Conclusions: It is relatively simple to find bacteria in soil and coax them to grow in the lab. It is not so simple to create an axenic culture and discover its identity with biochemical and molecular testing. It is more challenging still to determine if it is a potential antibiotic producer. Often microbes only make antibiotics as a secondary metabolite in certain phases of their growth curves, or only when forced to do so when in competition with other members of their ecosystem. Microbes competing in nature also are under pressure from viruses that control population size and shuttle genetic information around via horizontal gene transfer. Sawyer's mixture of soil microbes showed just this phenomenon: the even growth of bacteria began to show plaques, or evidence

Tiny Earth @ SSU 2019

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Tiny Earth
studentsourcing antibiotic discovery

Introduction to Tiny Earth:

'Studentsourcing Antibiotic Discovery'

Antibiotic resistance is a devastating issue that is affecting global health and development[1]. Antibiotics are medicines used to control bacterial growth in humans and other animals. The first antibiotic was discovered early in the 20th century when it was observed that the growth of a fungal contaminant on a plate with bacteria was able to stop the growth of the bacteria[2].

Bacterial infections are difficult to treat when the bacteria become resistant to the antibiotic, having evolved to grow in the presence of the drug. Because bacteria have rapid reproduction rates, and can evolve antibiotic resistance (AR) quickly, often due to horizontal gene transfer, the spread of AR infections has only accelerated since the discovery of the first antimicrobial drug. The outcomes of antibiotic resistance include higher medical costs, longer hospital stays, and increased mortality. The global spread of resistance mechanisms is hindering the ability to treat common infectious diseases. Respiratory, diarrheal, and sexually transmitted disease are getting harder to treat with the increase of antibiotic resistance[1,3].

While the incidence of AR microbes is on the rise, the discovery of new antibiotics has been on a constant downturn.

Due to the misuse and overuse of antibiotics, bacteria have grown resistant to treatments and continue to spread between humans and animals. The antibiotic resistance crisis will become life threatening if we do not get a grasp on the issue soon. The field of antibiotic stewardship has recently become important in clinical, veterinary, and agricultural areas alike.

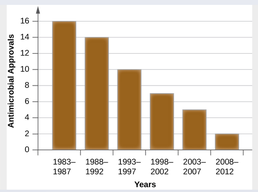


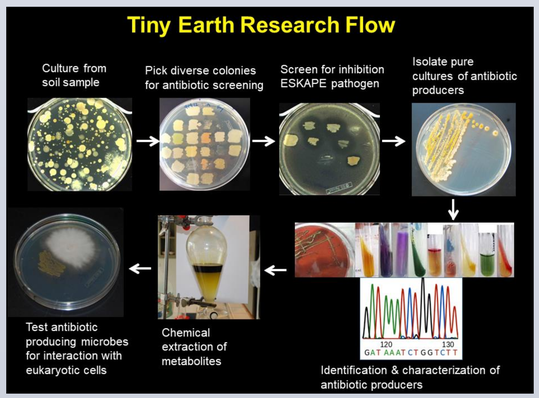
Figure 1: New Antimicrobials Approved by FDA 1983-2012

The One Health Organization[4] recognizes that these three elements: humans, animals and the ecosystem are inextricably interconnected and is a important leader in antibiotic stewardship.

Crowdsourcing antibiotic discovery from soil has emerged as an important technique in which the main goal is to find naturally occurring antibiotics that can be used to ameliorate the antibiotic resistance crisis. Soil is used since many antibiotics that are presently used have been derived from bacteria or fungi in soil. Large advancements in technology and medicine have been made from the discovery of antibiotics from soil such as penicillin, streptomycin, chloramphenicol, and tetracycline[5].

The Tiny Earth[6] program is a network of instructors and students focused on studentsourcing antibiotic discovery from soil. The mission of the program is two-fold:

- First, it seeks to inspire students to pursue careers in science through original laboratory and field research conducted in introductory courses with the potential for global impact.
- Second, it aims to address a worldwide health threat—the diminishing supply of effective antibiotics—by tapping into the collective power of many student researchers concurrently tackling the same challenge, living up to its motto "studentsourcing antibiotic discovery."



Characterization of Soil Isolates

- Our first soil isolates came from a plate that had been touched with a finger that had dirt from the floor on it.
- Axenic (without strangers) or 'pure' cultures are attempted using the quadrant streak plate method.

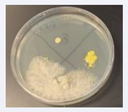


Figure 2: The crowded plate that the potential producer was first identified on. The producer can be seen in the center of the filamentous colony.

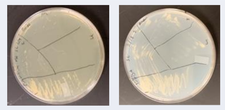


Figure 3: Two different isolates were purified with the streak plate technique. The growth medium was TSA (Trypticase Soy Agar incubated at 30˚C for 24 hours.

- A well isolated colony from a streak plate that has been repeated several times should yield colonies that are all descended from only one species of bacteria. That colony can be sampled in the Gram stain to determine the cell morphology and Gram staining reaction of the bacteria.
- Finally, growth of the soil isolate in the patch-patch method (see Tiny Earth Research Manual[7]) determines if it is producing a soluble secondary metabolite that inhibits the growth of other bacteria. We have a panel of ESKAPE safe relative strains to test in this way.
- Gram stains are performed on growth from the streak plate

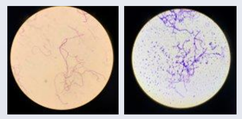


Figure 4: 1000X oil immersion photomicrograph. Gram stain of the different colony types. The isolates are Gram positive filamentous bacteria.

Figure 5: The isolates tested together against known ESKAPE safe relatives showing inhibition of growth against Gram positive bacteria on the right side of the 100 mm TSA plate, but not the Gram negative strains on the left.

- Upon reanimating the culture stored on a slant over winter break, it became clear that this producing culture was NOT a single species! We continued to try to isolate the strains from each other and tested them in a modified patch/patch method on a larger (150mm diameter) plate.

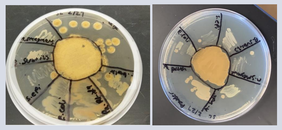


Figure 6: The white and beige isolates tested against known ESKAPE safe relatives NOT showing any inhibition of growth.

- Only the combination of the beige and white strains resulted in the inhibition of growth in other bacterial species.

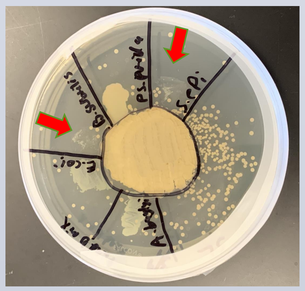
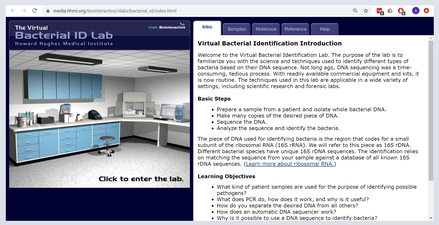


Figure 7: The isolates tested together against known ESKAPE safe relatives showing inhibition of growth against Gram positive bacteria.

Molecular Identification of Soil Isolates

In the Tiny Earth Research Guide[7], purified soil bacteria are intended to be identified using 16S rRNA PCR. Our soil isolates are Gram positive filamentous bacteria which puts them in the larger family of *Actinomycetes*, but the molecular ID allows identification to at least the genus level.

- The applications of Polymerase Chain Reaction (PCR) in modern biology are numerous. PCR allows for the exponential replication of a specific sequence of DNA. The application of 16s PCR for our research is to amplify the 16srRNA gene so that it may be sequenced. When sequenced, we may associate sequence characteristics similar to that of known bacteria to determine the genus to which the bacterium is related. The 16s Tiny Earth Colony PCR primers utilize target sequences for amplifying the 16rRNA gene.
  - "16S ribosomal RNA (or 16S rRNA) is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding for it are referred to as 16S rRNA gene and are used in reconstructing phylogenies, due to the slow rates of evolution of this region of the gene. Carl Woese and George E. Fox were two of the people who pioneered the use of 16S rRNA in phylogenetics in 1977."[8]
- The 16s Colony PCR method employs the use of a master mix reagent containing forward primer, reverse primer, polymerase buffer, dNTPs, $MgCl_2$, H_2O and DNA polymerase. The beauty of this method is the ability to inoculate the master mix directly with a small amount of cells picked directly from an isolated colony.
- Due to CoViD-19, we were unable to complete the colony PCR, but HHMI's Virtual Bacterial ID Lab allows us to understand the steps behind the process, from lysing the bacteria, through PCR and sequencing, and finally using NCBI BLAST to determine the phylogenetic relationship of the isolate.



https://media.hhmi.org/biointeractive/vlabs/bacterial_id/index.html

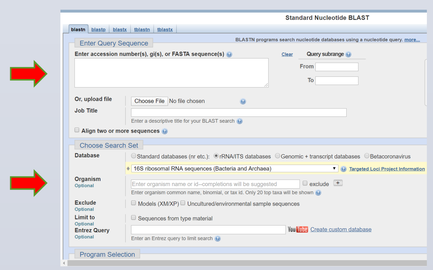


Figure 8: Basic Local Alignment Search Tool (BLAST) is a web-based tool from the NCBI (National Center for Biotechnology Information). A text file (FASTA) is pasted into the top window, and searched against a database of 16S ribosomal sequences.

https://blast.ncbi.nlm.nih.gov/

Conclusions

It is relatively simple to find bacteria in soil and coax them to grow in the lab. It is not so simple to create an axenic culture and discover its identity with biochemical and molecular testing. It is more challenging still to determine if it is a potential antibiotic producer. Often microbes only make antibiotics as a secondary metabolite in certain phases of their growth curves, or only when forced to do so when in competition with other members of their ecosystem. Microbes competing in nature also are under pressure from viruses that control population size and shuttle genetic information around via horizontal gene transfer. Sawyer's mixture of soil microbes showed just this phenomenon: the even growth of bacteria began to show plaques, or evidence of bacteriophage (viral) activity seen in Figure 9. Our work so far has given us intimate knowledge of these difficulties, and we can only continue to try to optimize our protocols to continue the search.

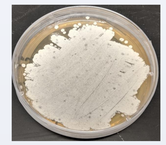


Figure 9: The plate prepared to attempt to make a chemical extract of any potential antibiotics showed evidence of bacteriophage activity. The faint 'holes' in the white growth are plaques, where a virus has lysed the bacterial cells.

Future Directions

The Tiny Earth Network continues to offer new ways to further identify potential producers and study the metabolic networks that exist to create the antibiotic. The Tiny Earth Database is a repository for the microbes found by students in the program, and the Tiny Earth Chemistry Hub will take a proven potential producer and further characterize the antibiotic with regard to its chemical structure. Promising microbes will have their entire genome sequenced. Bacteria are efficient in their regulation of metabolism, and often networks of genes regulated together can be discovered using bioinformatic approaches to search the genome.

The Tiny Earth Network CURE, or Course-based Undergraduate Research Experience, was introduced along with SEA-PHAGES in the Biology curriculum in 2018 as described in volume XXV of the Sextant[9]. The goal of the Tiny Earth and SEA-PHAGES CUREs are to provide an authentic research experience to undergraduate science majors and by doing so, inspiring them to pursue a career in science. CoViD-19 certainly put a cramp in our style this semester with trying to accomplish much in the lab for Tiny Earth, but so far, has supported the Thesis Project of Madison Angelli, and Research Projects for Frankie Cervone and Sawyer Ludwig. Additionally, during the Fall semester of BIO 304, Microbiology and Its Applications, the course home of Tiny Earth @ SSU, Melissa Pimentel submitted to the Tiny Earth Antibiotic Awareness Challenge and won third place in the annual national competition. I look forward to continuing to provide these opportunities for Salem State students for a long time to come.

Figure 10: Award winning entry to the Tiny Earth Antibiotic Awareness Challenge by Melissa Pimentel.

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