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Tiny Earth: Reverse Antibiosis Approach

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Abstract

The Tiny Earth project is a global effort to address the growing antibiotic resistance crisis, and aims to find new antibiotic producing bacteria within soil. The crisis has been attributed to the misuse and overuse of this type of drug. This has produced strains of bacteria that can resist most drugs known today, and a few of the more notable of these bacteria are known as the ESKAPE strains (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species). These pathogens are in the top three threats to global health, and finding new types of antibiotics are paramount to stopping them as well as preventing more like them from emerging. Here, we will cover the results of using a unique technique in finding new antibiotic producers.

Introduction

A nosocomial infection, an infection acquired at the hospital, can be transmitted directly or indirectly between patients, hospital staff, or visitors. With an estimated 1.7 million patients infected with hospital acquired infections (HAIs) in 2002, 99,000 patients died. Meanwhile, in an article from early 2021 the World Health Organization (WHO) stated only 11 new antibiotics had been approved since 2017, and of those only two were novel (meeting a requirement yet unmet).

Prokaryotes are both the most diverse as well as the most abundant form of life on the planet. Within a single gram of soil, it is thought that up to 10 billion cells could be contained, and within 30

grams of rich forest top soil there could be a million species, with up to a billion worldwide. In addition, genome mining indicates that less than 10% of the genetic potential of antibiotic producers is used. Actinomycetota is the phylum known for having the most antibiotic producers in it, and has contributed to each important class of drug used today.

Methods

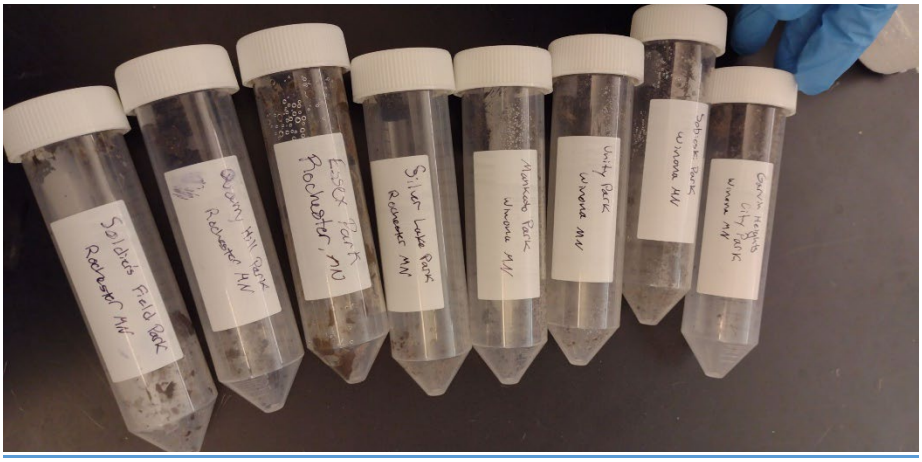


Figure 1

Our process began with the collection of soil samples from eight different public parks within Minnesota. One from each of Winona's Sobieski, Unity, Mankato and Garvin Heights parks (see Figure 1). As well as one from each of Rochester's Silver Lake, Quarry Hill, Soldier's Field and Essex parks for a total of eight locations. Soil was collected in late January when the sky was clear and the ground was capable of excavation. We sought out soil away from nearby trails, where contamination was less of a risk, but near vegetation, where diversity might be larger. Soil was collected with a fresh plastic spoon and placed into a sterile 50mL conical tube to reduce contamination chances. Data including the current date, temperature, and GPS coordinates were recorded. The soil was preserved until ready for use at -18 degrees celsius.

After the collection process, 2L of Reasoner's 2A (R2A) Agar was created using 18.2g agar powder and 2L milliQ water. From this, we poured fifty 100mm dishes and fifteen 150mm dishes. Each 150mm plate was to eventually be used for screening against tester strains, and each 100mm plates were used for determining dilutions or substituting one 150mm plate for two 100mm plate, if needed. We applied cycloheximide topically by sterile spreader after determining roughly how much agar went into each plate (~400uL agar / 1 cm^2 dish, with 56.7cm^2 and 145cm^2 per 100mm and 150mm dish, respectively) and then dissolving 0.2g of cycloheximide into 20mL of sterile water. This was then aliquoted out roughly proportional to the amount of agar estimated to be contained in each plate (150uL and 450uL per 100mm and 150mm plate, respectively). Plates were then left at room temperature on the lab bench for 72 hours to allow the cycloheximide to diffuse throughout the agar.

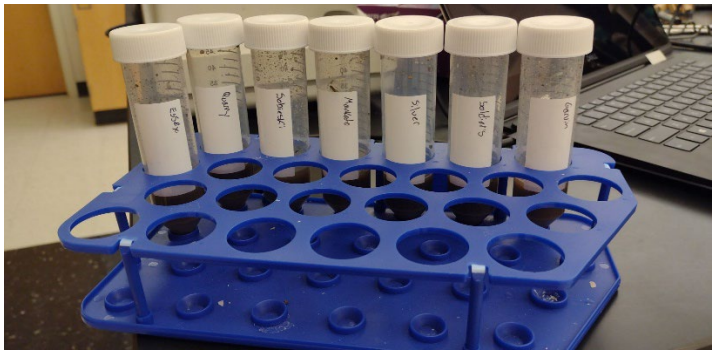


Figure 2

Next, the proper serial dilution was determined. Using the Tiny Earth's manual for guidance, 1g of soil was diluted into 9mL of sterile water and vortexed for 60 seconds (see Figure 2). Dilutions of 1:100 and 1:1,000 were created and 100uL of each were separately applied to sixteen 100mm plates, two for each soil sample, using a sterile spreader. Each plate was labeled for record keeping. The plates were put into a 37 degrees celsius incubator for 72 hours. Afterwards, photos were taken and it was determined that a dilution of 1:100 was the proper dosage (see Figure 3).

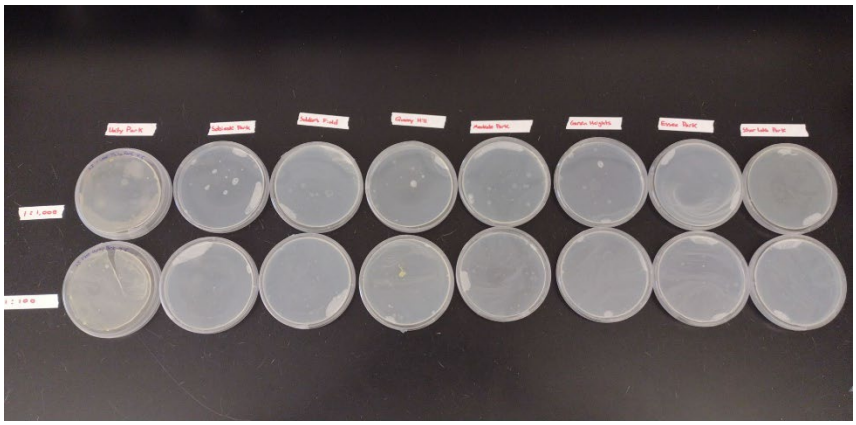


Figure 3

To simplify the use of our agar plates, 17 more 150mm R2A plates were made from 1L of milliQ water and 15.2g agar powder while adding 80mg cycloheximide to the agar solution. This brought us to 32 of the 150mm plates, dividing evenly into four plates per soil location.

Next, all 32 R2A 150mm plates were inoculated, four plates for each location's soil sample at a 1:100 dilution, with 270uL of dilution (scaled up proportionately from 100uL for a 100mm plate) using a sterile spreader. Each plate again labeled and given a number 1-4 to be later assigned to an ESKAPE strain. Plates were incubated at 30 degrees celsius, a temperature more acceptable for slow growing actinomycetes, overnight.

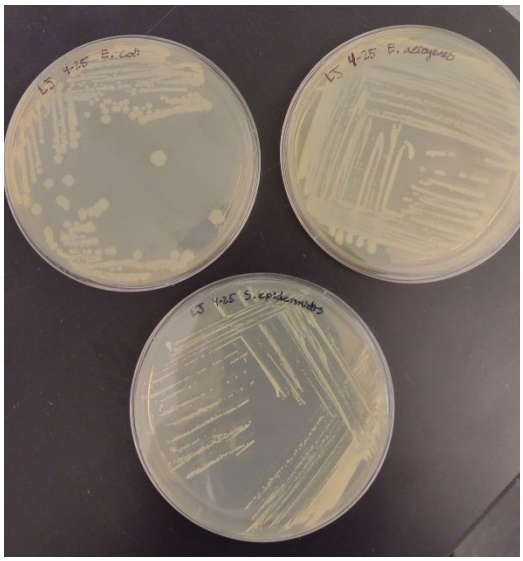


Figure 4

At the same time, ten different ESKAPE relative tester strains were retrieved from the freezer and plated onto tryptic soy broth agar (TSA) plates and grown at 37 Degrees Celsius overnight (see Figure 4). We chose Erwinia carotovora, Bacillus subtilis, Escherichia coli, and Staphylococcus epidermidis as our four ESKAPE relative strains to use for screening as their colonies grew quickly and were available for use the next day.

After retrieving the inoculated R2A plates from the incubator, photos were taken and the plates were wrapped with Parafilm and placed in cold storage overnight. During this time the ESKAPE relative strains were also retrieved, a broth culture was made for each and left to grow on a shaking incubator overnight.

The next day, all R2A plates and ESKAPE broths were collected. The colony forming units (CFU) per gram soil was determined (# of colonies * dilution factor / 0.45mL) per plate using the quadranting technique, and averaged per soil location. Each R2A agar was carefully flipped over, using a sterile metal spatula, upside-down into the lid of its plate so that the bacterial colonies were now pressed up against the plastic. The newly exposed “top” side of the agar (that was previously attached to the bottom side)

was swabbed with a single tester strain using a sterile cotton swab. Four plates per soil and four tester strains meant one plate per tester, per soil. Finally, all 32 plates were left to incubate at 30 degrees celsius overnight again.

Plate-Tester Designation:

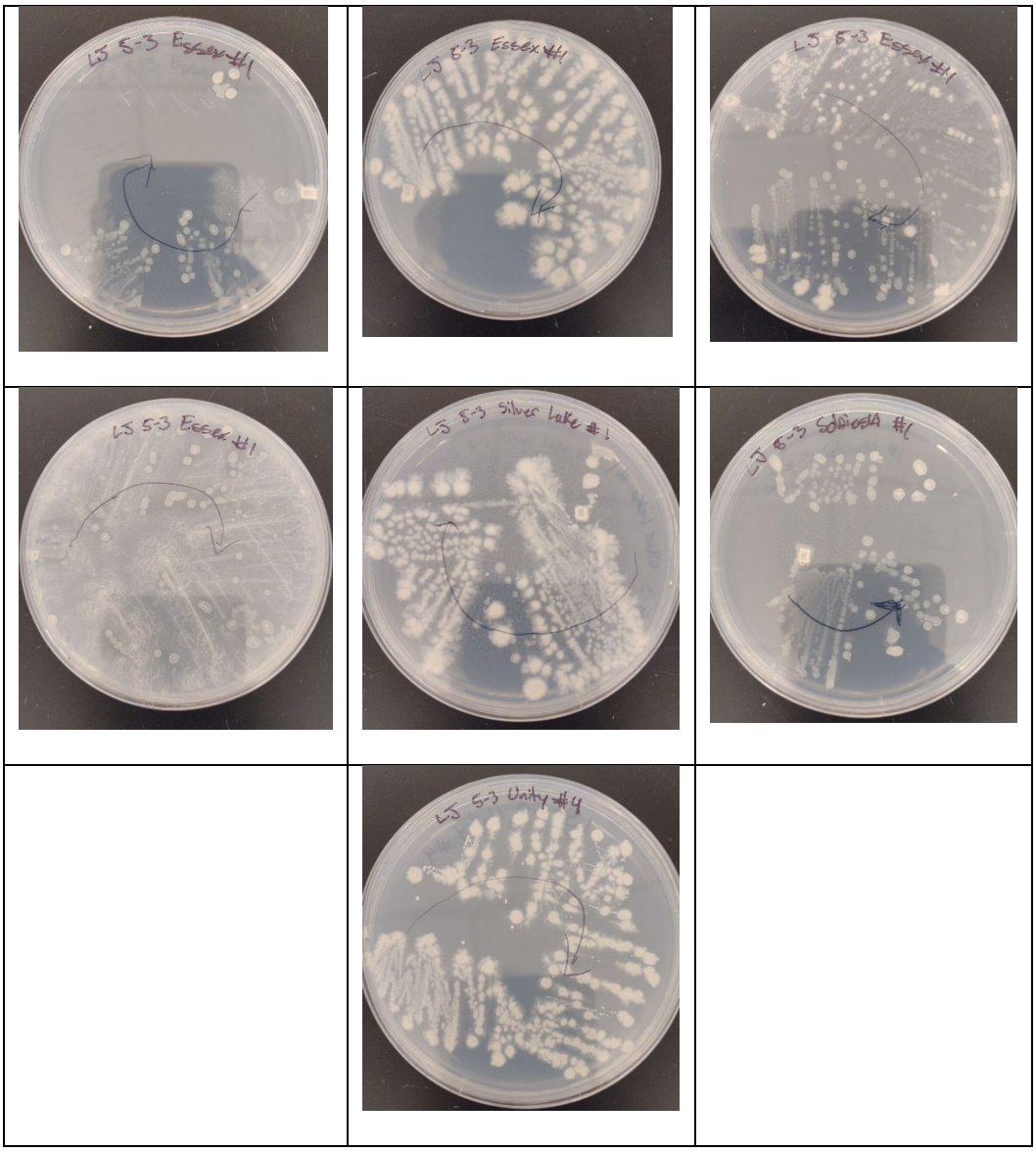
#1 - B. subtilis

#2 - E. carotovora

#3 - E. coli

#4 - S. epidermidis

The following day all plates were again retrieved and photographed. Each was observed for the appearance of zones of inhibition above a bacterial colony within the streaked tester strain indicative of an antibiotic producer. Each candidate was recorded and removed using a sterile plastic micropipette tip that was cut above the tip so that the opening's circumference was roughly that of the colony to be removed (like using a cookie cutter). These columns of agar were transferred to their own R2A plate, streaked for single colonies, and left to incubate at 37 degrees celsius overnight (see Figure 5). All R2A and tester plates were re-wrapped and put back into cold storage. Finally, after 24 hours, the re-streaked colonies were observed and recorded, while isolated colonies were put into a broth culture for potential refreezing.


LJ 5-3 Essex #1
LJ 5-3 Essex #1
LJ 5-3 Essex #1
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LJ 5-3 Silver Lake #1
LJ 5-3 Unity #4

Figure 5

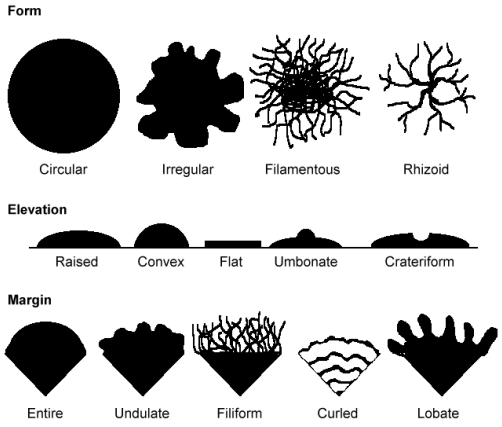


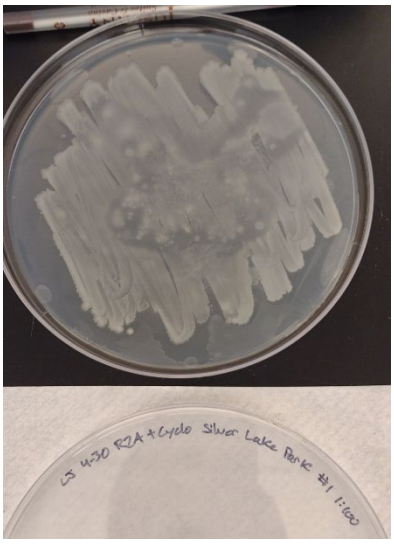
Figure 6

Results

We studied soil colonies from eight different locations, each came from a unique environment with varying vegetation, access to sunlight and water, and possible nearby pollutants. Plating on larger dishes allowed for less risk of overcrowding of colonies. There were three more commonly noticed colony morphologies: circular, irregular, and filamentous. All colonies were white in color, with one greenish exception from the Sobieski #1 plate. Only seven screened plates resulted in a visible halo (see Figure 6).

A table containing all numerical data including total colonies per plate, total producers per plate, hit rate and CFU can be found here. Despite our best efforts, four plates were still overrun by some highly motile filamentous colony. Only four plates produced observable possible antibiotic producers.

Figure 7



Discussion

Actinomycetes are more likely to grow under specific circumstances, and not all of those circumstances were met in this experiment which may contribute to the lack of candidate producers in my results. The flipping technique used proved to be easier to execute than expected, but did still run a risk of contaminating or even destroying the agar and its colonies with it. Furthermore, the extraction method used alongside the flipping technique pulled up a column of agar with the desired colony on one side but the tester strain on the other side. This meant

that re-streaking for a pure colony would have to be done more times, as the first re-streak would still have some tester strain on it which would inevitably inoculate the fresh plate. The idea of diluting the tester strain broth came too late, but may have been helpful in the case of B. subtilis, as it tended to grow very thick very quickly even with just a light swab making it difficult to observe the smaller candidates. There were hopes to create an equation (dubbed “The Joseph Formula”) using our data that would accurately determine the likelihood of finding antibiotic producers in the soil of a given area, but with such scarce producers the accuracy of such an equation may be too low to depend on.

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