Bacillus* sp. isolate. The hypothesis, that the *Bacillus* sp. strain synthesized BPA, was based on evidence of BPA degradation by a Gram-negative aerobic microorganism (71, 93) and other microorganisms in the environment (94, 95). Possibly, the *Bacillus* sp. strain might be capable of BPA biosynthesis by reversing the degradation pathway. As BPA is a major industrial chemical, the discovery of an organism capable of BPA biosynthesis would be desirable. However, in cultures of the same *Bacillus* sp. strain grown under identical conditions in a second experiment, BPA was not detected, nor was it detected in the sterile medium. This lack of reproducibility of BPA production, coupled with the possibility that the BPA could be a metabolite of that *Bacillus* sp. strain, stimulated us to devise experiments to determine the source of BPA in microbial cultures. One clue that served as the starting point of the experiments was that new

polycarbonate flasks had been used for the growth of the *Bacillus* sp. strain in the first experiment, but only repeatedly washed flasks used in the second.

BPA is used widely in the production of polycarbonate, epoxy resins, and flame retardants, and in 1993, 640,000 metric tons of BPA were produced (94). It is used in the manufacture of polycarbonate flasks and bottles used in research laboratories. BPA was detected in water contained in polycarbonate flasks and subjected to 30 min autoclaving at 121° C (63), twice the length of autoclaving used typically for media preparation in microbiology laboratories. A great deal of attention has been focused upon measurement of BPA in foods packaged or stored in polycarbonate vessels (16, 17, 61), because BPA has antibiotic (94) and estrogenic activities (9, 63, 94). Recently, concern over the presence of BPA in plastic bottles has prompted a shift to glass bottles in the San Francisco area (35).

Our investigations were triggered by the absence of BPA in microbiological medium autoclaved 15 min at 121° C. If microbial growth results in release of BPA from polycarbonate, BPA could be a contaminant in cultures of recombinant microorganisms producing proteins of human or animal origin or, with respect to our own vein of research, in microorganisms probed for the discovery of new antibiotics. Herein we report the microbial-mediated release of BPA from polycarbonate.

Materials and methods

Bacterial strains

Escherichia coli strain C (ATCC 13706) was used in the study. The *Bacillus sp.* strain A460-4-2-7 was isolated from a soil sample collected in February, 2004, approximately 100 m from the shore of the Dead Sea in the Hashemite Kingdom of Jordan (N 31° 41.973', E 35° 34.974'; elevation -374 m) as a colony producing a zone-of-inhibition on a lawn of *Micrococcus luteus* spread on one-tenth-strength BHIB agar medium (Becton Dickinson & Co., Sparks, MD). *Bacillus* strain A460-4-2-7 is a non-obligate predator strain based upon its ability to grow on *M. luteus*, *S. aureus*, and other microorganisms in the absence of other nutrient and on normal laboratory medium in the absence of prey microorganisms.

Growth medium and growth of bacteria

Bacillus strain A460-4-2-7 and *E. coli* strain C were grown in ¼-strength Tryptic Soy Broth (Becton Dickinson & Co., Sparks, MD) containing 0.2 % (w/v) glucose (TSB+G). Single isolated colonies on ¼-strength TSB+G agar were picked and used to inoculate 10 ml of TSB+G medium in 125 ml glass Erlenmeyer flasks and incubated for 2 days without shaking at 30°C. Those 10 ml cultures were used to inoculate 90 ml of TSB+G medium in new, thoroughly washed, baffled 250 ml polycarbonate flasks and incubated for 2 days with aeration (60 rpm) at 30°C. The thorough washing was adapted from (17) and consisted of: (1) wash with detergent (Bio-Clean Detergent, Stanbio Laboratory, Boerne, TX) and water, (2) rinse 3-times with 20 %

flask volume of tap and then 3-times with 20 % flask volume of distilled H₂O, (3) fill with distilled H₂O and autoclave (15 min at 15 psi), and (4) rinse 3-times with 20% flask volume of distilled H₂O. Fifty ml of the resulting 100 ml cultures were used to inoculate 450 ml of TSB+G medium contained in new polycarbonate flasks (Nalgene, Rochester, NY) rinsed 3-times with 100 ml distilled H₂O. The inoculated cultures were incubated for 5 days with aeration (60 rpm) at 30°C. After incubation, the contents of the flasks were transferred to either new, rinsed or new, thoroughly washed 500 ml centrifuge bottles, frozen at -70°C, and shipped to Research Triangle Institute for fractionation and identification of compounds with antimicrobial activity. At each transfer, the purity of the culture was assessed by streaking on TSB + G agar medium; for all results reported herein, the cultures were pure and not contaminated.

General chemistry procedures

Preparatory HPLC was carried out on a Varian Prostar HPLC system (Walnut Creek, CA), equipped with Prostar 210 pumps operating at 10 ml min⁻¹ and a 330 photodiode array detector, with data collected and analyzed using a Star Chromatography Workstation; the column was a YMC ODS-A (5 µm; 250×25 mm; Waters; Milford, MA). Analytical HPLC utilized the same HPLC system at 1 ml min⁻¹ with a Inertsil ODS-3 (5 µm; 250×4.6 mm; Metachem Technologies; Torrance, CA), with chromatograms analyzed at 270 nm. All NMR experiments were performed in CDCl₃ with TMS as an internal standard using a Varian Unity Inova-500. Low-resolution ESIMS and APCIMS were determined on an Applied Biosystems/MDS Sciex API 150 EX single quadrupole LC/MS system (Applied Biosystems, Foster City, CA).

Results

Isolation and identification of Bisphenol A from *Bacillus* strain A460-4-2-7

A culture of predator *Bacillus* sp. strain A460-4-2-7 was processed for the identification of anti-microbial compounds using a modification of procedures described previously (20). Briefly, the entire 500 ml culture was subjected to one freeze-thaw cycle before being freeze dried. The resultant powder was stirred with 1:1 chloroform: methanol overnight, the solution was filtered to remove insoluble materials, and the volume of the filtrate was reduced in vacuo. The resulting extract was then partitioned between 4:1:5 chloroform:methanol:water in a separatory funnel. Upon settling, the bottom layer (organic) was collected, and the solvent was removed under reduced pressure to generate the organic extract (35.0 mg). An aliquot of that organic extract was purified via reverse phase HPLC using an acetonitrile:water gradient (20:80 to 40:60 over 30 min, then up to 100:0 over 20 min) to yield 1.8 mg of BPA. The structure of BPA was confirmed by analysis of both spectroscopic (^1H , ^{13}C , DEPT, HSQC, and HMBC NMR data) and spectrometric (low resolution ESI and APCI MS) data and by comparison with an authentic sample of BPA (Alfa Aesar, Pelham, NH; Lot GBFA028690), which co-eluted on HPLC (isocratic acetonitrile:water, 40:60, R_t 18.5 min).

Isolation and identification of Bisphenol A from *E. coli* strain C

Repeated and independent cultures of *Bacillus* strain A460-4-2-7 grown under the same conditions failed to yield BPA. A clue to its source came from observation that new

polycarbonate flasks were used in the first culture of *Bacillus* strain A460-4-2-7, whereas old, repeatedly washed polycarbonate flasks had been used in the subsequent cultures where no BPA had been detected. That observation led us to test the hypothesis that BPA was being released from new flasks as a consequence of microbial growth. *E. coli* strain C was used for these experiments because there has been no report of BPA production by this species, in spite of its widespread use. Further, *E. coli* is often the choice for production of recombinant chemotherapeutic proteins, whose detection might be hindered by the presence of BPA. For all the experiments, a single large volume of TSB+G medium was prepared in a thoroughly washed glass flask and dispensed into different flasks as described in Table 2.1. To analyze for the presence of BPA in the *E. coli* extracts, the method described above for *Bacillus* strain A460-4-2-7 was used and a seven-point standard curve was generated over the range of 5 µg to 62.5 ng using triplicate injections of 50 µL for each point ($r^2 > 0.99$ for the equation $y = 3.76 \times 10^3 x + 2.77 \times 10^5$). The limits of quantitation (LOQ) and detection (LOD) were 170 and 51 µg of BPA per L of culture broth (respectively).

The only *E. coli* culture combination yielding BPA was Flask 4 (2.8 mg L⁻¹ of culture); namely *E. coli* strain C grown in 500 ml of TSB+G medium for 5 days with aeration (60 rpm) at 30°C in a new, rinsed polycarbonate flask (Table 2.1). BPA was not detected in either the *E. coli* or *Bacillus* cultures grown in thoroughly washed, new polycarbonate flasks (data not shown). A repeat of that experiment with *E. coli* strain C gave the same result; only Flask 4 yielded BPA (data not shown). BPA was not detected in the medium in an uninoculated flask incubated in parallel for 5 days with aeration (60 rpm) at 30°C (i.e., Flask 3, Table 2.1). Further, cultures of *E. coli* grown in glass flasks and transferred to new, rinsed centrifuge bottles for storage and transfer (Flasks 5-7, Table 2.1), did not yield any BPA, demonstrating that microbial growth or

metabolism, not simply autoclaving and storage, led to the appearance of BPA. In contrast to the work of Krishnan et al. (63), BPA was not detected in the autoclaved medium (Flask 2). That is likely due to the fact that autoclaving time used here was 15 min, half the time used by Krishnan et al. (63).

Discussion

The results of the experiments described herein identify the source of BPA in cultures of the nonobligate predator *Bacillus* sp. strain A460-4-2-7. Although the results do not rule out the possibility that a microorganism might exist that can synthesize BPA, particularly in light of the fact that microbial-mediated BPA degradation has been reported (71, 93-95), the experiments make it likely that the appearance of BPA in the *Bacillus* cultures was due to microbial-mediated release from inadequately washed polycarbonate flasks. This is the first report demonstrating that growth or metabolism of either *E. coli* or *Bacillus* sp. was required for BPA release. Other factors, such as alkalinity could also promote BPA release (17). Autoclaving and incubation of uninoculated medium for 5 days with aeration at 30°C was insufficient for release of BPA. However, autoclaving for periods of 30 min at 121°C does result in BPA release in the absence of microbial growth (63). Fortunately, that length of autoclaving is usually avoided by microbiologists, since this could cause hydrolysis of proteins and polysaccharides and caramelization of sugars.

Our objective in writing this report is to alert microbiologists to the possible appearance of BPA in cultures of microorganisms in inadequately washed polycarbonate containers. This amount of a nuisance material has the potential to derail a natural products chemistry investigation of such a culture, where amounts of promising, biologically-active secondary

metabolites could be less than 3 mg L^{-1} , especially before the culture conditions are optimized (19, 20). BPA has both antimicrobial (37, 94) and estrogenic activities (63, 94), and thus its appearance in cultures could lead bioassay-based drug discovery in the wrong direction. Because the polycarbonate flasks ordered at two different times released BPA upon cultivation of either *Bacillus* sp. or *E. coli*, this phenomenon evidently reflects a general characteristic of the polycarbonate flasks. However, it is possible that not all polycarbonate flasks share this characteristic, and we did not perform a survey of different sources to explore this question. Further, growth or metabolism of some microorganisms may not provide the conditions for extraction of BPA from polycarbonate flasks. Rather, our suggestion is to thoroughly wash all polycarbonate containers as described in the materials and methods and avoid high temperature (63) and caustic conditions, because BPA extraction is higher at alkaline pH (17). The microbial-mediated release described here was not due to alkalinity, because the pH of the *Bacillus* sp. or *E. coli* cultures following growth was 6.5. Finally, one thorough washing is sufficient to reduce BPA release to undetectable levels (17).

Table 2.1 Bisphenol A detection experiments with *E coli* strain C

Flask No.	Growth Flask	Centrifuge Bottle	Medium, Inoculation, and Incubation Conditions	Concentration of BPA [§]
1	Rinsed [*]	Washed [†]	TSB+G medium, never in flask, not autoclaved, transferred to centrifuge bottle and frozen	ND ^{**}
2	Rinsed	Washed	TSB+G medium, autoclaved in flask, cooled and immediately transferred to centrifuge bottle and frozen	ND
3	Rinsed	Washed	TSB+G medium, autoclaved in flask, incubated at 30° C for 5 days, transferred to centrifuge bottle, frozen	ND
4	Rinsed	Washed	TSB+G medium, autoclaved in flask, cooled, inoculated with <i>E. coli</i> , incubated at 30° C for 5 days, transferred to centrifuge bottle, frozen	2.8
5	Glass [‡]	Rinsed	TSB+G medium, never in flask, not autoclaved, transferred to centrifuge bottle and frozen	ND
6	Glass	Rinsed	TSB+G medium, autoclaved in flask, cooled, immediately transferred to centrifuge bottle and frozen	ND
7	Glass	Rinsed	TSBG medium, autoclaved in flask, incubated at room temperature for 5 days, transferred to centrifuge bottle, and frozen	ND

^{*} Rinsed = New flasks and centrifuge bottles rinsed 3-times with 100 ml distilled H₂O

[†] Washed= Centrifuge bottles washed thoroughly as described in Materials and Methods

[‡] Glass=Glass flasks washed thoroughly as described in Materials and Methods

[§] mg of BPA per L of culture

^{**} ND = Below the limit of detection (0.05 mg BPA L⁻¹)

Chapter 3: Proliferation of Antibiotic-Producing Bacteria and Concomitant Antibiotic Production as the Basis for the Antibiotic Activity of Jordan's Red Soils

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Abstract

Anecdotes, both historical and recent, recount the curing of skin infections, including diaper rash, by “Red Soils” in the Hashemite Kingdom of Jordan. Following inoculation of Red Soils isolated from geographically separate areas of Jordan, *Micrococcus luteus* and *Staphylococcus aureus* were rapidly killed. Over the 3 week incubation period, the number of specific types of antibiotic-producing bacteria increased and high antimicrobial activity (MIC ~ 10 µg/ml) was observed in methanol extracts of the inoculated Red Soils. Antibiotic-producing microorganisms whose numbers increased during incubation included actinomycetes, *Lysobacter*, and *Bacillus*. The actinomycetes produced actinomycin C₂ and actinomycin C₃. Analysis of the microbial populations by ribosomal RNA gene Internal Transcribed Spacer Restriction Fragment Length Polymorphism (rRNA ITS-RFLP) showed that bands attributable to *M. luteus* and *S. aureus* disappeared and other bands, possibly attributable to the antibiotic-producers, increased after 3 weeks incubation. No myxobacteria or lytic bacteriophage with activity against either *M. luteus* or *S. aureus* were detected in either soil before or after inoculation and incubation. Although

protozoa and amoebae were detected in the soils, the numbers were low and did not increase over the incubation period. These results suggest that the antibiotic activity of Jordan's Red Soils is due to the proliferation of antibiotic-producing bacteria.

Introduction

There is a growing recognition of the pressing need for new antimicrobial agents for the treatment of infectious diseases (18, 92). As just one cogent example, new antibiotics are in high demand for the treatment of *Staphylococcus aureus* infections (54), particularly due to the emergence of methicillin-resistant *S. aureus* (MRSA) in communities and hospitals (54, 100). Further, providing effective and affordable antibiotics to people in the epidemic-prone Third World remains a major challenge (91).

Historically, natural products have played a key role in the discovery and development of many antibiotics (81). In particular, soil-based actinomycetes have been the source of countless drugs, such as streptomycin, actinomycin, erythromycin, and vancomycin, to name only a few (26). One approach to the discovery of new antimicrobial agents from natural sources has been to use folklore or historical records to guide the collection of samples (31).

Through our ongoing studies of the biodiversity of the Hashemite Kingdom of Jordan (Jordan) (3-8, 82, 83), we were intrigued by anecdotes of the antibiotic-like properties of "Red Soil", used historically for treating skin infections and diaper rash and still in use in some communities as an inexpensive alternative to pharmaceutical products. Within Jordan there are four major biogeographic regions (2) (39), and Red Soils are most commonly found in the Mediterranean region, of the northwestern portion of Jordan, near cities such as Irbid, Ramtha, and Ajloun. An area away from housing, preferably not touched by feet and thus considered

“clean” is chosen, and soil below the surface is collected, as the surface is considered contaminated or “not clean.” After washing and drying the infected area, the sieved soil is applied daily as either a powder or paste until the infection subsides. The basis for the antimicrobial activity of Red Soils is not known.

Antimicrobial activity of soils against inoculated microorganisms has been attributed to abiotic or biotic factors. Abiotic activity has been shown to be responsible for the antimicrobial activities of clay minerals used in the treatment of a mycobacterial skin infection, “Buruli Ulcer” (57). Soil texture was found to influence survival of *Pseudomonas fluorescens* and *Bacillus subtilis* in soil (99), while soil temperature and pH and the presence of roots affected the leaching of a genetically modified strain of *P. fluorescens* in soil (62). Biotic factors, including predation and antimicrobial-producing or lytic microorganisms, were suggested as mechanisms of killing microorganisms introduced into soils (1, 22, 68, 69). Prior inoculation of soil with one strain of *P. fluorescens* reduced the ability of a second *P. fluorescens* strain to colonize (30). A phenazine pigment produced by a *P. fluorescens* strain was shown to be responsible for biological control of a root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* (97), and it has been shown that filaments of the biocontrol fungus *Trichoderma* grow towards fungal pathogens and release antibiotics and lytic enzymes (12). With that background as a guide, we undertook an investigation to identify the basis for the antimicrobial activity of Jordan’s Red Soils.

Materials and Methods

Sample collection. Red Soil samples were collected from two separate sites, and the geographic relationship of these and other soil samples collected by our team in Jordan in 2004 are described

and mapped in a recent manuscript (83). Sample A460-9-3 was collected from an agricultural field on the grounds of the National Center for Agricultural Research and Technology Transfer (NCARTT) north of Amman at N 32° 04.699 min and E 35° 50.553 min (elevation 633 m).

Sample A460-14-2 was collected from a farm in the northeastern portion of Jordan near the village of Hoffa Alwestyia at N 32° 34.109 min and E 35° 42.946 min (elevation 345 m). As a control non-“Red Soil”, sample A460-2-2 was collected from a citrus orchard in the northern portion of the Jordan River Valley at N 32° 37.378 min and E 35° 35.914 min (elevation – 219 m).

Growth and enumeration of Micrococcus luteus and Staphylococcus aureus. *M. luteus* is a normal inhabitant of soil (22) and can be easily identified and enumerated because of its yellow colony pigmentation and resistance to 7.5 % (wt/vol) NaCl. Although *S. aureus* is not a normal inhabitant of soil, it is a member of the human skin microbiota (45) and involved in skin infections (100). *M. luteus* and *S. aureus* cells were grown in 10 ml ¼-strength Tryptic Soy Broth (TSB, BBL Microbiology Systems, Cockeysville, MD) in 125 ml cotton-stoppered flask with aeration (120 rpm) at 30° C for 3 days. Cells were collected by centrifugation (5,000 × g for 10 min) and washed 2× with an equal volume of sterile tap water and suspended in 10 ml of sterile tap water (approximately 2×10^8 CFU/ml). Colonies of *M. luteus* were enumerated on ¼-strength TSB agar containing 5 % (wt/vol) NaCl (TSB+Salt). Colonies of *S. aureus* were enumerated on Mannitol Salt Agar (Beef Extract, 1 g; Proteose Peptone No. 3, 10 g; NaCl, 75 g; mannitol, 10 g; agar, 15 g; phenol red, 0.025 g in 1 l of distilled water).

Measurement of M. luteus and S. aureus killing by Red Soils. Samples (5 g, each) of Red Soils A460-9-3 and A460-14-2 and control agricultural soil A460-2-2 were added to sterile 50 ml

screw capped centrifuge tubes. In this manner soil moisture content and headspace could be maintained throughout the duration of the experiment. One set of soils were not inoculated and employed as negative controls for antibiotic measurement. *M. luteus* (0.5 ml) and *S. aureus* (0.5 ml) were added to the soil samples and mixed thoroughly (approximately 2×10^7 CFU of *M. luteus* and *S. aureus*/g soil). Immediately, a 0.5 g sample was removed aseptically, weighed, transferred to a sterile 50 ml centrifuge tube, suspended in 5 ml of ¼-strength TSB, vortexed at the highest setting for 60 sec, and shaken at 1 reciprocation per sec for 30 min at room temperature. The resulting suspension was diluted in ¼-strength TSB and 0.1 ml of the undiluted and diluted soil suspensions were spread (in duplicate) on TSB+Salt (*M. luteus*) and Mannitol Salt Agar (*S. aureus*). Inoculated soil suspensions were incubated at room temperature and a 0.5 g soil sample was removed and processed to enumerate *M. luteus* and *S. aureus* as described above after 1, 2, and 3 weeks incubation. The results are reported as mean colony-forming units (CFU)/g (\pm standard deviation) of inoculated soil samples from duplicate, independent experiments. Identity of *M. luteus* (yellow pigmented colonies on ¼-strength TSB Salt agar) and *S. aureus* (orange colonies surrounded by a yellow halo on Mannitol Salt Agar) colonies was confirmed by subculture and cultural, biochemical, and enzymatic tests.

Measurement of MICs of methanol extracts of soils. A 0.5 g sample of each uninoculated soil (negative control) and inoculated and uninoculated soils after 3 weeks incubation was suspended in 5 ml of methanol, vortexed at the highest setting for 60 sec, and shaken at 1 reciprocation per sec for 30 min at room temperature. The soil was pelleted by centrifugation ($5,000 \times g$ for 10 min) and the supernatant methanol solution was collected without collecting any soil. The methanol extract was evaporated to dryness overnight in a beaker at room temperature, and the residual extract was dissolved in 5 ml of DMSO. The dry weight of the DMSO solution was

measured by drying 2 ml in a tared aluminum pan overnight at 105°C. The minimal inhibitory concentration of the DMSO solutions were measured by broth microdilution in 96 well plates against *M. luteus*, *S. aureus*, *M. smegmatis*, *S. cerevisiae*, and *A. niger*. The target microorganisms were grown and prepared for MIC measurement as described previously (19). The results are reported as the minimal concentration as dry weight completely inhibiting growth of the target microorganism. To serve as quality assurance standards, MIC values for known antibiotics were included (Table 3.1).

Enumeration, isolation, and identification of antimicrobial-producing microorganisms in inoculated Red Soils. Antimicrobial-producing colonies from the inoculated soils were enumerated on lawns of target microorganisms. Cultures (0.1 ml) of *M. luteus*, *S. aureus*, *C. albicans*, and *A. niger* (spore suspension) grown as described (19) were spread on 1/10-strength Brain Heart Infusion Broth agar (BBL Microbiology Systems, Cockeysville, MD). Immediately, 0.1 ml of the undiluted and diluted soil suspensions (in duplicate) were spread, the plates allowed to air dry and incubated at 30° C for 2-5 days. In addition, 0.1 ml of the undiluted (supernatant) and diluted suspensions spread on copper agar [i.e., 4.63 g Brain Heart Infusion Broth (Difco, Detroit, MI) (one-tenth strength), 0.04 g CuCl₂ (anhydrous), 7.5 g agar, and 500 ml distilled water)] and the medium was incubated at 30°C (24). Empirically, colonies appearing on copper agar are likely to be antibiotic-producers (23, 24). Colonies of bacteria appearing on the copper agar or colonies surrounded by zones of inhibition on the lawns were picked, and isolated colonies were used to inoculate 2 ml of ¼-strength TSB and incubated 3 days at 30°C. The antimicrobial-producing strains were identified by colony and microscopic morphology (i.e., actinomycetes and *Bacillus*) and profiles of cellular fatty acids (Midi Labs, Newark, DE). To determine the range of antimicrobial activity of the isolates, 10 µl of a cell-free filtrate of the 2

ml cultures was spotted on lawns of the target microorganisms (above). Clearing of the lawn was taken as evidence of antimicrobial activity (+ in Table 3.1). To determine the susceptibility of *Propionibacterium acnes* ATCC strain 11827 to the isolates, 100 µl of each cell-free filtrate or of a methanol-extract of the inoculated soils was added to 1.9 ml of Reinforced Clostridial Medium (RCM, Oxoid, UK). Each was then inoculated with 100 µl of a *P. acnes* culture grown at 37°C for 3 days under anaerobic conditions (Gas Pak Plus™, Becton Dickinson, BBL, Sparks, MD) and after 3 days scored visually for a reduction in turbidity (+ in Table 3.1) by comparison to a control.

Detection of enzymatic activity. Protease (25), lipase (72), and alkaline phosphatase activities (101) were measured in untreated and boiled cell-free culture filtrates and in organic and aqueous fractions of chloroform-methanol (1:1) extracts of representative *Lysobacter* isolates from Red Soils.

Detection and enumeration of myxobacteria, protozoa, amoebae, and bacteriophage. Samples (0.5 g) of uninoculated and inoculated and incubated 3 wks soils were suspended in 10 ml sterile tap water and vortexed at the highest speed for 60 sec. The suspension was allowed to settle for 1 hr, and 1 ml was filtered through a 0.22 µm pore size filter. To detect myxobacteria, 0.1 ml samples of the diluted and undiluted unfiltered suspension were spread on CF agar with and without a lawn of *Escherichia coli* strain C (55). After 1 wk incubation at 30°C, the agar medium was examined under 10-fold magnification for evidence of fruiting bodies. Protozoa and amoebae were detected by mixing 3 ml of molten (45° C) 1/10th-strength BHI Broth containing 0.7 % agar (top agar) with 0.1 ml of the filtrate and 0.1 ml of either *M. luteus* or *S. aureus* and pouring the suspension on the surface of BHI agar. After 1-3 days incubation at 30°C, cleared zones were inspected under 100-fold magnification to determine whether protozoa

or amoebae were present. For bacteriophage enumeration, 0.1 ml of the filtrate and 0.1 ml of either *M. luteus* or *S. aureus* was added to 3.0 ml of top agar, the contents mixed, and poured over the surface of BHI Agar. After overnight incubation at 30°C, the number of plaques was counted.

Detection of antimicrobial activity of organic extracts of isolates. Isolates were grown, extracted, and tested for antimicrobial activity using a modification of procedures described previously (20). Briefly, isolates were grown to 500 ml volumes in ¼-strength TSB+S broth, frozen, thawed, and the entire culture was freeze-dried. The resultant powder was extracted by stirring overnight in either methanol or chloroform: methanol (1:1). The resultant slurry was filtered to remove solids, and the solvent was removed in vacuo. This extract was then partitioned between chloroform:methanol:H₂O (4:1:5). The organic fraction (lower portion) was removed and dried in vacuo. A measured aliquot of this fraction was dissolved in 1 ml of DMSO, and the antimicrobial activity was measured as described previously (3).

Isolation of actinomycins from isolate 14-2-1. A culture of isolate 14-2-1 was extracted as described above. The organic fraction, showing antimicrobial activity, was purified further using a Varian Prostar HPLC systems (Walnut Creek, CA, USA) equipped with Prostar 210 pumps and a 330 photodiode array detector (PDA), with data collected and analyzed using Star Chromatography Workstation software (version 5.52), via an ODS-A column (250 × 25 mm, i.d., 5 µm; YMC, Wilmington, NC); the CH₃CN:H₂O gradient solvent system initiated at 1:1 and increased to 8:2 linearly over 70 min. Two major compounds (coded 11065-92-1 and 11065-92-2) were isolated that eluted between approximately 40 and 50 min, and these were identified via the below experiments as actinomycin C₂ and actinomycin C₃, respectively. These assignments were deduced via ¹H- and ¹³C-NMR using a Varian Unity Inova-500 instrument with a 5 mm

broad-band inverse probe with z-gradient. They were confirmed by high resolution mass spectrometry [performed on a Finnigan MAT 95Q hybrid-sector instrument (ThermoFinnigan; San Jose, CA)], where compound 11065-92-1 (1.5 mg) yielded a sodiated molecular ion that correlated with actinomycin C₂ (1291.63192 amu measured vs 1291.633346 amu calculated for C₆₃H₈₈N₁₂O₁₆Na) and where compound 11065-92-2 (3.0 mg) yielded a sodiated molecular ion that correlated with actinomycin C₃ (for 1305.65237 amu measured vs 1305.648996 amu calculated for C₆₄H₉₀N₁₂O₁₆Na). Finally, these assignments were verified by comparison to actinomycin C reference standards (Axxora, LLC, San Diego, CA). Compound 11065-92-1 eluted with the same retention time (8.75 min) and same UV spectrum as actinomycin C₂ and compound 11065-92-3 eluted with the same retention time (9.59 min) and same UV spectrum as actinomycin C₃; both were measured using the aforementioned HPLC system with a gradient solvent system of CH₃CN:H₂O that initiated at 60:40 and increased linearly to 80:20 over ten min on an ODS-A column (150 × 4.6 mm, i.d., 5 μm; YMC, Wilmington, NC).

Isolation of DNA from cultures and soils. DNA isolation from inoculated Red Soils immediately following inoculation and after 3 wks incubation was carried out using the FastDNA[®] SPIN[®] Kit (For Soil) from Q^BIOgene (Carlsbad, CA). DNA isolation from all cultures was carried out using the UltraClean[™] Microbial DNA Isolation Kit from Mo Bio Laboratories, Inc (Solana Beach, CA). The manufacturers' directions were followed for both kits.

PCR amplification of rRNA internal transcribed spacer (ITS) sequence and acrylamide gel electrophoresis. PCR amplification of the ITS sequences was performed in 50 μl reaction volumes using GoTaq[®] Green Master Mix from Promega Corp. (Madison, WI) following the manufacturer's directions. Primer L (5'-GCTGGATCACCTCCTTTCT-3') and Primer R (5'-CTGGTGCCAAGGCATCCA-3') were used for all reactions (65). Acrylamide gels (10 %) were

prepared from a 40% acrylamide/bis (29:1) stock solution (VWR International, West Chester, PA).

Results

Red Soils kill M. luteus and S. aureus. Following inoculation of *M. luteus* and *S. aureus* into the Red Soils their numbers fell dramatically by 12 days (Figure 3.1). Numbers did not fall in the inoculated agricultural soil (Figure 3.1). The values in the figure represent averages of duplicate counts from two independent experiments. The surviving CFU/g of both *S. aureus* and *M. luteus* were significantly below those of corresponding inoculum densities and the agricultural soil after 12 and 22 days of incubation ($P < 0.05$ by one-tailed Student's T-test). Separate or combined inoculation of the two bacteria yielded the same results (data not shown). Autoclaved Red Soils did not demonstrate any killing (data not shown), illustrating that biotic factors were responsible for killing. One consequence of using small amounts of soil was that 10 % of soil was removed for analysis at each sampling point. The benefit was that distribution of the inoculum was more uniform using the small soil volumes, thus reducing variation in recovery of bacteria. Further, soil texture, moisture content, and head space was maintained throughout the 3 wk incubation period. Experiments with larger amounts of soil led to the formation of aggregates of entirely different soil texture.

Increased antimicrobial activity in Red Soils following M. luteus and S. aureus inoculation.

Three weeks incubation of the *M. luteus*- and *S. aureus*-inoculated Red Soils led to the appearance of significantly higher levels of antimicrobial activity of methanol extracts of the inoculated soil (Table 3.1, $P < 0.05$, Student's T-test). The data reported in Table 3.1 was taken from one of the two independent experiments in which the results were nearly identical. There

was no detectable antimicrobial activity recovered from either of the uninoculated Red Soils incubated in parallel for 3 weeks (negative control). Soil samples were inoculated with *M. luteus* and *S. aureus* and immediately, or after 3 weeks incubation at room temperature, methanol extracts were obtained as described. Although there was no detectable antimicrobial activity of the methanol extract in the two Red Soils at the time of inoculation, there was broad spectrum antimicrobial activity in the methanol extracts obtained after 3 weeks incubation (Table 3.1). The methanol extracts of both inoculated soils also exhibited antibiotic activity against *P. acnes* (Table 3.1), a member of the normal microbiota of skin implicated in dermal infections (100). Although the MIC values for the methanol extracts are moderate (i.e., 0.003 – 0.150 mg/ml, Table 3.1) compared to pure antibiotics, it is to be understood that the values are for crude extracts, not purified compounds, and antibiotics are subject to adsorption by soils and thereby difficult to extract (53).

Absence of myxobacteria, protozoa, amoebae, or bacteriophages. Neither myxobacteria-like fruiting bodies, nor bacteriophage plaques were detected in Red Soils, examined prior to and following inoculation and 3 wks of incubation. Protozoa and amoebae were detected at low levels in the uninoculated Red Soils ($\leq 10/g$); however, their numbers did not increase over the course of 3 wks of incubation.

Increased number of antibiotic-producing bacteria following M. luteus and S. aureus inoculation in Red Soils. Coincident with the fall in numbers of *M. luteus* and *S. aureus* (Figure 3.1) was a moderate increase in the number of colonies producing zones of inhibition against *M. luteus* and *S. aureus* (Table 3.2). Only a few of the increases were significantly higher as indicated in the footnote to Table 3.2. There were no differences in the number of colonies producing zones of inhibition in the un-inoculated Red Soils over the course of the incubation period (data not

shown). The data in Table 3.2 were from experiments where *S. aureus* and *M. luteus* were separately inoculated into the Red Soils to demonstrate that the increase in zones of inhibition against both *S. aureus* and *M. luteus* occurred when just one was inoculated into soil. Almost identical results were obtained from experiments where both were inoculated. Inoculation of the Red Soils with either *M. luteus* or *S. aureus* also increased the number of antibiotic-producing microorganisms against *C. albicans* (3- and 4.5-fold, respectively) and *A. niger* (3.5- and 13-fold, respectively) for Red Soil A460-9-3. Although the data reported in Table 3.2 list the total number of colonies surrounded by zones of inhibition in the target microbial lawns, certain types predominated. For example, in Red Soil A460-9-3 inoculated with *M. luteus*, the total number of colonies with zones of inhibition was $2.0 \times 10^7/\text{g}$, of which $0.3 \times 10^7/\text{g}$ were *Bacillus*, $0.2 \times 10^7/\text{g}$ were actinomycetes, and $1.5 \times 10^7/\text{g}$ had a transparent slimy or transparent mucoid colony type, whose zones of inhibition on lawns of either *M. luteus* or *S. aureus* lacked any clear evidence of a colony.

Antimicrobial-producing bacteria from Red Soils. Table 3.3 lists the antibiotic-producing isolates, their identification, spectra of antimicrobial activities for cell-free culture filtrates and, in some cases, the minimal inhibitory concentration (MIC) of the organic fraction of individual cultures. Identification of actinomycetes was based on colony and microscopic morphology, whereas *Bacillus* and *Lysobacter* were identified by cellular fatty acid analysis (MIDI, Newark, DE). The *Lysobacter* isolates had either a transparent mucoid or transparent slimy colony morphology and differed in colony pigmentation (i.e., yellow, orange, red, and brown). Cell-free, culture filtrates of *Lysobacter* isolates 9-3-16 (mucoid, yellow), 9-3-17 (slime, brown), and 14-2-10 (slime, orange-red) all had protease, lipase, and alkaline phosphatase activities that were absent in boiled cell-free culture filtrates (data not shown). Significantly, the boiled cell-free

culture filtrates retained antimicrobial activity and the organic and aqueous fractions of chloroform-methanol extracts of the *Lysobacter* cultures lacked enzymatic activities. These data demonstrate that the antimicrobial activity of the cell-free culture filtrates or culture extracts was not due to extracellular *Lysobacter* enzymes.

Evidence that isolates from the Red Soils produce compounds with antibiotic activity. One actinomycete, isolate 14-2-1, identified on the basis of colony and microscopic morphology, was examined using a bioactivity-directed fractionation approach for compounds with antibiotic activity (20, 67). Briefly, an organic extract of a 500 mL culture of this isolate grown in ¼-strength TSB containing 0.5 % (w/v) sucrose exhibited MIC values of 5 and 1 µg/mL against *M. luteus* and *S. aureus*, respectively. Upon further purification using reverse phase HPLC, two compounds were isolated (11065-92-1 and 11065-92-2), and these were identified as actinomycin C₂ and actinomycin C₃, respectfully. These structures were elucidated using a suite of spectroscopic and spectrometric tools, particularly NMR and high resolution mass spectrometry (HRMS). Moreover, the chromatographic profiles of the isolated compounds were compared directly with actinomycin C reference standards.

Changes in pattern of amplified rRNA ITS products in inoculated Red Soils. The feasibility of employing a non-cultural method for following changes in the Red Soils' microbiota was investigated. DNA was isolated from the soils immediately following inoculation and after 3 wks of incubation. The DNA was subjected to rRNA ITS-RFLP analysis using PCR and products were separated by electrophoresis in polyacrylamide gels as described. Bands representing the amplified ITS products for individual strains (as markers) and inoculated soils are shown in Figure 3.2. The *S. aureus* and *M. luteus* strains and the three antibiotic-producing isolates yielded multiple ITS-RFLP bands (Figure 3.2, lanes 2, 3, 6, 7, and 8) in agreement with

variation in length of internal spacers reported for bacteria (60). *Lysobacter* isolates A460-9-3-16 and A460-9-3-17 shared identical ITS profiles (Figure 3.2, lanes 6 and 7) although they have different colony morphologies (Table 3.3). Because multiple amplified ITS products were produced (Figure 3.2), assignment of bands in the inoculated soils to individual isolates is problematic. In spite of that constraint, it does appear that three *S. aureus* bands (i.e., 750, 600, and 375 bp) disappeared after three weeks of incubation (Figure 3.2, lanes 2, 4 and 5). One of the *M. luteus* bands (i.e., 1,000 bp) appeared to decrease in intensity upon incubation (Figure 3.2, lanes 3, 4, and 5). Although new bands appeared (e.g., 500 and 250 bp) in the inoculated and incubated soils (Figure 3.2, lane 5), their mobilities were not the same as those for the antibiotic-producing isolates (Figure 3.2, lanes 6, 7, and 8).

Discussion

The data herein strongly suggest that the killing of inoculated *S. aureus* and *M. luteus* by Jordan's Red Soils was due to the proliferation of antibiotic-producing bacteria and their concomitant production of antimicrobial compounds. Abiotic factors do not appear responsible because autoclaved Red Soils failed to kill the inoculated bacteria, unlike the case of the antimicrobial clays (57). Rather than due to protozoan, bacteriophage, or myxobacterial predation, killing by Red Soils appears to be mediated by consortium of antibiotic-producing bacteria; namely actinomycetes, *Bacillus*, and *Lysobacter*. That conclusion was supported by evidence that Red Soil A460-14-2 harbors an actinomycete, which synthesizes at least a pair of antibiotic compounds of the actinomycin structural class. Although it is likely that the enzymatic activities of the *Lysobacter* in the native Red Soils contributed to killing (13), members of this genus have been reported to also produce antimicrobial compounds (56). Likewise, the absence of the three extracellular enzyme activities of the *Lysobacter* strains, coupled with the presence

of antimicrobial activity in the boiled cell-free culture filtrates and the organic and aqueous fractions of chloroform-methanol extracts, argues strongly that the antimicrobial activity of the *Lysobacter* strains was due to production of compounds rather than enzymes that exhibit antimicrobial activity. Studies are ongoing to isolate and elucidate compounds with antibiotic activities from some of these bacterial isolates from Red Soil.

The magnitude of the killing of *S. aureus* appears to be similar to that noted by Liang et al. (68), who showed that killing was due to unidentified biotic factors as *S. aureus* grew in autoclaved soil (68). In another study demonstrating killing of microorganisms inoculated into soils, it was proposed, but not proven, that predatory bacteria were responsible for killing (22). In a third study, eukaryotic predation was shown to be involved in killing, although antimicrobial-producing or lytic microorganisms could have been involved as well (1). Although there were only modest increases in the number of colonies surrounded by zones of inhibition after incubation of the inoculated Red Soils (Table 3.2), their ability to kill would be expected to be magnified by the production of extracellular antimicrobial compounds (Table 3.1).

To complement the results of colony counts, ITS-RFLP was chosen as a culture-independent means for assessing both disappearance of prey microbes and emergence of antibiotic-producing bacteria. A similar approach has been employed to identify different members of microbial consortia and to assess the impact of predation by protozoa (86, 90). Although there could be differences in the total amount of DNA extracted, the decrease in intensity of the *M. luteus* and *S. aureus* bands in parallel with the decrease in colony counts coupled with the increase of other bands (Figure 3.2) demonstrates the feasibility of this approach.

These data provide a rationale for the traditional use of Jordan's Red Soils for the treatment of skin infections, including diaper rash. In the absence of any inherent, abiotic antibiotic activity of Red Soils, and as *S. aureus* is a normal inhabitant of human skin microbiota (45), we hypothesize that application of Red Soils to an infected area of skin (i.e., inoculation) leads to the proliferation of bacteria that produce antibiotic compounds, killing the infecting skin microbiota. This is similar to the killing of *Shigella paradysenteriae* by a colicin-producing strain of *E. coli* following co-infection in the peritoneal cavity of mice (43). *Shigella* killing coincided with the multiplication of the *E. coli* strain and an increase in colicin (antibiotic) concentration (43). Killing by Red Soils is probably not restricted to the infecting microorganisms, because inoculation of Red Soils with either *M. luteus* or *S. aureus* led to the appearance of broad spectrum antimicrobial activity (Table 3.1). This is likely due to the proliferation of a spectrum of microorganisms producing a suite of antimicrobial compounds (Tables 3.2 and 3.3).

Table 3.1. Antimicrobial activity of methanol extracts of Red Soils prior to and after inoculation with *M. luteus* and *S. aureus*.

Sample	Minimal Inhibitory Concentration (Fold Increase) ^a					
	<i>M. luteus</i>	<i>S. aureus</i>	<i>M. smegmatis</i>	<i>S. cerevisiae</i>	<i>A. niger</i>	<i>P. acnes</i>
Red Soil A460-9-3						
Before Inoculation	> 0.55	> 0.55	> 0.55	> 0.55	> 0.55	> 0.35
Three Weeks Later	0.003 (183)	0.006 (92)	0.040 (14)	0.23 (2)	0.007 (79)	0.050 (7)
Red Soil A460-14-2						
Before Inoculation	> 0.35	> 0.35	> 0.35	> 0.35	> 0.35	> 0.35
Three Weeks Later	0.004 (88)	0.011 (32)	0.011 (32)	0.150 (2)	0.011 (32)	0.050 (7)
Positive Controls^b	0.0001	0.0008	0.0002	0.025	0.025	ND

^aValues are reported in mg/ml (fold- increase). ^bPositive control data (from left to right) are for ampicillin, ampicillin, kanamycin, amphotericin B, and amphotericin B. These are typical average values, as reported previously (67).

Table 3.2. Increase in antibiotic-producing microorganisms following inoculation of Red Soils.

Soil Sample + Inoculum	Incubation Period	Predators/g soil (Fold Increase) ^a Against:			
		<i>M. luteus</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>A. niger</i>
A460-9-3 + <i>M. luteus</i>					
	0 days	3.2×10^5	1.0×10^7	9.0×10^4	2.0×10^5
	20 days	6.8×10^5 (2.1)	2.0×10^7 (2.0)	2.7×10^5 (3.0)	7.0×10^5 (3.5)
A460-9-3 + <i>S. aureus</i>					
	0 days	2.4×10^6	1.0×10^6	2.0×10^5	2.0×10^5
	20 days	9.1×10^7 (38) ^c	9.0×10^6 (9.0) ^c	9.0×10^5 (4.5)	2.6×10^6 (13) ^c
A460-14-2 + <i>M. luteus</i>					
	0 days	3.4×10^5	3.0×10^5	ND ^b	ND
	20 days	1.2×10^6 (3.5)	3.3×10^6 (11) ^c	ND	ND
A460-14-2 + <i>S. aureus</i>					
	0	7.7×10^5	4.0×10^5	ND	ND
	20 days	1.4×10^6 (1.8)	1.1×10^6 (2.8)	ND	ND

^aData from the experiment where *M. luteus* and *S. aureus* were inoculated separately into the same soil samples.

^bND = Not Determined.

^cSignificantly higher numbers after incubation (P < 0.05, Student's T-test)

Table 3.3. Antimicrobial-producing isolates recovered from two different Red Soils and the relative activity of cell-free culture filtrates against a panel of microorganisms. MIC values for organic extracts of some of the isolates are provided in mg/ml.

Isolate Number	Identification	Colony Morphology	Antimicrobial Activity of Cell-Free Culture Filtrates by Spot Plate (MIC of Organic Extracts of Some Isolates in mg/ml) ^a						
			<i>M. luteus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>S. cerevisiae</i>	<i>A. niger</i>	<i>P. acnes</i>
Soil Sample A460-9-3									
9-3-12	<i>Lysobacter</i> ^d	Transparent	-	-	-	+	-	-	ND ^b
9-3-13	<i>Lysobacter</i> ^d	Slime	+ (1.85)	+ (0.93)	+ (0.93)	-	-	-	ND
9-3-14	<i>Actinomycete</i> ^d		+(0.35)	+	-	+ (0.05)	+ (0.05)	+ (0.20)	ND
9-3-15	<i>Lysobacter</i> ^d	Slime	+ (0.90)	+ (0.11)	-	-	-	-	ND
9-3-16	<i>Lysobacter</i> ^d	Mucoid	+ (0.09)	+ (0.04)	+ (0.35)	+ (0.35)	+ (0.18)	+ (0.09)	-
9-3-17	<i>Lysobacter</i> ^d	Slime	+ (0.19)	+	+	+ (0.03)	-	+ (0.08)	+
9-3-19	<i>Lysobacter</i> ^d	Mucoid	+ (0.34)	+ (0.17)	-	-	-	-	ND
9-3-20	<i>Lysobacter</i> ^d	Slime	+	+	+	+	-	-	ND
9-3-21	<i>Actinomycete</i> ^d		+ (0.14)	+ (0.14)	+ (0.58)	+ (0.29)	+	-	ND
9-3-22	<i>Lysobacter</i> ^d	Slime	- (>1.20)	- (>0.60)	- (>0.60)	- (>0.60)	ND	- (>0.60)	ND
9-3-23	<i>Lysobacter</i> ^d	Slime	+	+	-	-	+	+	ND
9-3-26	<i>Bacillus</i> ^d	Target	+	+	+	+	-	+	+
Soil Sample A460-14-2									
14-2-1	<i>Actinomycete</i> ^c	Yellow	+	+	+	-	-	-	+
14-2-3	<i>Actinomycete</i> ^c	Brown	+	+	+	-	-	-	ND
14-2-6	<i>Actinomycete</i> ^c		-	-	+	-	-	-	ND
14-2-10	<i>Lysobacter</i> ^d	Slime	+ (0.40)	+ (0.05)	-	-	-	-	-
14-2-13	<i>Lysobacter</i> ^d	Slime	+	+	+	-	-	-	ND

^aAntimicrobial activity expressed as clearing (+) or no clearing (-) on lawn of target microorganism spotted with 10 µl cell-free culture filtrate and as minimal inhibitory concentration (MIC) of organic extracts in mg/ml prepared as described in Experimental Procedures. ^bND = Not Determined. ^cIsolated on copper agar. ^dIsolated from a zone of inhibition.

Fig. 3.1. Killing of *M. luteus* and *S. aureus* by Red Soils A460-9-3 and A460-14-2. Soil A460-2-2 is an agricultural, non-Red Soil, which was used as a negative control. In all experiments, *M. luteus* and *S. aureus* were inoculated into the soil samples, and the number of colony forming units (CFU) of the inoculated bacteria per g of soil was measured at intervals to 22 days.

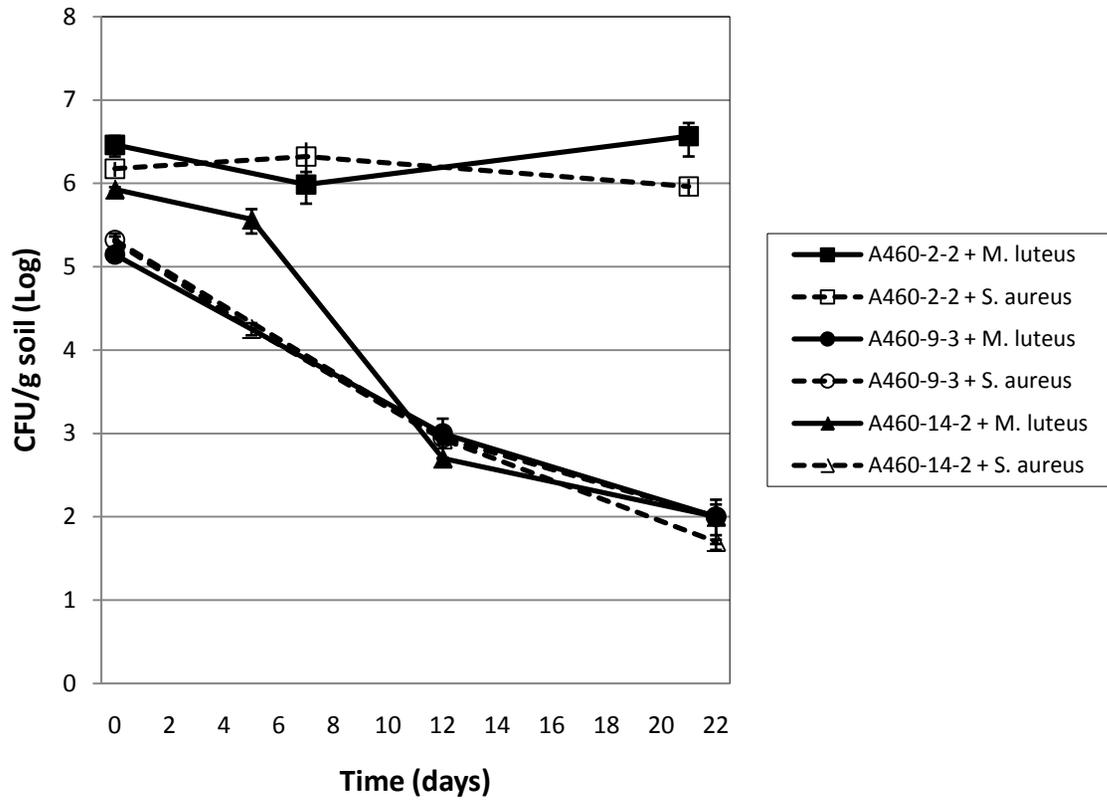
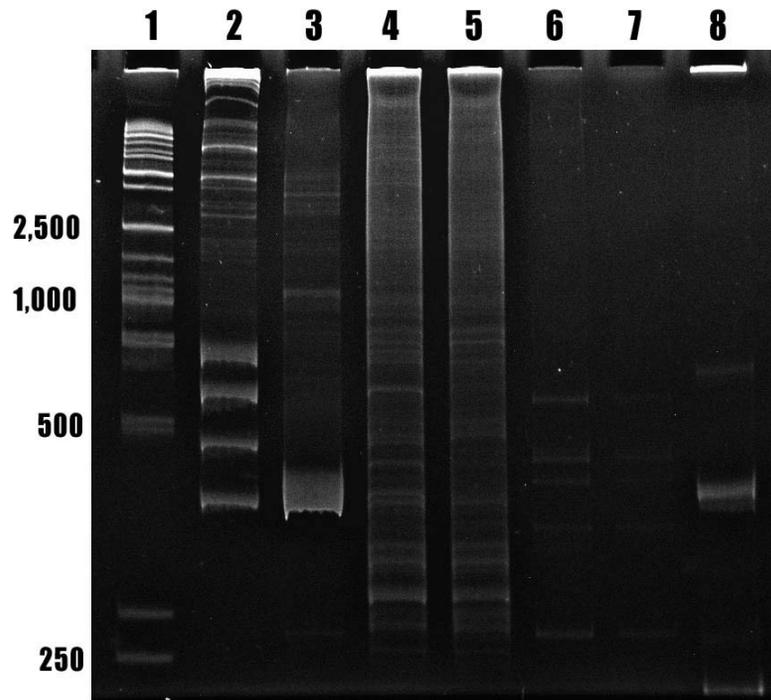


Figure 3.2. Acrylamide gel electrophoresis of products of PCR amplification of bacterial internal transcribed spacer (ITS) sequences of *M. luteus*, *S. aureus*, and “Red Soils”. Lane 1, molecular weight markers; lane 2, *S. aureus*; lane 3, *M. luteus*; lane 4, Red Soil A460-9-3 inoculated with *M. luteus* and *S. aureus* (immediately after inoculation); lane 5, Red Soil A460-9-3 inoculated with *M. luteus* and *S. aureus* (incubated 3 wk); lane 6, Red Soil isolate A460-9-3-16; lane 7, Red Soil isolate A460-9-3-17; lane 8, Red Soil isolate A460-9-3-26.



Chapter 4: Limitations to the Utility of Community Level Physiologic Profiling

Abstract

BIOLOG EcoPlate™ substrate arrays have been used to describe the functional diversity of microbial communities (i.e., community level physiologic profile) by measuring substrate utilization patterns. This system was employed to measure the range of substrates utilized by several different soil types. Replicate BIOLOG EcoPlate™ utilization profiles were not reproducible for either replicates of the same soil's suspension or for duplicate suspensions of the same soil. Specifically, absorbances reflecting substrate utilization varied over a 10-fold range. The repeated variability suggests that investigators using BIOLOG EcoPlates™ interpret substrate utilization patterns with caution.

Introduction

The biodiversity of soil microflora can be assessed by three interrelated measurements: genetic diversity, functional diversity, and taxonomic diversity (104). Toward measurement of soil microbial functional diversity, patterns of substrate utilization using arrays of different carbon sources (BIOLOG EcoPlate™) have been used (46, 50, 52). This approach has been called community level physiological profiling (CLPP) and is advantageous because it is reported to provide quantitative data that is rich in information and amenable to multivariate analyses as employed in taxonomic studies (104).

BIOLOG EcoPlate™ substrate arrays can be used to describe the range of substrates that can be metabolized by resident microbial populations and hence compare utilization profiles between populations. As such, BIOLOG EcoPlate™ substrate utilization profiles can be used to describe the functional diversity of resident microbial populations and describe the impact of environmental stresses, including temperature, on the population (27). To estimate

the proportion of microorganisms able to metabolize a range of substrates, BIOLOG 96-well plates were used with a most probable number approach (44). The basis for that approach was that metabolism of a particular substrate was dependent upon a small proportion of the microbial population.

Although replicate profiles of the same suspension yielded essentially the same results, replicate profiles of different suspensions of the same soil showed variability (52 {Balsler, 2002 #1). Further, suspended cells from a soil suspension may (76) or may not (11) yield identical patterns of substrate utilization for a soil slurry or particulate fraction from the same soil. Thus it is important when performing CLPP, to measure profiles of multiple suspensions of the same soil (11, 73) and to separate soil suspensions into the slurry (or intact), suspended and particulate fractions (11).

The CLPP for soils should vary according to the composition of the microbial community. The presence of antimicrobial-producing microorganisms or any predation (such as amoeba), should reduce the number of positive responses in a BIOLOG EcoPlate™, as the overall diversity of the community should be reduced as a result of selective pressures. These differences in soil microbial communities would yield different CLPP patterns that could be used to categorize soils with minimal effort and expense. This straight forward categorization procedure was proposed as part of a larger drug discovery project (38); the goal was to decide rapidly where to pursue sources of possible leads. If certain substrate utilization patterns had an increased likelihood of containing antimicrobial-producing organisms, those soils could be given priority over soil patterns that were not correlated with antimicrobial production.

To develop the use of BIOLOG EcoPlates™ for drug discovery, we first sought to identify the effect of types of soils and their diversity on EcoPlate™ results. Several soil types were selected for this study. These soils were chosen for a difference in soil conditions,

presumably leading to differences in the soil microbial community. Preliminary data indicated that if a soil sample suspension was diluted, then an increase in the number of substrates utilized was observed (Table 4.1). Specifically, a Jordanian soil used to examine the initial effect of dilution was collected from the ancient Roman city of Gedara (83). There was no metabolism of substrates using the initial suspension; however, 23 of 32 substrates were metabolized when a 10-fold dilution was made of that same soil. Subsequent investigation demonstrated that the soil had a higher concentration of NaCl. This prompted a more in-depth examination of the effect of dilution across several soil samples to determine if this was a general trend associated with all soils. We postulated that this could be due to the dilution of an inhibitory compound, such as high salt. Dilution would also allow us to identify the most probable number of microbes present needed to metabolize a particular substrate (44).

Materials and Methods

2.1 Soil Samples

The following soil samples were collected: (1) Virginia Tech campus agricultural, (2) saline soils from Saltville, Virginia, (3) river bed from the New River in Montgomery County, Virginia, (4) evergreen grove from Pandapas Pond in the Jefferson National Forest, and (5) and (6) soils from 2 separate residences in Montgomery County, Virginia (Table 4.1).

2.2 Soil Preparation and Dilution

Soil samples were mixed thoroughly and 2.5 g of soil was suspended in 50 mL sterile distilled water and vortexed at the highest setting for 30 sec. This was then allowed to settle at room

temperature followed by a repeat vortexing. A sample of the soil suspension was withdrawn immediately after vortexing, and a 10-fold dilution series in sterile distilled water was prepared.

2.3 Inoculation, Incubation, and Scoring BIOLOG EcoPlate™

From each dilution in the series, 150 µL was used to inoculate each of the 32 wells of the triplicate BIOLOG EcoPlate™. These plates were sealed with Parafilm® and incubated at 30° C for 7 days. Positive and negative wells were determined using a microplate reader at 595 nm.

2.4 Analysis of BIOLOG results

All microplate readings were normalized by subtracting the control well (no substrate) absorbance value to control for the absorbance of particulate matter. Any reading below 0.300 was scored as a negative, as this was the value that corresponded to the negative control well containing no substrate. Any value greater than or equal to 0.300 was scored as a positive as this value represented the point where a visual inspection would begin to be scored as a positive (visual purple color in the well).

2.5 Homogenization of soil samples

For homogenization experiment, soils were subject to 5 minutes of corresponding mixing method. Five grams of soil was suspended in 100 mL of sterile tap water. “Shaken” soils were placed in a 1 L baffled flask and placed on flatbed shaker at 120 rpm. “Vortexed” soils

were placed in a 250 mL bottle and vortexed on the highest setting. “Blended” soils were blended using a commercial blender (Waring, Model 33BL79) on highest setting.

Results

Following published use of BIOLOG EcoPlate™ profiling (44), it was discovered that a 1000-fold dilution provided the lowest background turbidity in each well. At higher soil densities, the color and/or turbidity of the soil suspension prevented detection or measuring the appearance of the purple color representing substrate hydrolysis. In particular, soils could be scored as metabolizing or not metabolizing for the same substrate in different replicates. Intensity of the response (measured the absorbance at 595 nm) varied for the same soil suspension as much as 10-fold in replicates (Figure 4.3). This variation for replicates of the same suspension was also found for different suspensions of the same soil sample and was not abolished by repeated thorough mixing of the soil. The variation in both positive/negative response and intensity was not limited to one soil but appeared in all soils tested (Table 4.2).

It had been reported that variation decreased when replicate suspensions of a thoroughly mixed and homogenized soil were prepared (11). Unfortunately, following the mixing and homogenization technique of Balsler *et al.* (11), the profile of substrates utilized was different for three replicate soil suspensions prepared from a single thoroughly mixed forest soil (Figure 4.3). Using other homogenization methods (Shake, Vortex, and Blend), the reproducibility could be improved but the variation did not abate completely (Table 4.3).

Discussion

In contrast to the results of Balser *et al.* (11), replicates of the same soil suspension did not yield the same results (Figure 4.1). For example, the absorbance (as a reflection of metabolism) of N-acetyl-D-glucosamine (wells E2, E6, and E10) varied over a 10-fold range in triplicate assays of the same suspension (Figure 4.1).

A number of phenomena could be responsible for the observed variation. Non-uniform distribution of microorganisms in soil and soil particulates would lead to an inability to assess accurately the CLPP on any soil microbial community. However, even thorough mixing of a soil failed to result in soil suspensions yielding the same substrate utilization profile. Perhaps if soils are sieved before water suspensions are prepared, the utilization profile variation would be reduced within the particle size fractions. Differences in the suspending of soil particles or rapid rates of soil particle settling during pipeting and inoculation of BIOLOG EcoPlate™ wells could have also affected the results. If that were the case, it would be expected that samples pipetted into the EcoPlate™ wells would be higher amongst substrates whose wells that were inoculated last. In the experiments reported here, the order of inoculation was from A1 to A4, then B1 to B4, etc. However, the data in Figures 4.1, 4.2, and 4.3 do not show that trend. Differences in substrate utilization are not clustered in the lower right hand corner in each set of 32 wells; rather the variation appears to be substrate-specific.

Even after thorough blending of the soil using a commercial food blender, the variation between replicates is still present (Table 4.3). While blending did reduce the amount of variation in some cases we feel it most likely that soil microbial spatial heterogeneity, that cannot be overcome by thorough mixing and homogenization, is likely responsible for the variation in substrate utilization profiles. Matsumoto *et al.* (75) observed differing populations of bacteria in soil aggregates while looking at both the surface and inside of the

aggregates after blending. They observed the inside of the aggregate after dispersion using an ultrasonic processor. This could be used to further homogenize the soil sample but it still may not eliminate the variation seen on the EcoPlates™.

Unfortunately, the lack of reproducibility shown by variation in absorbances and utilization patterns prevented the use of BIOLOG EcoPlates™ to characterize the functional diversity of soil samples. Being that this effect was present across several soil types, we believe this to be a general trend present in most soils and should be taken into account whenever the BIOLOG EcoPlate™ system is being utilized.

Table 4.1. Description of Soils

Soil Collection Site	GPS Coordinates	Soil Type	Characteristics
Residence 1	N 37° 13.325' W 080° 24.842'	Lawn – Clumpy Clay	Mowed, seeded, fertilized
Residence 2	N 37° 13.297' W 080° 22.313'	Lawn – Loose Top Soil	Mowed, seeded, fertilized
New River Shore	N 37° 15.049' W 080° 36.553'	Flood Plain	General use area
Pandapas Pond	N 37° 16.854' W 080° 28.271'	Undisturbed	Natural Succession Forest
Saltville Marsh	N 36° 52.296' W 081° 46.425'	Saline	Marsh beside salt well heads
Corn Field	N 37° 13.006' W 080° 25.869'	Agricultural	Fertilized and tilled

Table 4.2. +/- variation across replicate plating of soils.

	Plating 1	Plating 2	Plating 3
Pandapas Pond	22/32	10/32	15/32
Saltville Marsh	28/32	20/32	7/32
Residence 1	10/32	11/32	13/32
Residence 2	17/32	23/32	20/32
New River Shore	4/32	7/32	8/32
VT Corn Field	26/32	9/32	2/32

Table 4.3. Variation in positive/negative response per substrate (Δ) and number of positive wells for different soil homogenization methods.

Soil Type	Mixing Method	Δ	# Positive Responses
Residence 1 Clumpy Clay	Shaker	17	12.0 \pm 1.73
	Vortex	13	23.3 \pm 2.52
	Blend	4	27.0 \pm 1.73
Residence 2 Loose Top Soil	Shaker	14	11.7 \pm 2.52
	Vortex	7	19.3 \pm 2.52
	Blend	13	23.0 \pm 2.00

Figure 4.1. Same suspension, replicate plating of New River Shore. Highlighted cells are examples of variability among replicate plating as each color corresponds to the same substrate in each plating.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	-0.01	-0.007	-0.014	0	0.778	1.493	0.145	0	0.056	0.294	0.166
B	1.859	0.198	-0.012	0.008	0.001	0.008	0.033	0.05	1.078	0.006	1.681	0.063
C	-0.003	-0.012	-0.012	-0.013	0.011	0.037	0.021	0.128	0.009	0.003	-0.004	0.007
D	-0.002	-0.013	0.019	0	0.887	0.004	0.154	0.168	0.792	0.219	-0.006	0.157
E	0.078	1.569	-0.008	-0.012	0.029	0.123	0.463	0.09	0.136	0.532	0.004	-0.006
F	0.039	-0.01	0.007	0.069	0.06	-0.001	0.132	0	0.002	-0.006	0.018	0.08
G	1.191	-0.011	-0.011	-0.009	0.796	0.025	0.041	0.001	0.865	1.104	0.011	-0.004
H	-0.002	0.679	-0.007	-0.013	1.009	0.003	0.81	-0.001	2.889	0.003	-0.007	0.461

Figure 4.2. Variability in substrate utilization in replicates of a homogenized soil sample. (Pandapas Pond). Colored squares represent a positive reaction (>0.3 absorbance readings at 595nm).

	1	2	3	4	5	6	7	8	9	10	11	12
A		Blue	Blue	Blue		Green		Green				Orange
B	Blue	Blue			Green		Green		Orange	Orange	Orange	Orange
C	Blue	Blue		Blue	Green				Orange			Orange
D	Blue			Blue	Green		Green		Orange		Orange	Orange
E		Blue		Blue						Orange		
F	Blue	Blue	Blue	Blue	Green				Orange			Orange
G	Blue		Blue	Blue	Green							
H		Blue	Blue	Blue	Green					Orange	Orange	

Figure 4.3. Illustration of substrate variability using Saltville Marsh. Highlighted cells are examples of variability among replicate plating as each color corresponds to the same substrate in each plating.

	Replicate 1				Replicate 2				Replicate 3			
	1	2	3	4	5	6	7	8	9	10	11	12
A	0	2	1.222	2.371	0	1.856	0.582	1.891	0	-0.014	-0.015	-0.015
B	1.243	2.343	1.701	2.202	2.089	2.257	2.111	2.678	0.102	-0.002	0.049	0.384
C	2.238	1.834	2.575	2.095	0.109	0.527	0	0.438	1.252	-0.013	0	-0.015
D	2.247	2.44	2.212	2.832	1.175	1.627	0.615	2.558	-0.002	-0.014	-0.012	-0.015
E	2.071	1.908	1.873	1.898	0.047	2.823	0.102	0.222	-0.014	-0.014	0.495	-0.013
F	2.406	2.201	2.102	0.899	0.767	0.013	1.242	0.807	0.049	-0.014	-0.014	0.018
G	2.072	1.412	0.77	1.913	1.682	2.043	0.584	-0.015	-0.014	-0.007	-0.006	-0.014
H	2.823	0.808	2.157	1.008	0.004	0.021	0.896	0.758	0	-0.015	-0.014	0

Chapter 5: Speeding Isolation of Novel Antibiotics

Abstract

Adsorbent resins bind compounds in liquid media, through either hydrophobic or ionic interactions, and can subsequently release these compounds when an eluting agent is used. This property has allowed for these resins to be used to recover known antibiotics from broth culture. They have been shown, in some cases, to increase production of these antibiotics and also to remove impurities that inhibit antimicrobial activity when included in cultures. It is these properties which make adsorbent resins a possible tool to speed isolation of compounds in natural product drug discovery. When adsorbent resins are added to a bacterial culture during growth, an increase in antimicrobial activity (when compared to no resin addition) can be seen in some cases. The antimicrobial activity can also be readily eluted from the resin at higher activities. The results suggest that discovery of novel antibiotics from bacterial cultures and, by extension, to other bioactive drugs from different starting materials, would be significantly sped up and simplified by the use of adsorbent resins.

Introduction

At the beginning of the 20th century, infectious diseases were the leading cause of death worldwide. Tuberculosis, pneumonia, and diarrheal diseases caused 30% of deaths, and the average life expectancy was 47, due largely to the high rate of infant mortality from childhood infections (29). As the 20th century progressed, deaths due to infectious diseases decreased dramatically as a result of a number of public health initiatives: better nutrition and housing, safer food and water, improved hygiene and sanitation, vaccination, and the discovery and widespread use of antibiotics. Life expectancy continued to rise in the United States, reaching a record high of 77.8 years in 2004, and chronic diseases like heart disease,

25 cancer, and stroke, replaced infectious diseases as major killers (79). Society had distanced
26 itself from the threats of smallpox, paralytic poliomyelitis, and tuberculosis that had so
27 dramatically impacted previous generations. Unfortunately, that success contributed, in part,
28 to major pharmaceutical companies' reducing antibiotic development programs and
29 redirecting resources toward "blockbuster" diseases (34) (33). Consequently, the pipeline for
30 new antibiotics has nearly dried up.

31 Isolation and identification of antimicrobial compounds from cultures of antimicrobial
32 producing bacteria is problematic because of the low antibiotic yields (47). In addition,
33 fermentation broths may contain many compounds that could interfere with isolation and
34 purification. A current strategy for simplifying and speeding the isolation and purification of
35 known antibiotics is the use of adsorbent resins (21, 47, 66, 74, 98). Resins offer two
36 advantages in antibiotic discovery projects involving antibiotic-producing bacteria. (1)
37 Adsorbent resins selectively bind antibiotics in fermentation broths (21), and (2) inclusion of
38 adsorbent resins during growth may relieve feedback repression of antibiotic production (66,
39 74, 98).

40 Adsorbent resins have also been shown to bind and thus recover a variety of
41 antibiotics from fermentation broths, including kirromycin (47), teicoplanin (66), and
42 geldanamycin (21), but because not all compounds in fermentation broths are bound by the
43 resins (ideally only the active antibiotics) the level of extracellular, non-antibiotic cellular
44 products is reduced. Adsorbent resins have also been employed to sequentially with one resin
45 removing non-antibiotic fermentation metabolites and impurities and a second resin to
46 selectively bind and isolate the active antibiotic (49). Consequently, purification protocols
47 deal with fewer competitors. A key requirement of adsorbent resins is that once the
48 antimicrobial has bound, it must be eluted from the resin and isolated. As studies of antibiotic
49 production genes have identified linked regulatory gene sequences, it has been expected that

50 the synthesis of the novel metabolites (e.g., antibiotics) would be subject to regulation within
51 the cellular machinery. In many instances this would be expected to involve negative
52 feedback by the antibiotic itself. Binding of the antibiotic to adsorbent resins would remove
53 the antibiotic from the culture medium and thus relieve feedback repression resulting in
54 increased production of antibiotic. It has been suggested that increased levels of production
55 could be due to the binding of antibiotics by the adsorbent resins, thus overcoming feedback
56 inhibition or repression (47).

57 Although adsorbent resins have been used to increase production and enhance
58 isolation of known antibiotics, they have not been employed with unknown antibiotics
59 produced by bacteria. Quite possibly, resins could relieve feedback repression and selectively
60 bind antibiotics to simplify and speed antibiotic discovery, thereby overcoming the challenge
61 of the low yield of antibiotics in any type of natural product, whether microbial cells or plants.

62 The objectives of this project were to: (1) measure the ability of resins to bind the
63 structurally or chemically unidentified antimicrobial agents in cultures, (2) elute unknown
64 antibiotics from resins and measure their activity, and (3) incorporate resins into growing
65 cultures to increase production of antibiotics.

66

67 **Materials and Methods**

68 **Antimicrobial-Producing Bacteria and Targets (Table 5.1).** The antibiotic-producing
69 strains used were *Streptomyces griseus* strain A460-14-2-1, *Bacillus cereus* strain 1-1-11,
70 *Brevibacterium halotolerans* strain 4-2-7, *Bacillus subtilis* strain 9-3-26, and *Burkholderia*
71 *ambifaria* strain 2.2N (28). Antimicrobial activity was tested against strains of *Micrococcus*

72 *luteus*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Candida albicans*, *Mycobacterium*
73 *smegmatis*, and *Aspergillus niger*.

74 **Adsorbent Resins.** Both hydrophobic [Amberlite XAD-2, Amberlite XAD-4, Amberlite
75 XAD-7, Amberlite XAD-16, Amberlite XAD-1180 (Rohm and Haas; Philadelphia, PA)] and
76 ionic [Sepabeads SP-850 and Diaions HP-20 and HP-21 (Mitsubishi Chemicals; White Plains,
77 NY)] resins were employed.

78 **Preparation of Resins.** The resins were washed by adding 10 grams of resin to 100 ml of tap
79 water in a 500 ml flask and mixed for one hour by a magnetic stir bar at 60 rpm. After one
80 hour, the resin was allowed to settle; the water was decanted. Two more washes were
81 performed by adding water, allowing resin to settle, and decanting the water. Next, the slurry
82 was sterilized in the autoclave for 15 minutes at 15 psi. After autoclaving, the resins were
83 placed in a small oven to finish drying.

84 **Measurement of Dry Weight.** Aluminum pans were labeled with strain and source, and dried
85 in a 100° C oven overnight. The aluminum pan was removed from the oven, cooled, and
86 weighed. A sample of cell-free filtrate was added to the aluminum pan, and it was dried in a
87 100° C oven overnight. The aluminum pan was removed from the oven, cooled, weighed and
88 weight was recorded. The dry weight was then calculated.

89 **Growth Medium and Conditions.** Each strain was grown in stages (colony in 2.5 mL, 2.5
90 mL in 25 mL) to 250 ml in ¼-strength TSB + 0.2 % sucrose (TSB+S) in a 1 liter baffled flask
91 for 7 days at 30° C and 120 rpm (stationary phase).

92 **Measurement of Minimal Inhibitory Concentration (MIC).** All extracts were resuspended
93 in 1 mL of 1/10th-strength BHIB + 0.1% DMSO. 50 µl of sterile 1/10th-strength Brain Heart
94 Infusion Broth (BHIB) was added to all wells of a 96 well microtiter plate and 50 µl of the
95 resuspended extract or cell-free filtrate was added to the wells in column 1. Two-fold

96 dilutions were made by withdrawing 50 μ l from the well in column 1 and adding it to the well
97 in column 2. This two-fold dilution procedure was repeated in series through well 11. The
98 final 50 μ l from well 11 was discarded to even all the well volumes (final volume = 50 μ l).
99 No extract or fraction was added to the wells in column 12 (i.e., no compound, positive
100 control). Wells 1 through 12 in a single row (e.g., A) were inoculated with 50 μ l of a
101 suspension of target microorganism (e.g. *M. luteus*) that had been diluted to equal a
102 McFarland Standard of 1. The plates were incubated at 30° C for 72 hours and the results
103 were reported as the lowest concentration of extract or fraction completely inhibiting growth
104 of the target microorganism.

105 **Identification of Resin(s) that Bind Antimicrobial Compounds.** Two approaches have
106 been used for measuring the binding of antibiotic activity in cultures. **Approach 1.** For each
107 resin, 10 mL of each cell-free culture filtrate was transferred to four (4) sterile 16 x 150 mm
108 screw capped tubes and no resin added or 0.02, 0.05 or 0.10 gm resin added and the tubes
109 were agitated on a rotator at 120 rpm at 30 ° C overnight. The tubes were removed from the
110 rotator, the resin allowed to settle, 1 mL of the supernatant collected without resin, and 1 ml
111 filter-sterilized by passage through a 0.22 μ m pore size Spin Filter. The MIC of each filtrate
112 was measured. **Approach 2.** For each resin, four 125 ml flasks containing 10 ml of ¼-strength
113 Tryptic Soy Broth with 0.2 % sucrose (TSB+S), and either no resin, 0.02 gm, 0.05 gm, or 0.10
114 gm of a particular resin, were inoculated with a single colony of the antibiotic-producing
115 strain. The cultures were incubated on a shaker (60 rpm) at 30°C for 7 days. The purity of
116 every culture was checked by streak plate. Cell-free supernatants were prepared from 1 ml of
117 culture using a 0.22 μ m pore size spin-filter and 10 μ l spotted on a lawn of every target for the
118 antibiotic. The spot plates were incubated at 30°C for two days and examined for evidence of
119 clearing (zones of inhibition) to confirm antibiotic production. Presence of clearing in the
120 resin-free control coupled with the absence of a cleared zone was taken as evidence of resin

121 binding of the unknown antibiotics. In addition, the lowest resin amount resulting in complete
122 removal of antibiotic activity was identified.

123 **Elution of Antimicrobial Activity from Resins.** Upon identification of those resins able to
124 bind the antibiotic of each strain, resins with bound antibiotic activity were collected by
125 vacuum filtration through Whatman No. 1 filter paper. The filtrate; namely the resin-treated,
126 cell-free culture broth was collected and refrigerated. The resin and filter paper were dried in a
127 Petri dish and the antibiotic eluted from the resins with 5 ml of a 50:50 mix of
128 acetonitrile:water by shaking for one hour. The acetonitrile:water mixture was transferred
129 from the Petri dish to a beaker without removal of resin beads, the beaker covered with cheese
130 cloth, and the solution evaporated to dryness overnight. The Petri dish containing the dry resin
131 was sealed and stored at room temperature. Once the extract had completely evaporated, the
132 dried material was suspended in 1mL of 1/10-strength Brain Heart Infusion Broth containing
133 0.1 % (vol/vol) dimethyl sulfoxide (BHIB + 0.1% DMSO). The medium was filter-sterilized
134 and 10 μ l spotted on lawns of appropriate target microorganisms, plates incubated at 30°C for
135 2-3 days, and scored for the presence or absence of clearing (zones of inhibition). In some
136 instances 100% acetonitrile was used to elute the antibiotic activity.

137 **Scale up of Antibiotic Production Coupled with Resin-Binding (Figure 5.1).** Three 1 liter
138 baffled flasks containing 500 ml of $\frac{1}{4}$ TSB+S and either no resin (2 flasks) or resin at the
139 lowest concentration able to completely bind antibiotic were inoculated with 5 ml of an
140 overnight culture and incubated on a shaker (60 rpm) at 30°C for 7 days. Following growth,
141 resin was added to one of the two cultures grown without resin and shaken at room
142 temperature for 1 hr. Resin from both cultures was recovered by filtration, dried, and eluted.
143 The antibiotic activity of the resin eluates and the cell-free culture filtrate of the one culture
144 grown without resin were measured by broth microdilution (Cain et al., 2000).

145 **Lyophilization and Extraction of Cultures.** Culture material was frozen, thawed, and the
146 entire culture was freeze-dried. The resultant powder was extracted by stirring overnight in
147 either methanol or chloroform: methanol (1:1). The resultant slurry was filtered to remove
148 solids, and the solvent was removed in vacuo. This extract was then partitioned between
149 chloroform:methanol:H₂O (4:1:5). The organic fraction (lower portion) was removed and dried
150 in vacuo (38).

151

152 **Results**

153 **Resin Binding.** The first objective was to determine whether resins bound the putative
154 antimicrobial compounds in the cell-free culture medium of the strains. The results (Table
155 5.1) show that Amberlite XAD-7HP bound antibiotic activity of all of the five strains tested.
156 Also, small amounts (eg. 0.02 gm/10mL) of XAD-7HP were able to bind all activity (Table
157 5.1). Amberlite XAD-4 and XAD-1180 bound the activity of only two of the five strains, and
158 Sepabeads SP-850 only bound the activity of one (Table 5.2). Amberlite XAD-16 and Diaion
159 HP-20 apparently did not remove antimicrobial activity from any of the strains (Table 5.2).
160 As XAD-7HP was able to bind activity of all of the strains, it was used in all further steps of
161 the project.

162 **Elution of Antibiotic Activity from Resins.** All the antimicrobial agents bound to resin
163 XAD-7HP could be eluted by 50:50 acetonitrile:water (Table 5.3). The antimicrobial activity
164 of the resin-eluate was substantially higher (lower MIC) than that of the untreated cell-free
165 culture filtrate (Table 5.3). Antimicrobial activity was not detected in every resin-treated
166 culture filtrate. Further, resin beads placed on a lawn of the target microorganisms after
167 elution lacked any inhibitory activity as well. As antimicrobial activities could be eluted from
168 XAD-7HP, we chose to use it for all large volume cultures.

169 **Effect of Resin Inclusion during Growth on Antibiotic Activity.** Following demonstration
170 that resins reversibly bound antibiotic activity, resin XAD-7HP was incorporated in cultures
171 to see if it increased activity or yield of antimicrobial. One of the strains (4-2-7) produced a
172 resin-eluate with a lower MIC value (i.e., higher antimicrobial activity) for the resin present
173 during growth, when compared to the four other strains (9-3-26, 1-1-11, 14-2-1 and 2.2 N)
174 that had a lower MIC value for resin added after growth (Figure 5.2).

175 Additionally, we compared the antimicrobial activity of resin eluates from resins
176 added throughout growth with activities of lyophilized culture extracts. The resin-eluates of
177 resins present during growth for two strains (1-1-11 and 2.2 N) had higher antimicrobial
178 activity (i.e., lower MIC) compared to the lyophilized culture extracts. The antimicrobial
179 activity of the lyophilized culture extracts of three strains (4-2-7, 9-3-26, and 14-2-1) was
180 higher (lower MICs) compared to the resin eluates of resins present during growth (Figure
181 5.3). Lyophilization and chloroform-methanol extraction lyses cells and thus, the resulting
182 extract contains both intracellular and extracellular antimicrobial activity. In contrast, the
183 resin eluates contain only extracellular antimicrobial activity.

184

185 **Discussion**

186 As others have shown, resins can reversibly bind antimicrobial compounds (21, 47, 66,
187 74, 98). Here however, the objective was to isolate unidentified antimicrobial compounds.
188 Based on the antimicrobial compounds produced by our strains of bacteria, the hydrophobic
189 resins (e.g., XAD) bound the antimicrobial compounds to a greater extent than the ionic resins
190 tested (Table 5.2). Although the resin is not likely to exclusively bind the antimicrobial
191 compounds, we found that the presence of resin in a culture during growth will increase or
192 decrease antimicrobial activity (Figure 5.2). This suggests that the binding and elution of
193 antimicrobial compounds from resin “purifies” the product. As the structure and properties of

194 the antimicrobials produced by these bacteria are unknown, this finding may not be true for all
195 antimicrobials. Further testing with a larger variety of resins with different properties and
196 strains producing a different spectrum of antimicrobial compounds will reveal if this trend is
197 common.

198 The concentrating of the antimicrobial activity after the addition of resin, versus the no
199 resin cell-free filtrate, suggests that the resin is selectively binding the antimicrobial and
200 thereby “purifying” the compound(s) (Table 5.2). The resin is also likely binding other
201 materials in the culture which would not result in a pure compound(s) but rather purer
202 material when compared to the whole culture filtrate. This selective binding could be further
203 exploited with a series of resins in order to obtain this purer material with considerably less
204 time and expense when compared to the previous freeze-dry, fractionation method of culture
205 extraction.

206 It would be suspected that presence of resin during growth would result in higher
207 antimicrobial activity of eluate due to binding of antimicrobial compounds by the resin, thus
208 preventing feedback inhibition (66, 74, 98). However, the antimicrobial producing strains
209 used in this experiment did not show this trend (Figure 5.2). Instead, they showed a higher
210 level of antimicrobial activity in resin added during the last hour of growth, when compared to
211 the resin present during growth. It is possible that this is due to the need for an extracellular
212 modification of the compound, which is being prevented by binding to the resin. Use of
213 different unknown antimicrobial producing strains would need to be tested to see if this is a
214 common trend.

215 Using resins to isolate antimicrobial compounds from culture is beneficial. It reduces
216 the time required to prepare a cell-free extract with substantial antimicrobial activity to 24
217 hours, whereas lyophilization and chloroform-methanol extraction may take 3-4 days (Figure

218 5.4). However, eluates from the resin only include extracellular antimicrobial, whereas the
219 lyophilized, chloroform-methanol treated extract includes both extracellular and intracellular
220 products. Also, resins are fairly cost effective tools. Amberlite XAD-7HP was the resin that
221 bound the most antimicrobials from the unknown strains and cost \$0.06 per liter of culture.
222 Though the prices between different resins vary, using resins is a very cost effective
223 technique.

224

225 **Table 5.1.** Antimicrobial-producing bacteria and targets.

Antimicrobial Producing Strains	Target Organisms
<i>Burkholderia ambifaria</i> strain 2.2 N	<i>M. luteus</i> , <i>E. coli</i> , <i>S. cerevisiae</i>
<i>Brevibacterium halotolerans</i> strain 4-2-7	<i>M. luteus</i> , <i>S. aureus</i> , <i>E. coli</i> , <i>A. niger</i>
<i>Bacillus cereus</i> strain 1-1-11	<i>M. luteus</i> , <i>S.aureus</i> , <i>E.coli</i> , <i>M. smegmatis</i> , <i>C. albicans</i> , <i>A. niger</i>
<i>Bacillus subtilis</i> strain 9-3-26	<i>M. luteus</i> , <i>E. coli</i> , <i>S. cerevisiae</i>
<i>Streptomyces griseus</i> strain 14-2-1	<i>M. luteus</i> , <i>E. coli</i> , <i>S. cerevisiae</i>

226

227 **Table 5.2.** Testing of adsorbent resin to determine ability of resin to bind antimicrobial
 228 activity.

Resin Type	Antibiotic-Binding (Resin gm/10 mL culture) ^a				
	<i>B. ambifaria</i> Strain 2.2N	<i>B. subtilis</i> Strain 9-3-26	<i>B. halotolerans</i> Strain 4-2-7	<i>B. cereus</i> Strain 1-1-11	<i>S. griseus</i> Strain 14-2-1
XAD-4	+ (0.10)	-	-	+ (0.04)	+ (0.10)
XAD-7HP	+ (0.02)	+ (0.05)	+ (0.02)	+ (0.04)	+ (0.10)
XAD-16	-	-	-	-	-
XAD-1180	+ (0.05)	-	-	+ (0.04)	-
Diaion HP20	-	-	-	-	-
Sepabeads SP850	+ (0.02)	-	-	-	-
	^b <i>S. cerevisiae</i>	^b <i>M. luteus</i>	^b <i>S. aureus</i>	^b <i>A. niger</i>	^b <i>M. luteus</i>
<p>^aPlus (+) indicates binding of antibiotic as evidenced by lack of activity in culture medium. Minus (-) indicates an absence of binding. Value in parenthesis indicates the lowest concentration of resin resulting in complete removal of antimicrobial activity.</p> <p>^bAntimicrobial activity target strain</p>					

229

230 **Table 5.3.** Effect of resin binding on concentration of antimicrobial activity when tested
 231 against *M. luteus*.

Material	Minimal Inhibitory Concentration (µg/mL)			
	<i>B. ambifaria</i> Strain 2.2 N	<i>B. subtilis</i> Strain 9-3-26	<i>B. halotolerans</i> Strain 4-2-7	<i>B. cereus</i> Strain 1-1-11
Cell free culture filtrate	120	200	100	100
XAD-7HP-treated cell free culture filtrate	>500	>500	>500	>500
Acetonitrile: Water eluate	30	50	20	50

232

Figure 5.1. Diagram of experimental setup for testing of resin binding. (1) Adsorbent resin added to the initial culture, (2) Adsorbent resin added for 1 hr. after culture growth, (3) No resin was added and the culture was lyophilized and extracted into organic and aqueous fractions.

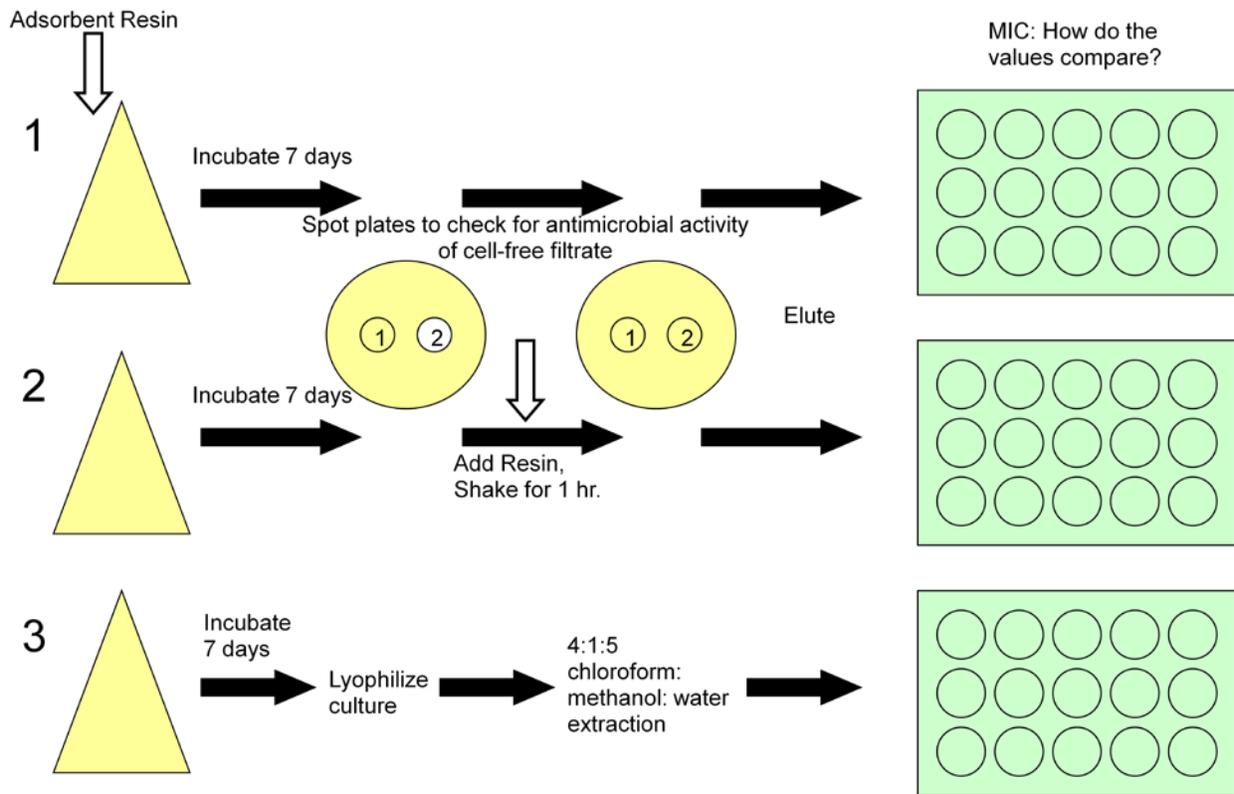


Figure 5.2. Antimicrobial activity of extracts for the three extraction methods. Antimicrobial activity was tested against a target organism for each strain. Strain 1-1-11 against *A. niger*, strain 4-2-7 against *S. aureus*, strain 9-3-26 against *M. luteus*, strain 2.2N against *S. cerevisiae*, and strain 14-2-1 against *M. luteus*.

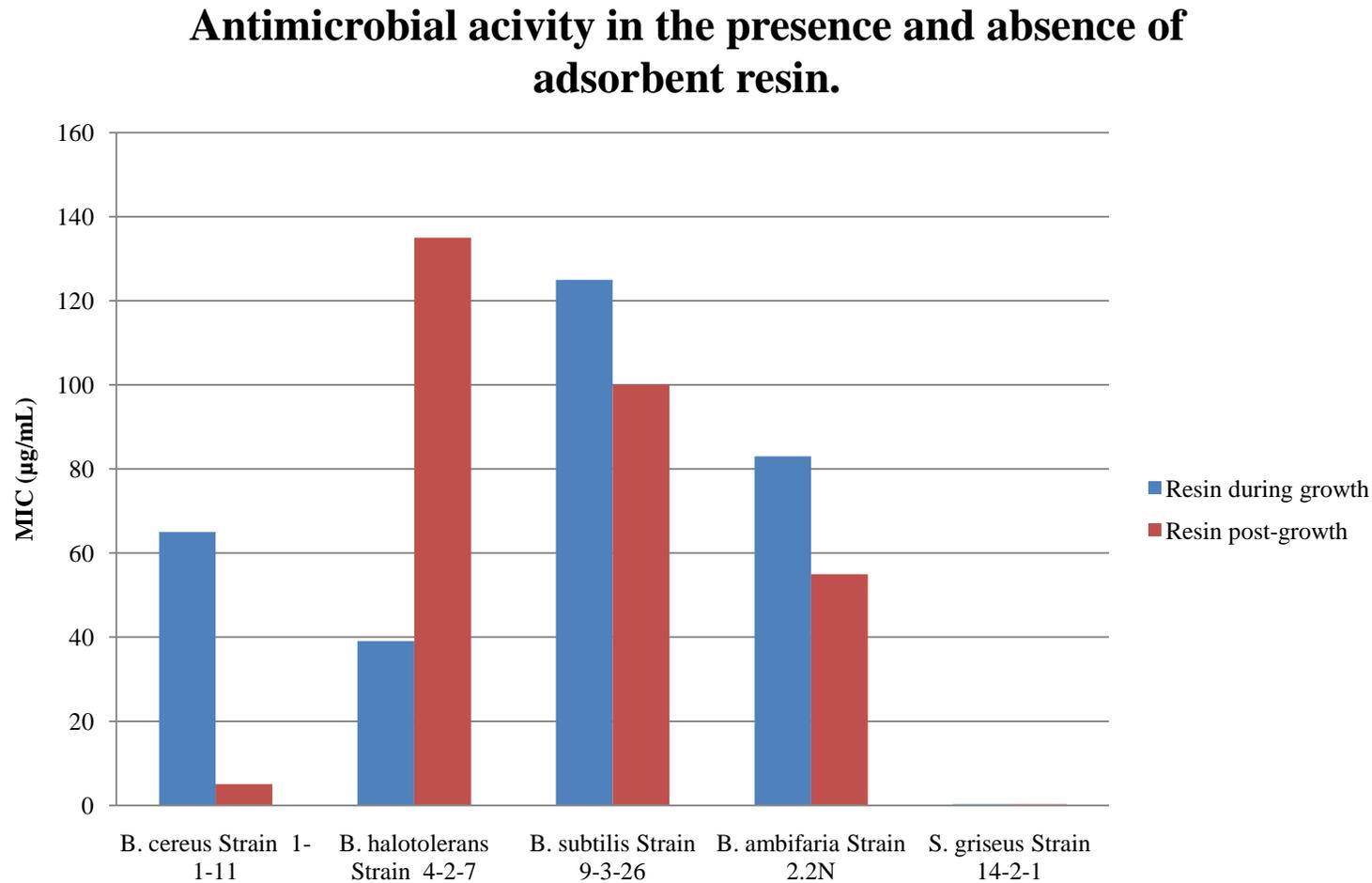


Figure 5.3. Comparison of antimicrobial activity of resin extraction and extraction via lyophilization. Strain 1-1-11 against *A. niger*, strain 4-2-7 against *S. aureus*, strain 9-3-26 against *M. luteus*, strain 2.2N against *S. cerevisiae*, and strain 14-2-1 against *M. luteus*.

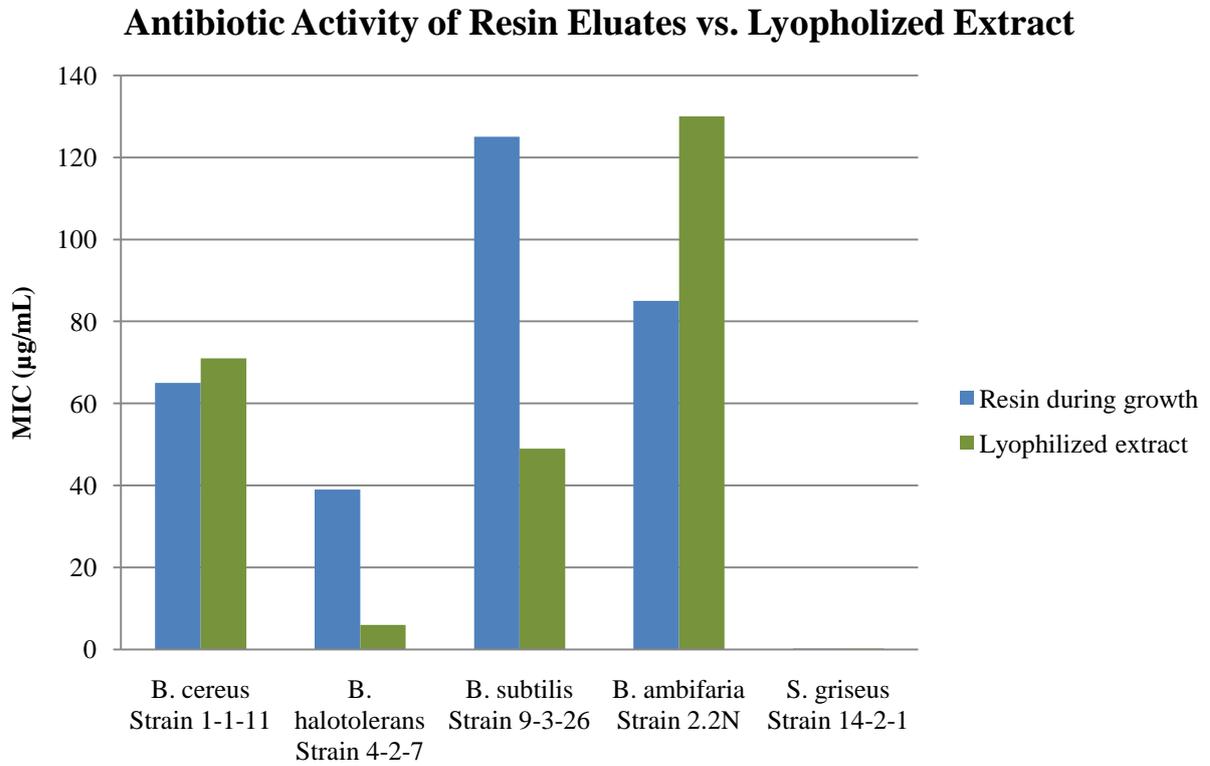
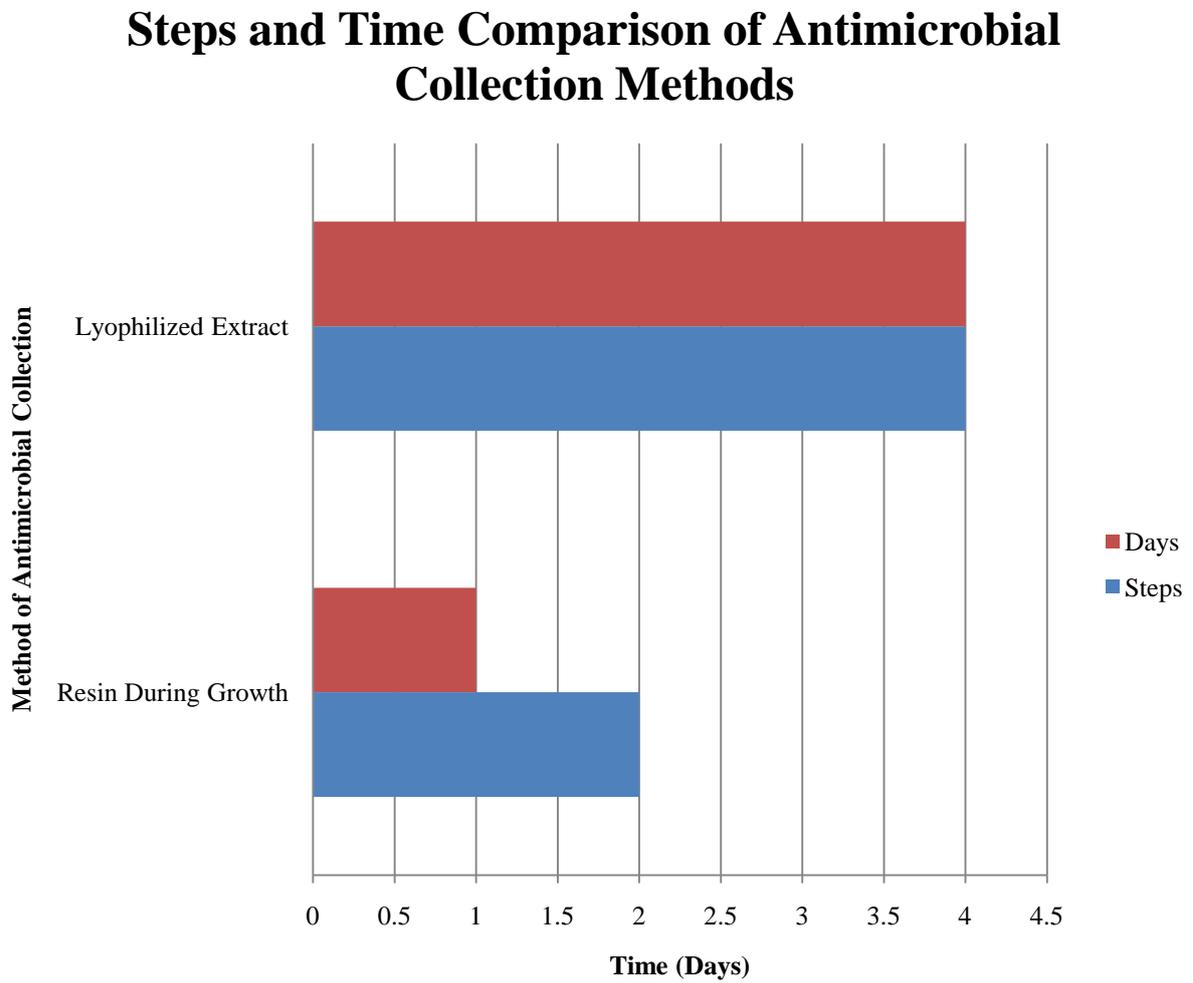


Figure 5.4. Step and time comparison of collection antimicrobial via resin addition vs. lyophilization.



Chapter 6: Antimicrobial-producing Bacteria functioning as a Driver of Diversity within an Artificial Microbial Community

Abstract

While the role of predatory animals in shaping the diversity of their communities has been a focus of considerable study, studies of the role that antimicrobial-producing bacteria have on their surrounding microbial community has largely been confined to microbe-plant interactions. Antimicrobial-producers should affect the surrounding microbial assemblage either through predatory or allelopathic interactions. In order to examine this effect, an artificial community was established in a sand microcosm in both the presence and absence of an antimicrobial producing bacterium. DNA was isolated from the community and examined for changes over time in the community structure. While changes could be seen over time in the communities, it was not possible to assign these differences to an effect of the antimicrobial-producer.

Introduction

Studying microbial communities has long been challenging to accomplish due to the limited number of microorganisms that can be cultivated on laboratory media. With the advent of genomics, microbial communities have become more accessible although current genomic strategies are biased towards known phyla based on primer design (58). It is also challenging to study microbial diversity since you cannot be sure you are accounting for all members of the community (14). It is also thought that the typical drivers of macro organism diversity do not apply to microorganism diversity (41) and there are a number of variables that could affect the community at a given time point such as: pH, organic matter input, and moisture level.

Microbial assemblage structure is often examined in the context of variations in communities in the rhizosphere (10, 89) (32) (77). These studies have largely focused on the effect that different microbial assemblages have on: the health of nearby plants (32), the plants ability to ward off disease (10), and the plants ability to adapt to rapidly changing conditions (10). Spatial heterogeneity and the point of sampling have also been a focus of study when microbial assemblage structure has been examined previously (51) (88). These studies indicate that soil physical structures and bacterial sizes play a role in determining the microbial assemblage structure as they affect the spatial homogeneity of a sample. Changes in microbial assemblages have also been observed in a cyclic nature based on changes in environmental conditions due to seasonal changes and availability of nutrients (40).

The role of predatory animals in shaping the populations of organisms that live in the same habitat has been a focus of considerable study. It has been shown that a keystone predator, even in low numbers, can increase population diversity by reducing competitive exclusion (78). Gause's Law of competitive exclusion is the theory that states that two organisms that compete for the same resources cannot stably coexist if all other factors remain constant. One organism will inherently have an advantage and will dominate the other to the point of its extinction in the long term. A predator can alleviate this competition if it reduces competitor numbers to below a threshold at which point resources are no longer a limiting factor.

It is unknown if antimicrobial-producing bacteria act to competitively exclude others by using antimicrobials as an advantage (allelopathy) or if they actively look to kill other microorganisms as a source of nutrients (predation). It has been shown that low levels of an added antibiotic produces little effect in the microbial community whereas high levels of antibiotics produces large shifts in the microbial community (80).

In the absence of an antimicrobial-producer it would be expected that a subset of the community members would become dominant as they would exclude other members due to nutrient competition. If the dominant community members were sensitive to an antimicrobial, then it would be expected that the other members of the community would increase with the addition of the antimicrobial-producing organism as competitive exclusion should be reduced. Conversely, if the dominant member is resistant to the produced antimicrobial then no shift in community structure should be observed. Interactions in this manner would indicate antimicrobial-producers act in a predatory fashion whereas other changes in the microbial assemblage structure would indicate an allelopathic relationship between antimicrobial-producers and the surrounding microbial community.

Methods and Materials

We chose to examine an artificial microbial community in a sand microcosm in the presence and absence of an antimicrobial producing bacterial strain. This sand microcosm eliminated the variables of soil aggregates, organic matter content, and some sterilization problems. The artificial community allowed for all community members to be known and accounted for during subsequent analysis steps whereas a natural microbial community would contain many unknown members. The antimicrobial-producing strain we selected was one which we have used previously and we know the antimicrobial compounds it produces (96).

Sand sterilization and Soil extract preparation. Sand was rinsed twice in sterile water and then autoclaved for 30 min. After being dried over at 70° C, sand was stored in a sterile container. Pea gravel was washed and autoclaved following the same procedure.

Soil extract was used to return moisture to the microcosms because it contains many of the nutrients that are essential for survival without providing an abundance of nutrient which would allow for growth independent of any competition. Soil extract was prepared by

suspending 400 g of soil in 1 L of distilled water and autoclaving on liquid cycle for 60 min. It was allowed to cool and settle overnight after which the supernatant was filtered through Whatman #1 filter paper (or 10 µm pore size). The filtrate was again allowed to settle overnight after which the supernatant was filtered through Whatman #3 filter paper (or 5.0 µm pore size paper). The resultant filtrate was then autoclaved in bottles for 15 min on liquid cycle.

Sand Jar Setup (Figure 6.1). A small layer of autoclaved pea gravel was placed in the bottom of each jar to avoid having standing liquid in the bottom layer of sand. A double layer of cheesecloth was placed on top of the pea gravel on top of which was placed 200 g of autoclaved sand (premixed with 1 mL / 10 g of liquid). The jar was covered with cheesecloth held on by a rubber band (to facilitate air flow but restrict dust getting in) and the jars were incubated on the lab bench at room temperature (~21° C). The jars were weighed immediately after inoculation so that sterile soil extract could be added each day to replace the liquid lost to evaporation.

Sand Acclimation. 40 g of soil was suspended in 100 mL distilled water and vortexed for 1 min, and then allowed to settle for 1 hr. 20 mL of the supernatant was removed and added to a sand jar after which the jar was incubated at room temperature (21° C) for 7 days. 0.5g of sand was removed and plated (in duplicate) on PCA. After incubation at 30° C for 48 hr, various colonies were picked to separate plates for further testing.

Isolate Testing. Isolates were grown on PCA plates and kept to be used throughout testing. Each isolate was grown in 5 mL ¼-strength Tryptic Soy Broth containing 0.2% sucrose (TSB+S) at 30° C for 48 hr. The isolates were tested for antimicrobial activity by spot plates on lawns of various target organisms (*Staphylococcus aureus*, *Micrococcus luteus*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Candida albicans*, *Aspergillus niger*, and *Mycobacterium smegmatis*) with any isolates that exhibited antimicrobial activity being

discarded. Resistance and sensitivity to *Burkholderia ambifaria* strain 2.2N was tested by spot plate. Isolate DNA was isolated using Ultra Clean Microbial DNA Isolation Kit (Mo Bio) and PCR was performed as described below.

Preparation of isolates and antimicrobial-producer. Isolates were streaked onto PCA plate to confirm purity and morphology after which they were used to inoculate 5 mL of ¼ TSB+S which was incubated at 30° C for 48 hr on a rotator. The cells were harvested by centrifugation and resuspended in sterile tap water before addition to sand jars. Antimicrobial-producing strain *B. ambifaria* strain 2.2N (96) was chosen as the producing strain due to its regular use in the laboratory. From the isolates tested, 6 were chosen for addition to the sand microcosm based on their characteristics of being able to grow in the sand, 4 being sensitive to strain *B. ambifaria* 2.2N, 2 being resistant to *B. ambifaria* strain 2.2N, and being able to be readily cultured. The strains chosen were: strain 123-2, strain 129, strain 131, strain 231, strain 331-1, and strain 331-2 (strain designations based on numbering system used during isolate testing).

Community setup. For each isolate to be used in the community, 1 mL was added to 200 g of sterile sand and mixed thoroughly before being added the sand to the jar. Sterile soil extract was added up to a total of 20 mL (isolates + soil extract) before addition to the sand jar. Each jar was weighed with the weight being recorded on the side of the jar. This weight was used to add sterile soil extract each day to account for moisture loss due to evaporation. The community was allowed to establish for 48 hrs before the addition of strain 2.2N. 1 mL of *B. ambifaria* strain 2.2N was added after 48 hr. and the sand was thoroughly mixed with sterile spatula to incorporate strain *B. ambifaria* 2.2N evenly across the established community.

Sampling and DNA analysis. Before each sample, the sand in the jar was mixed thoroughly with a sterile spatula and 0.5g of sand was removed to use for DNA isolation starting on the

date of *B. ambifaria* strain 2.2N addition (Day 0). DNA was isolated using FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Ohio) following manufacturer's directions. PCR amplification was carried out using universal 16S rDNA primers (70) (Primer 527f – 5'-ACCGCGGCCCKGCTGGC – 3', Primer 1406r – 5'-ACGGGCGGTGTGTRC – 3') and GoTaq (Promega, Wi) following manufacturer's suggested guidelines. The PCR product banding patterns and peak area were compared using 1.5% agarose gels stained gel in EtBr and GelCompar II software (Applied Maths, NV). Samples were taken to 14 days, as after 14 days most bands were no longer obtainable from any of the strains (data not shown).

Results

Absence of B. ambifaria strain 2.2N. In the absence of *B. ambifaria* strain 2.2N, strains 129 and 231 maintained the relative peak area throughout the sampling period (Figure 6.2). Strain 331-1 reduced in peak area until it was below the limit of detection in all sampling jars (Figure 6.2). Strains 123-2, 131 and 331-2 maintained peak area in one sampling jar but declined in the others (Figure 6.2).

Presence of B. ambifaria strain 2.2N. In the presence of *B. ambifaria* strain 2.2N, no single strain maintained peak area throughout the sampling period in all sampling jars (Figure 6.3). Strains 131, 231 and 331-2 maintained their peak area throughout the sampling period in one sampling jar (Figure 6.3). Strains 331-1, 129 and 123-2 declined in peak area in all sampling jars (Figure 6.3).

Resistant strains against B. ambifaria strain 2.2N. Strains 129 and 331-1 were determined to be resistant to the antimicrobial activity of *B. ambifaria* strain 2.2N. Strain 129 maintained peak area in the absence of *B. ambifaria* strain 2.2N but was reduced to an undetectable level in the presence of *B. ambifaria* strain 2.2N (Figure 6.4). Strain 331-1 had the same decline pattern in both the presence and absence of *B. ambifaria* strain 2.2N (Figure 6.4).

Sensitive strains against B. ambifaria strain 2.2N. Strains 331-2, 131, 231, and 123-2 were determined to be sensitive to the antimicrobial activity of *B. ambifaria* strain 2.2N. Strains 331-2 and 231 maintained peak area in the absence of *B. ambifaria* strain 2.2N as well as in the presence of *B. ambifaria* strain 2.2N in one sampling jar (Figure 6.5). Strains 331-2 and 231 were present for part of the sampling period in both other sampling jars but declined by the end of the sampling period (Figure 6.4).

Discussion

The microbial communities did change over the sampling period with some microorganisms reducing in number to the point of appearing absent in the banding pattern and others becoming numerous enough to identify bands (Tables 6.1 and 6.2). In the absence of the antimicrobial-producer, *B. ambifaria* strain 2.2N, most strains seem to be present for the first 7 days with varied band results up to 14 days (Table 6.1). Strain 129 appeared to retain the most band peak area throughout the sampling period (Figure 6.2). While strain 129 was resistant to *B. ambifaria* strain 2.2N, in the presence of the antimicrobial-producer it was observed that strain 129 declined. This suggests another interaction within the microbial assemblage that is causing strain 129 to decline. This interaction is not present in the absence of *B. ambifaria* strain 2.2N suggesting that strain 129 and *B. ambifaria* strain 2.2N are competing for limiting nutrient with *B. ambifaria* strain 2.2N having a competitive advantage.

In the presence of strain 2.2N there was a decrease in the number of visible bands for each of the microcosms (Table 6.2). The maintenance of the peak area for sensitive strains 231 and 331-2 (Figure 6.3) suggests a community interaction that is protective against the antimicrobials produced by *B. ambifaria* strain 2.2N.

The data obtained suggests that while microbial community diversity is not being increased by the presence of an antimicrobial producing bacterium, community interactions

are changing the characteristics of the individual isolates. This most evident when looking at the survival of the resistant and susceptible isolates in the presence of *B. ambifaria* strain 2.2N. The survival patterns indicate that *B. ambifaria* strain 2.2N is probably interacting with the microbial assemblage in an allelopathic manner. This competition would allow for the survival of sensitive strains by the sensitive strains utilizing nutrients that *B. ambifaria* strain 2.2N is not utilizing. An allelopathic interaction also helps to explain the disappearance of a resistant strain as a purely predatory interaction would leave the resistant strain unharmed.

These interactions could be due to the limited community size that was used in this study or may be specific to the antimicrobial-producer chosen. An antimicrobial-producer with a different range of active agents could have a different affect on the community. Community interactions would appear to have a larger effect in this study than the antimicrobials being produced. This study's microcosms may have misrepresented this aspect as artificial communities are not as robust as natural communities.

The goal of this study was to ascertain if antimicrobial-producing bacteria have the ability to increase diversity in their microbial community, but the limited artificial community does not appear to have indicated that this is the case. A larger microbial community in the microcosm may help to resolve this issue but would also complicate the DNA based comparison due to the increase in band numbers. Further work with larger microcosm communities as well as varied antimicrobial producers would need to be done in order to determine if these antimicrobial producers are helping to drive microbial community diversity through either a predatory or allelopathic interactions.

Table 6.1. Changes in sand microbial communities over time without the presence of *Burkholderia ambifaria* strain 2.2N.

Jar 1 - No 2.2N

	Day 0	Day 2	Day 5	Day 7	Day 9	Day 14
123-2	+	+	+	+	+	-
129	-	+	+	+	+	+
131	-	+	+	+	-	-
231	+	+	+	+	+	-
331-2	+	+	+	+	-	-

Jar 3 - No 2.2N

	Day 0	Day 2	Day 5	Day 7	Day 9	Day 14
123-2	+	+	+	+	+	-
129	+	+	+	-	+	+
131	+	+	+	-	-	+
231	+	+	+	+	+	+
331-2	+	+	+	+	+	+

Jar 5 - No 2.2N

	Day 0	Day 2	Day 5	Day 7	Day 9	Day 14
123-2	-	+	+	+	+	+
129	+	-	-	+	+	-
131	+	-	-	+	+	-
231	+	+	+	+	+	+
331-2	+	-	-	+	-	-

Table 6.2. Changes in sand microbial communities over time in the presence of *Burkholderia ambifaria* strain 2.2N.

Jar 2 - Yes 2.2N

	Day 0	Day 2	Day 5	Day 7	Day 9	Day 14
123-2	-	-	-	-	-	-
129	-	-	+	-	-	-
131	-	-	+	-	-	-
231	+	+	+	+	+	+
331-2	-	-	-	-	-	-

Jar 4 - Yes 2.2N

	Day 0	Day 2	Day 5	Day 7	Day 9	Day 14
123-2	+	+	+	+	+	-
129	-	-	-	-	-	-
131	-	-	-	-	-	-
231	-	-	+	+	+	+
331-2	+	+	+	+	+	+

Jar 6 - Yes 2.2N

	Day 0	Day 2	Day 5	Day 7	Day 9	Day 14
123-2	-	+	+	+	+	+
129	-	+	+	-	-	+
131	-	+	+	-	-	-
231	-	+	+	+	+	-
331-2	+	-	+	+	+	-

Figure 6.1. Sand jar diagram.

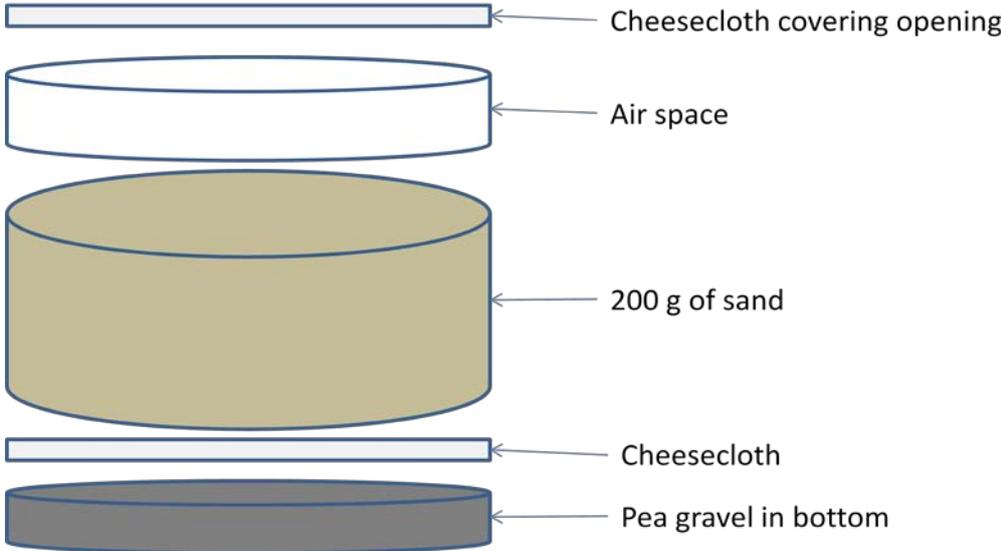


Figure 6.2. Overlay plots of survival of isolates in the absence of antimicrobial-producing strain *B. ambifaria* strain 2.2N.

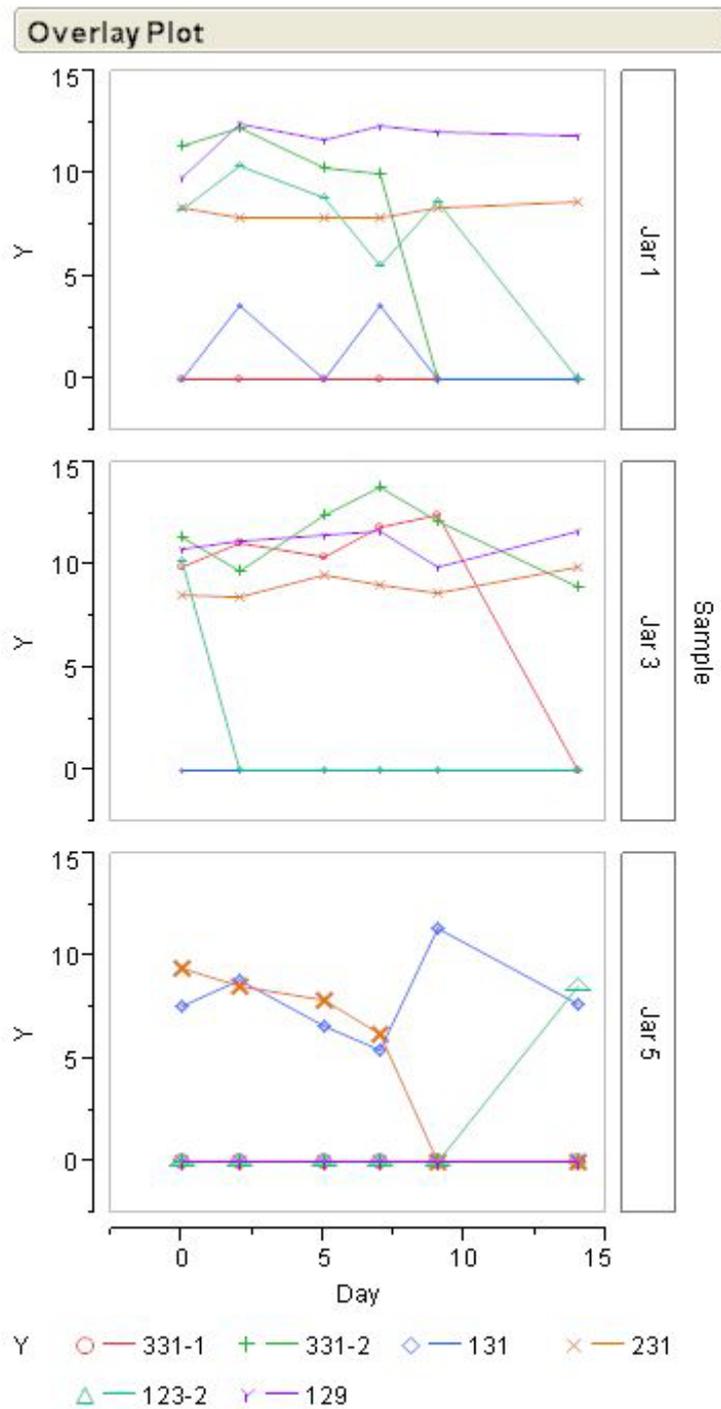


Figure 6.3. Overlay plots of survival of isolates in the presence of antimicrobial-producing strain *B. ambifaria* strain 2.2N.

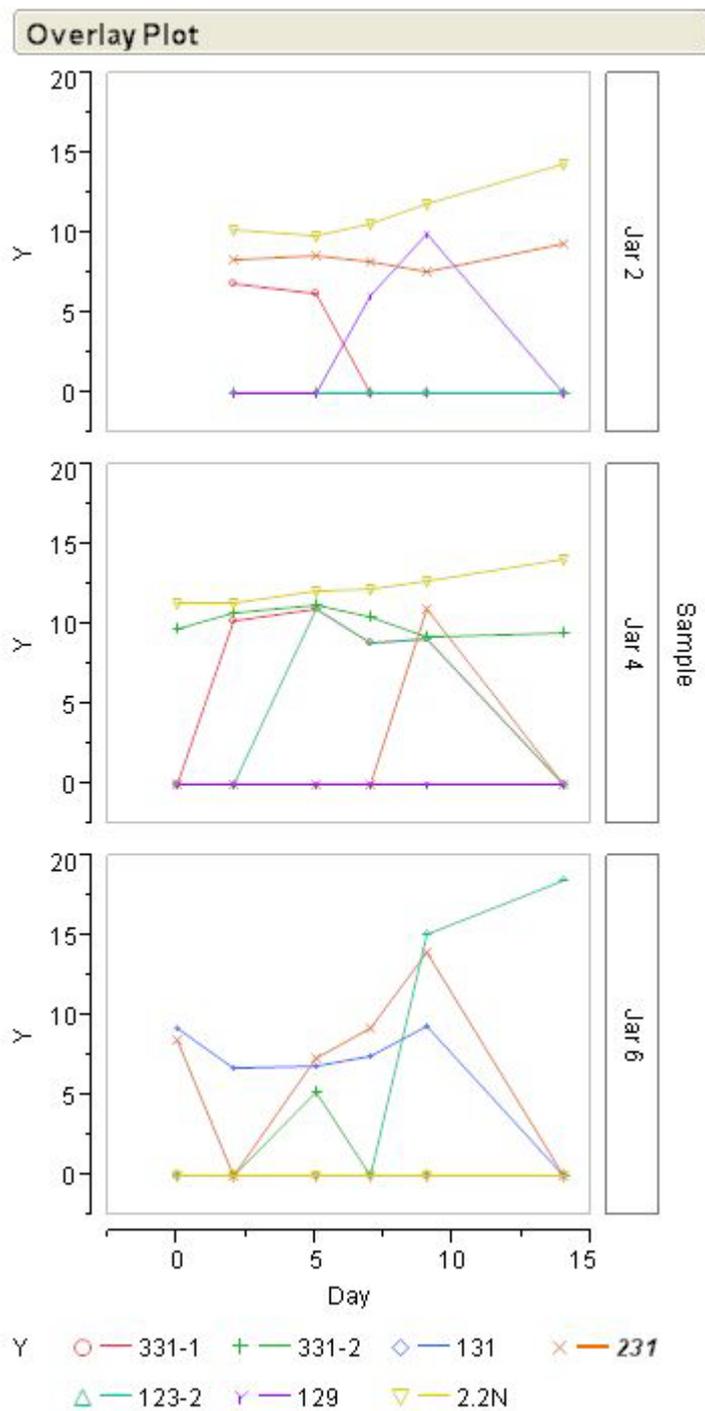


Figure 6.4. Overlay plots of survival of isolates resistant to *B. ambifaria* strain 2.2N. Jars 1, 3, and 5 do not contain *B. ambifaria* strain 2.2N.

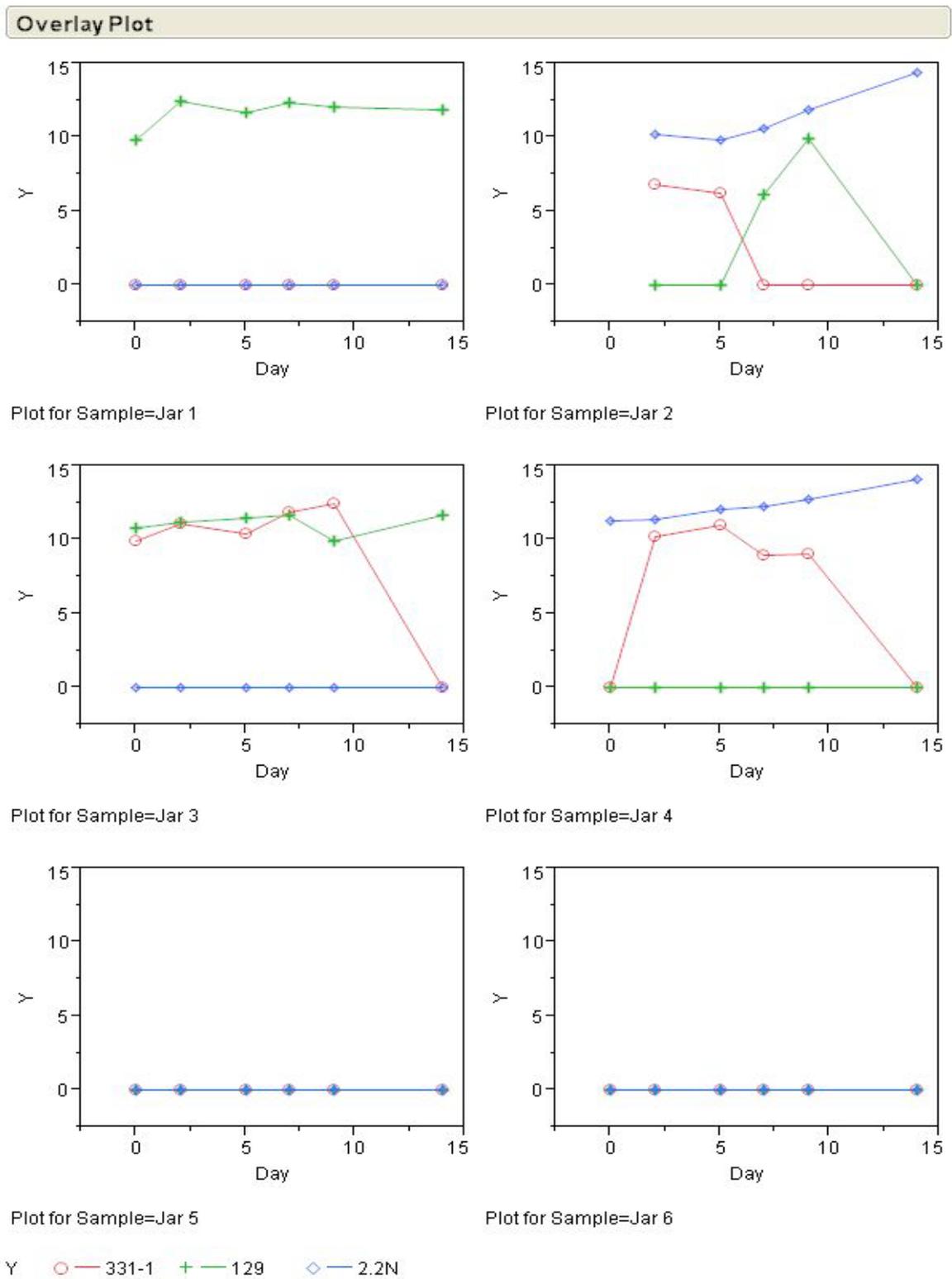
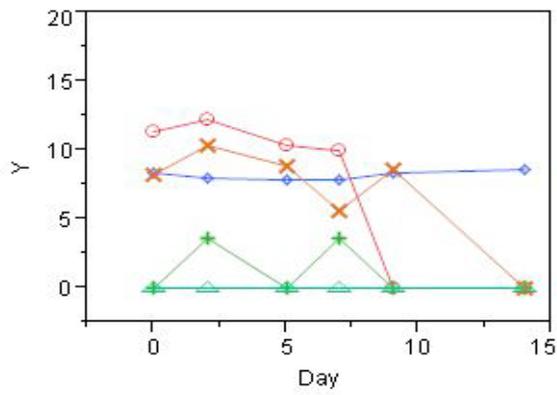
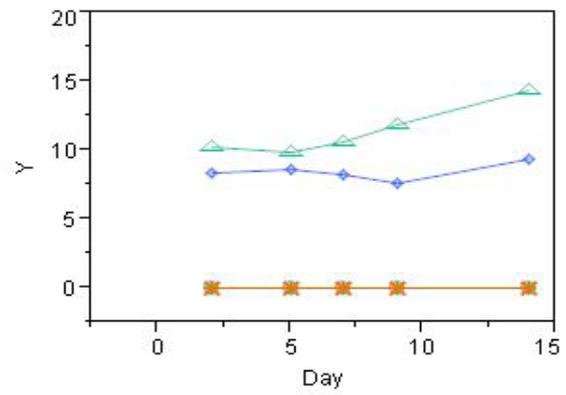


Figure 6.5. Overlay plots of survival of isolates sensitive to *B. ambifaria* strain 2.2N. Jars 1, 3, and 5 do not contain *B. ambifaria* strain 2.2N.

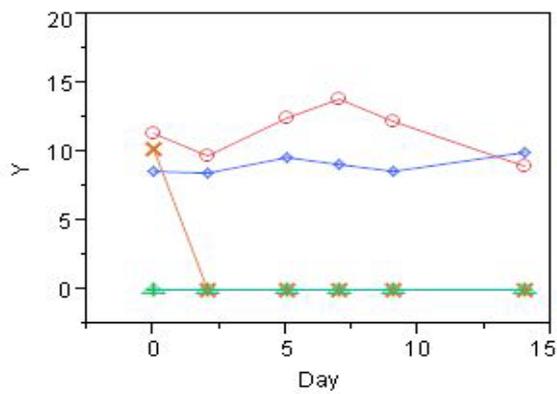
Overlay Plot



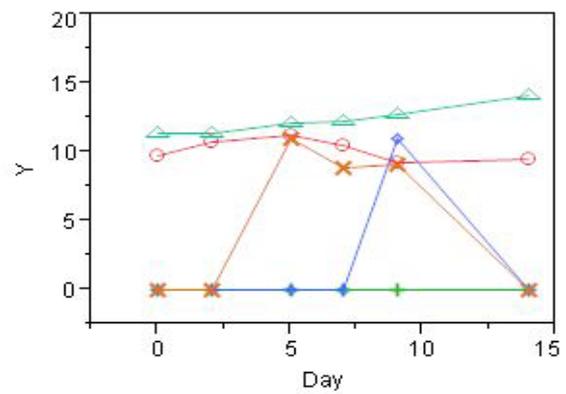
Plot for Sample=Jar 1



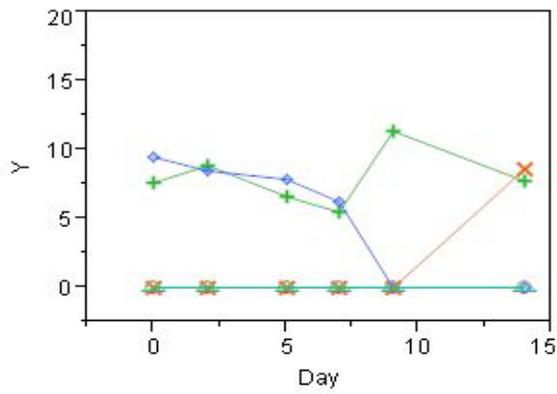
Plot for Sample=Jar 2



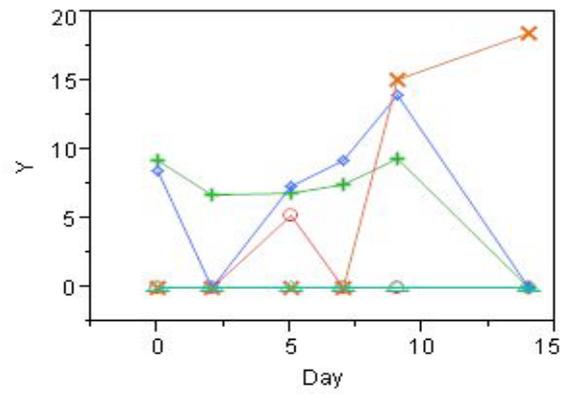
Plot for Sample=Jar 3



Plot for Sample=Jar 4



Plot for Sample=Jar 5



Plot for Sample=Jar 6

Y ○ 331-2 + 131 ◇ 231 × 123-2 △ 2.2N

Chapter 7: Conclusions and Future Directions

The work presented here illustrates the complexities of any drug discovery project. Chapter 2 illustrated that extraneous compounds can be introduced through means other than production from your desired organism. Leaching of compounds from growth vessels and utensils is likely common and generally undetected. It is possible that these leached compounds could be responsible for degradation of desired cellular products and are a contributing factor in the low yields associated with natural products from otherwise “pure” cultures.

While Chapter 3 shows that a soil can have antimicrobial properties that are based on the resident microorganisms, it also illustrates that there are many microorganisms involved producing a variety of compounds. These compounds are difficult to separate from each other to test individual activities and it is likely that many of these antimicrobials work synergistically with one another to achieve the effect seen in the “Red Soils.” Individual isolates can be obtained, having antimicrobial activity, from these soils but the challenge then becomes obtaining the antimicrobial in quantities where it can be identified and tested.

Chapter 5 was focused on obtaining these antimicrobial compounds in a manner that was both as efficient and simple as possible. Adsorbent resins provide a method by which extracellular compounds can be sequestered from the rest of the media constituents and then readily desorbed in a purer form than in the original culture. We were able to remove the antimicrobial activity from culture material using adsorbent resins and then recover that activity. It may also be possible to use a sequence of resins in order to remove compounds with no activity from the culture material before removing the antimicrobial in an effort to obtain even purer material for further studies. Testing this effect using resin known not to bind the antimicrobial activity might allow for a reduction in the number of activity directed

fractionation steps. This would lead to less compound loss and could allow for a greater yield thereby allowing for structural identification or further testing.

Characterizing the soils used in these drug discovery studies would be useful in that it could allow for decisions to be made on which soils to pursue as the most likely candidates to contain antimicrobial producers. Chapter 4 focused on an effort to develop a method for looking at the CLPP of each soil in such a way as to where it could be compared against the number of antimicrobial producing microorganisms. After sufficient sampling had been done, this would establish a likelihood of a soil containing antimicrobial producers and rapid screening of soils to test more extensively. Unfortunately, the Biolog EcoPlate™ system did not yield results that were reproducible so no such conclusions were able to be drawn. It is still possible that the CLPP could be used in this manner but the heterogeneity issue, along with any other reasons for the large variations in results, would need to be overcome before this method could be employed.

The role of antimicrobial producing bacteria in the microbial community has not been studied previously and was the focus of Chapter 6 of this dissertation. This study was not designed to be a definitive answer to if bacteria functioned as a Keystone Species within their communities but rather a study on “can” they function this way. More studies would need to be undertaken using both artificial and natural microbial communities to determine if these bacteria are true drivers of diversity within their communities. While changes were seen in the microbial community as time progressed, I was unable to assign these differences due to the presence of an antimicrobial producing bacterium. It is possible that antibiotic producing bacteria do shape the structure of their natural microbial community and this ecological study should be examined in the future.

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