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Antimicrobial Properties of an Unknown Microorganism Isolated From the Local Environment

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ANTIMICROBIAL PROPERTIES OF AN UNKNOWN MICROORGANISM ISOLATED FROM THE LOCAL ENVIRONMENT

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ABSTRACT

Bacterial species that cause disease can usually be combatted with antibiotics; but as the years pass, more and more bacterial pathogens are becoming resistant to these treatments. In fact, the Center for Disease Control has identified eighteen classes of bacteria ranging from urgent to concerning threats due antibiotic resistance (2019), warning the advance of an antibiotic resistance crisis in which untreatable bacterial infections will become a leading cause of death (Bennadi, 2014). The Small World Initiative is a program created in 2012 at Yale University to address the antibiotic crisis through a crowdsourcing effort where undergraduate students are encouraged to sample their local environments for antibiotic producing organisms. This study, although it is not coordinated through the Small World Initiative, is inspired by that effort and its methodology. The local environment of Otterbein University in Westerville, Ohio was sampled and a possible antibiotic producer was isolated. The bacterium of interest was characterized using microbiology techniques and identified using molecular genetics and bioinformatics approaches.

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INTRODUCTION

The term “antibiotics” was first used by the microbiologist Selman Waksman to describe molecules produced by microorganisms that have antagonistic effects on the growth of other microorganisms, excluding synthetic compounds and molecules of non-microbial origin from this definition. Today the term is used broadly to represent any molecule that kills or inhibits the growth of bacteria, whether it be synthetically or naturally derived (National Health Society). Antibiotics are produced naturally by microorganisms at levels much lower than the therapeutic dose. They are secondary metabolite molecules that are produced through a multi-step process involving multiple genes (Sengupta, Chattopadhyay, & Grossart, 2013). Some scientists theorize that natural antibiotic production is a bacterial defense mechanism in response to interactions with other bacteria (Clardy et al. 2009). However, Selman Waksman did not agree, stating that the ability of these microbes to produce antibiotics that kill surrounding bacteria under unnatural conditions did not imply such a role existed in nature. Some studies (Clardy et al, 2009) have shown that microorganisms produce these ‘antibiotic’ molecules at very low concentrations, changing the transcriptomes of their neighboring bacteria rather than inhibiting their growth. These findings have provided evidence that what we refer to as ‘natural antibiotics’ are actually signaling molecules that, when produced at very high concentrations, can kill other bacteria.

Antibiotics are natural and have been produced by microorganisms since the early beginnings of life, leading naturally to the evolution of antibiotic resistance in various bacteria. Antibiotic resistance genes were maintained through functional conservation because they act as protection from natural antibiotics produced by pathogenic bacteria. Bacteria which evolved to resist

antibiotics from microbes within their natural community are referred to as intrinsically resistant (Munita & Arias, 2016).

Bacteria exchange genetic material via three mechanisms: transformation, transduction, and conjugation. Transformation is the direct incorporation of naked genetic material into the genome. Transduction involves the mediation of bacteriophages to exchange genetic information between cells. Conjugation is the direct exchange of genetic information from cell-to-cell contact, also known as bacterial "sex". The exchanged DNA is usually in the form of mobile genetic elements such as plasmids or transposons. Typically, antibiotic resistance in the clinical setting is spread through the method of conjugation (Munita & Arias, 2016).

The majority of antibiotics used clinically today are considered broad-spectrum, meaning they are active against both Gram-negative and Gram-positive bacteria, or in general they are active against multiple species of bacteria (Melander, Zurawski, & Melander, 2013). These are useful for empirical antibiotic therapy (cases where bacterial infection is suspected but the pathogenic bacteria has not been identified) or in scenarios where multiple pathogenic bacteria are present. Often, empiric therapy with broad-spectrum antibiotics is suggested in critical situations because the identification of the bacteria causing infection can take one to three days (Leekha, Terrell, & Edson, 2011). However, there are considerable drawbacks to the use of broad-spectrum antibiotics clinically. Since these drugs are non-selective, they will act against a number of bacteria within the host, including non-pathogenic bacteria that are a part of the host natural microbiome. The other major issue is that selection for resistance can occur in not only the infectious bacteria but also the natural bacteria being exposed to the antibiotic, and these bacteria

can become a resource for antibiotic resistance as well (Melander, Zurawski, & Melander, 2013). The other major type of antibiotics are narrow-spectrum, which are only active against a limited number of species of bacteria. Therapy with these antibiotics requires diagnostic testing to identify the pathogenic bacteria present (Melander, Zurawski, & Melander, 2013). Antibiotics may also be classified as bactericidal or bacteriostatic. Bactericidal refers to antibiotics that definitively kill bacteria, while bacteriostatic antibiotics inhibit the growth of bacteria by allowing the host immune system to clear the infection (Loree & Lappin, 2019).

Infectious diseases prevailed as a leading cause of death until the mid 1900s when antibiotics were first commercialized (Cunha, Fonseca, & Calado, 2019). The 1940s to 1970s were the most productive years for antibiotic development and are referred to as the 'golden era' of antibiotic discovery. The first antibiotic research in the 1920s involved studying the effects of synthetic chemicals on the growth of bacteria. During this time period, a scientist by the name of Alexander Fleming began examining a contaminating mold that inhibited the growth of the staphylococcus he had been studying. He identified the mold as a type of *Penicillium*, and his observations led to the discovery of the first natural antibacterial agent, penicillin. Fleming's discovery inspired other scientists to begin sampling their local environment for natural compounds which could inhibit the growth of pathogenic bacteria. In 1939 tyrothricin was the first antibiotic discovered from deliberate sampling of the local soil by scientist Rene Dubo. Many others were soon to follow such as the discovery of actinomycete antibiotic production by Selman Waksman in the 1940s as well as the discovery of streptomycin by a graduate from Waksman's lab, Albert Schatz, in 1943. Systematic soil collection and environmental sampling

became the traditional method of antibiotic discovery with pharmaceutical companies instilling programs to increase soil sample collection around the world (Dougherty & Pucci, 2012).

Very few antibiotics have been developed within the last two decades. Experts identify global lack of funding as one of the biggest challenges facing the antibiotic crisis today (Piddock, Garneau-Tsodikova, & Garner, 2016). ~~This can be attributed to a recent change in attitude from pharmaceutical companies on antibiotic discovery leading to a major decrease in funding toward infectious disease treatments.~~ Between 2003 to 2013, 38 billion USD of venture capital were invested into pharmaceutical developments, of which only 1.8 billion USD went toward development of antimicrobials, which is less than 5% (O'Neill, 2016). Some antibiotics currently used in the clinical setting are stored and saved as last resorts for patients with infections resistant to common therapies. This is in effort to delay resistance to these antibiotics for as long as possible. However, this is not ideal for pharmaceutical companies with the priority of high sales and income. Additionally, the treatment period for antibiotics is short, making them less profitable than long term treatments. The reality of antibiotic resistance only makes commercial antibiotic development even less appealing (Sukkar, 2013). This problem is demonstrated through the net present value (NPV) of developing antibiotics, which is currently estimated to be negative $50 million. According to the EU Office of Health Economics, for antibiotics to be competitive with other therapeutics in terms of profitability they would need to have a NPV of around $200 million (Renwick, Brogan, & Mossialos, 2015).

Antibiotic resistance is a growing problem within the healthcare industry. Bacterial species that cause disease can usually be combatted with antibiotics; but as the years pass, more and more

bacterial pathogens are becoming resistant to these treatments. In fact, at least three life-threatening species are already resistant to all known antibiotics (Levy, 1998), warning the advance of an antibiotic resistance crisis. Already an estimated 35,000 deaths a year in the United States alone can be attributed to bacterial infections that cannot be treated (Center for Disease Control, 2019), and it is estimated that by 2050 the number of global deaths related to antibiotic resistance will reach 10 million per year (O'Neill, 2016).

Overuse and misuse of antibiotics as treatment contribute to the increase in resistant bacteria. Antibiotics are overprescribed and quite often patients self-medicate using leftover antibiotics when they are feeling ill (Bennadi, 2014). Exposure to these antibiotics can increase the reservoir of resistance genes. Each emergence of resistant bacteria provides a possibility for a new totally resistant pathogen, putting today's public health at the edge of a global crisis in which bacterial infections will become a major cause of death. Therefore, there is an extensive and urgent need for novel antibiotic treatments.

In 2012, Dr. Jo Handelsman of Yale University decided to address the antibiotic resistance epidemic as well as the STEM deficit by kick-starting the Small World Initiative. This is a crowdsourcing effort intended to encourage students to pursue careers in STEM through hands-on experience attempting the traditional method of antibiotic discovery; swabbing and sampling their local environments and isolating antimicrobial-producing bacteria that could lead to novel antibiotics. Over 275 undergraduate programs participate, but the initiative is still in its early stages (smallworldinitiative.org). In June 2018, Dr. Handelsman started another program called Tiny Earth with the aim of replacing traditional microbiology laboratory courses with research-

based courses to “studentsource” the discovery of novel antibiotics. Versions of Tiny Earth courses are available in 15 different countries and almost 10,000 students are participating, performing the same soil sampling methodology that was used during the golden era of antibiotic discovery (tinyearth.wisc.edu).

This study, while it is not specifically coordinated through the Small World Initiative nor the Tiny Earth program, is inspired by that same effort and its methodology. The local environment at Otterbein University in Westerville, Ohio is investigated for bacteria displaying antimicrobial properties, and the microorganisms of interest are characterized and identified using microbiology and molecular genetics approaches as well as bioinformatics.

METHODS

Bacterial Strain Isolation

The local environment of Otterbein University in Westerville, Ohio was sampled for bacterial species. A sample of environmental bacteria was obtained using a sterile swab on the main door of the first floor of Otterbein's Science Building near the atrium. This sample was grown on trypticase soy agar (TSA) at 37°C for about 3 days, ~~so colonies on the plate appeared larger than usual~~. One microorganism in particular was very obviously inhibiting the growth of other bacteria. This specific strain of interest, which was labelled as JH1, was isolated as a single colony by dilution streaks onto TSA plates. A colony was picked to produce a nutrient broth culture in a test tube. One ml of culture and 700µl of 50% glycerol were added to a freezer tube and frozen at -80°C for storage. A second, presumably clonal stock was made from a second colony.

Characterization

A series of microbiology techniques were used to initially characterize JH1. JH1 was streaked on multiple variations of media including nutrient agar (NA) and TSA. A gram stain was performed to categorize JH1 as gram negative or gram positive. Gram positive bacteria have thick peptidoglycan walls that crystal violet will adhere to, while gram negative bacteria have a more complex cell wall with a thin layer of peptidoglycan that is surrounded on the outside by an outer membrane and on the inside by a periplasmic space. First, a sample of bacteria was heat fixed to a slide using a Bunsen burner. Crystal violet was used as a primary stain, which was followed by Gram's Iodine as a mordant, creating a crystal violet-iodine complex in both Gram-negative and

Gram-positive strains. Ethanol was used to decolorize, which will result in the loss of the crystal violet-iodine complex in Gram-negative bacteria, but not in Gram-positive bacteria due to their thicker peptidoglycan walls. Safranin was used as a counterstain to visualize cells in which the primary crystal violet was removed. The slide was rinsed with water and then observed under a light microscope at 1000x. The color of the observed bacteria indicates the gram variability.

Observation under light microscopy was used to determine the shape and arrangement of the bacterial cells, which can be used as additional information in the identification of the microorganism.

Some types of bacteria when introduced to environmental stress or nutrient deprivation will produce dormant, metabolically inactive structures called endospores as a survival technique. Staining procedures with malachite green were used to determine whether JH1 produces endospores. In order to test for this ability, cultures of JH1 were incubated at 37°C for three days instead of one to induce a nutrient deprived environment. Colonies from this sample were heat fixed to a slide and covered with blotting paper. The paper was saturated with malachite green and steamed over boiling water. This heat is necessary for the malachite green to adhere to the endospores because the spore coat is made of keratin-like protein and is highly resistant to staining. The slide was rinsed and counterstained with safranin, then visualized under a light microscope at 1000x total magnification.

Assessment of Antagonist Activity

JH1 was plated against common well-studied Gram-negative and Gram-positive bacteria. Specifically, its activity was studied against the pathogenic bacteria *Escherichia coli*, *Serratia marcescens*, *Micrococcus luteus*, and *Staphylococcus epidermidis*. Bacterial lawns were created of each on TSA plates, and JH1 was streaked in a singular thin line down the center of each plate. Bacterial lawns were created using sterile applicators in a cross-hatching pattern. ~~This process was repeated multiple times due to varying success among lawn creation.~~ The plates were incubated for 1-2 days at 37°C to allow time for sufficient bacterial lawn formation. Plates were then assessed for the presence of a zone of inhibition, indicating antagonistic activity of JH1. This process was repeated using similar technique but with an increase in JH1 streak thickness in order to assess antimicrobial activity at higher concentration. In this case, JH1 was tested against *Staphylococcus aureus* instead of *Staphylococcus epidermidis* due to availability.

Molecular Methods

Molecular methods were used to identify the taxonomy of the strain. Specifically, 16S rRNA gene amplification followed by Sanger sequencing was chosen. 16S rRNA is a part of the 30S small ribosomal subunit in the 70S prokaryotic ribosome. This section of the genome is commonly sequenced in the identification of pathogenic bacteria because it is highly conserved yet can contain sufficient variation to serve as a phylogenetic marker.

The JH1 strain was suspended in LB broth in 200mL flasks that were shaken overnight at 37°C. These flasks were then pipetted into conical tubes that were centrifuged at 4000rpm for seven minutes to create pellets. Next, the Wizard Genomic DNA Purification Kit (Promega) was used

for chromosome preparation, specifically using the Gram-negative protocol. Eppendorf tubes containing the purified DNA were rehydrated overnight at 4°C. Presence of chromosomal DNA was confirmed through gel electrophoresis of the samples against a 1kb ladder at 120V, followed by UV visualization.

After chromosome confirmation, four polymerase chain reactions (PCR) were run using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT -3') primers commonly used for 16S rRNA sequencing. PCR success was confirmed through gel electrophoresis at 120V against a 1kb ladder. Successful PCR is indicated by the presence of DNA bands around 1,500 base pairs. The concentration of these four samples was then evaluated using the NanoDrop One Spectrophotometer (Thermo Scientific). PCR product purification was attempted using the QIAquick PCR Purification Kit (Qiagen), however this process was continually unsuccessful as the products were over-diluted and not concentrated enough to be sent for sequencing. The PCR products were subsequently sent to Eurofins for sanger sequencing with the added service of purification.

Sanger sequencing involves a polymerase chain reaction using deoxynucleotides that commonly make up DNA, as well as dideoxynucleotides, which are specialized to terminate the reaction after addition. Each of the four possible types of dideoxynucleotides, which correspond to adenine, thymine, guanine, or cytosine, are tagged with a different color of fluorescent dye. Sanger sequencing will result in extension products that are fluorescently labeled and that vary in length, depending on when the reaction was terminated. These extension products are separated by capillary electrophoresis, which will categorize the fragments by size and excite the

fluorescently labeled DNA fragments to detect their color as they pass. A sensor detects the fluorescence signal and records it as a base call in an electropherogram.

Successful sanger sequencing data was identified by the presence of clear and individual peaks in the electropherogram data. The most highly successful sequences were evaluated and assembled using the program CodonCode Aligner. Successful sequences with incomplete ends were edited to remove incomprehensive data. An initial consensus sequence was obtained using CodonCode Aligner and aligned using the Nucleotide Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

In order to identify the species, more of the genomic sequence was required. To do this, custom DNA oligos were created using the initial consensus sequence as a template. These were designed to extend from the middle of the sequence outward, therefore obtaining the peripheral sequences that the polymerase did not anneal to in the initial sanger sequencing reaction. Custom oligos were ordered from Integrated DNA Technologies. These are referred to as 590R (5'-ACTCAAGTTTCCCAGTTC-3') and 906F (5'-GTTTAATTCGAAGCAACG-3').

The chromosomal preparation protocol was performed again and confirmed with gel electrophoresis and UV visualization. PCR was done using the same 27F and 1492R primers that are specific to the 16S rRNA part of the genome, and the success of PCR was confirmed through gel electrophoresis and UV visualization once again. The custom designed oligos, along with additional 27F primers, were rehydrated and diluted to be sent to Eurofins with PCR products to be used for Sanger sequencing. Sequencing data from the second round of sequencing was

assembled in conjunction with the initial sequencing data using the bioinformatics program Geneious Prime to create a longer consensus sequence. This sequence was aligned again using BLASTn from NCBI.

RESULTS

Identification of Primary Characteristics of JH1

The initial TSA plate on which JH1 was discovered included multiple strains of bacteria isolated from the environment through sterile swab. The bacteria corresponding to JH1 was obviously inhibiting the growth of nearby bacteria as indicated by the presence of a zone of inhibition. JH1 was isolated for individual study. Growth of JH1 onto agar plates revealed multiple characteristics of its colony morphology. The shape of individual colonies are circular. The elevation of the bacteria is slightly raised and margins are entire (smooth). The color of colonies is an off-white yellow, and the texture is smooth and moist (Figure 1). This is consistent when plated on multiple types of agar, including basic NA and TSA.

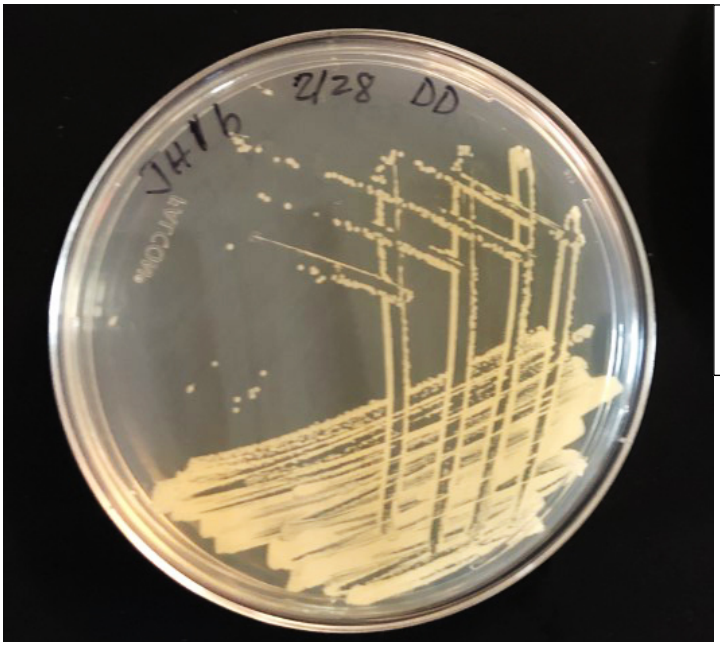

Figure 1. JH1 grown on TSA agar at 37°C for 3 days. The quadrant streaking method was used so that individual colony formation would be available for characterization.

The results of Gram-staining revealed that JH1 bacterial cells stained pink, indicating Gram-negative bacteria. Visualization under the brightfield microscope also revealed a bacillus (rod) - shaped bacteria that does not appear to be arranged in staph or strep formation (Figure 2a). The results of the endospore staining reveal both pink and green stained structures (Figure 2b). The

pink structures are vegetative cells and the green-stained spheres are endospores. JH1 can therefore be categorized as an endospore-forming species with bacillus-shaped cells.

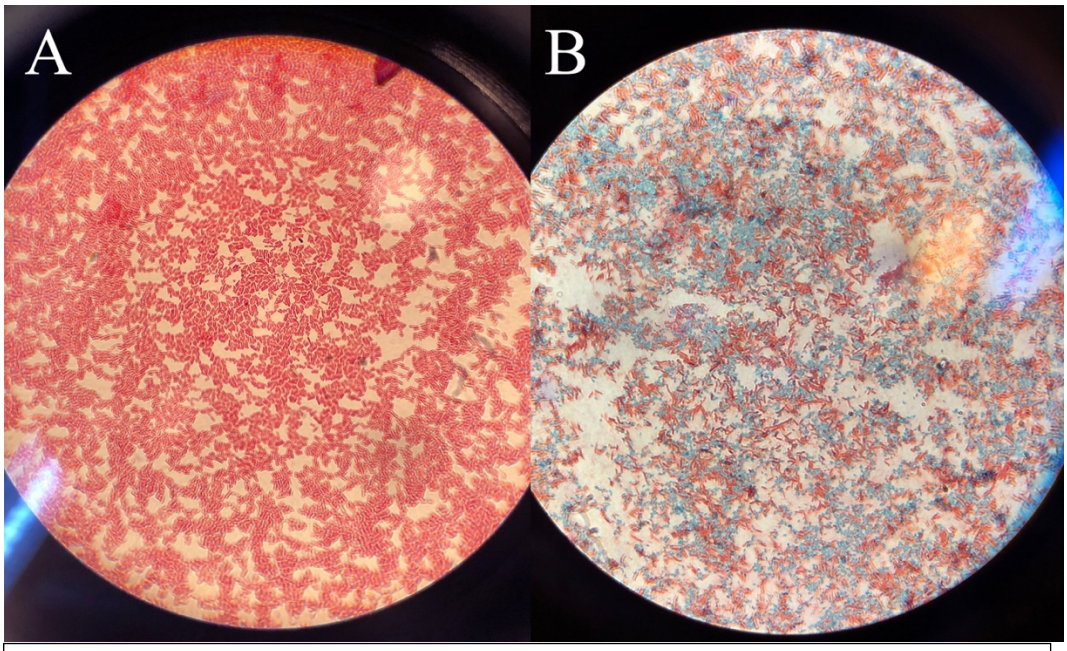

Figure 2. JH1 under brightfield microscopy at 100X total magnification after **A.** Gram staining and **B.** endospore staining procedures.

Variable Antagonistic Activity Dependent on Concentration

The initial results of streaking JH1 in a thin line against multiple pathogenic strains showed variable antagonistic activity. A zone of clearing is present against *M. luteus* (Figure 3a) and *S. epidermidis* (Figure 3b) in the first trial. In the second trial, a zone of clearing is again visible against *M. luteus* (Figure 3a), but this time there is no zone of clearing against *S. epidermidis* (Figure 3b) and a zone of clearing was present against *S. marcescens* (Figure 3c). No zone of clearing is present against *E. coli* in either trial (Figure 3d). The success of lawn creation was slightly inconsistent, most likely due to the concentrations of stock bacteria being sampled. The

most successful lawn creations were created by *S. marcescens* and *E. coli.* The inconsistencies in lawn creation were addressed through the attempt of multiple trials.

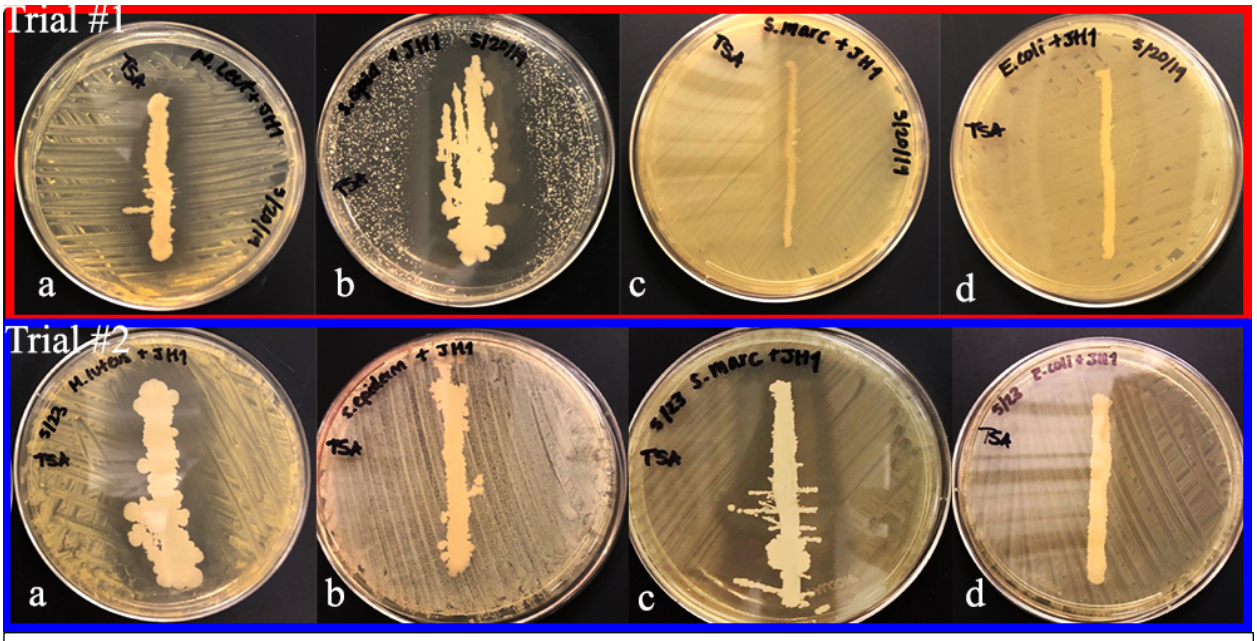

Figure 3. Two trials of streaking JH1 on TSA agar against **a.** *Micrococcus luteus*, **b.** *Staphylococcus epidermidis*, **c.** *Serratia marcescens*, and **d.** *Escherichia coli*. Plates were incubated for 1-2 days at 37°C. Multiple trials were done to address varying success among lawn creation and to confirm results.

Since the results of these trials were so variable, the plates were analyzed for possible irregularities in conditions. The ability for JH1 to inhibit the growth of other bacteria seemed to be present when streaks of JH1 were thickest. This hypothesis led to continued testing with purposefully thicker streaks of JH1 to assess antimicrobial activity at higher concentrations. Results of streaking JH1 in a thicker line against pathogenic strains showed higher antagonistic activity than under the previous conditions. Zones of clearing are evident against all four types of bacteria in this trial, although minimal against *E. coli* (Figure 4). The zone of clearing against *S. marcescens* is somewhat dim, which may indicate partial inhibition of growth (Figure 4c).

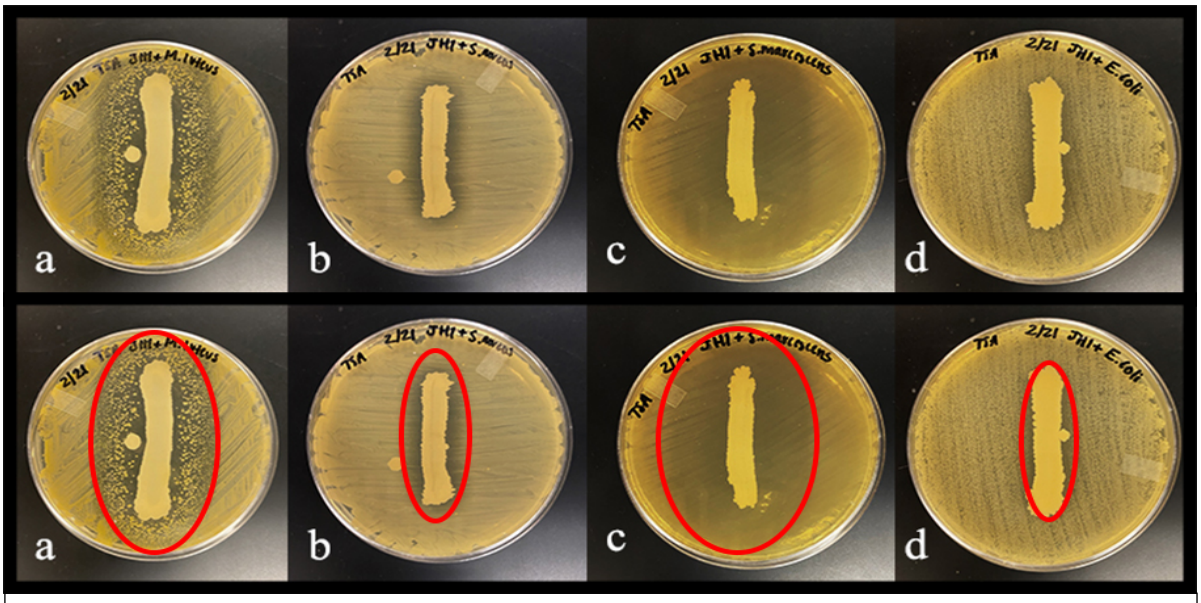

Figure 4. JH1 streaked in purposefully thicker lines onto lawns of **a.** *Micrococcus luteus*, **b.** *Staphylococcus aureus*, **c.** *Serratia marcescens*, and **d.** *Escherichia coli*. Lawns were grown on TSA agar for 1-2 days at 37°C. Red eclipses have been added to clarify zones of clearing in photos.

Identification of *Bacillus* Species

After DNA isolation using the Wizard Genomic DNA Purification Kit, the presence of chromosomal DNA was confirmed through gel electrophoresis against a 1kb ladder. UV visualization of this gel revealed a singular band with high intensity that remained relatively high on the gel. This is consistent with the size of chromosomal DNA being large and therefore inhibiting the ability to travel through the gel. With this confirmation, PCR using the 27F and 1492R primers could begin. PCR was done in quadruplicate to produce four products and maximize the possibility of success.

UV visualization of this gel revealed that the four bands corresponding to PCR products had migrated to equal distance, indicating equivalent length of fragments. The fragments align with the 1kb ladder at close to 1500 base pairs (Figure 5), which is consistent with the length of fragment expected using 27F and 1492R primers for 16S rRNA sequencing.

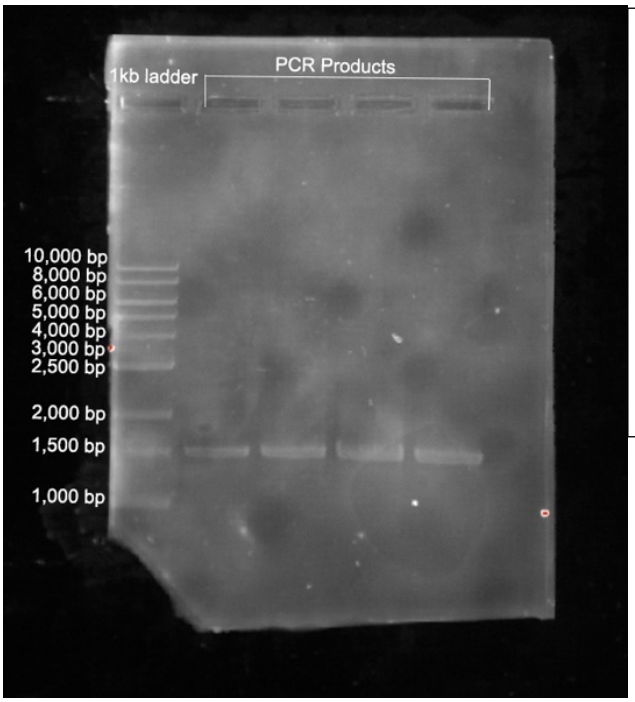

Figure 5. UV visualization of gel electrophoresis of initial PCR products using 27F and 1492R primers against a 1kb ladder. PCR was done in quadruplet to increase probability of success. PCR products are aligned and travelled to the area of gel corresponding with 1500 bps.

Electropherograms from the four PCR products using both 27F and 1492R primers were obtained, resulting in eight sequences of varying success. The electropherograms were evaluated and six out of eight sequences were productive enough for use. Both one sample using 27F and one using 1492R primers were unsuccessful and therefore not used in the creation of the consensus sequence. CodonCode Aligner was used to assemble an initial consensus sequence shown below (Figure 6). Results of using BLASTn from NCBI showed over 99% alignment with multiple *Bacillus* species, including *Bacillus pumilus*, *Bacillus safensis*, and *Bacillus zhangzhouensis*. In order to obtain more of the sequence and identify the species, custom oligos were then designed using common primer design rules. These primers were specifically designed to obtain the peripheral sequences where the polymerase may have been just attaching or not yet attached. The primers annealed to sequences in the middle of the sequence and extended outward, and are referred to as 590R and 906F.

Figure 6. Initial consensus sequence from primary sequencing data using 27F and 1492R primers with highlighted target sequences for new custom primer annealing. The yellow highlight indicates placement of the reverse primer, 590R, and the green indicates placement of the forward primer, 906F.

Chromosome purification was once again performed and confirmed using gel electrophoresis and UV visualization. Results of this visualization were consistent with the earlier extraction. PCR with 27F and 1492R primers was repeated in quadruplet again, with confirmation by gel electrophoresis and UV visualization producing results equivalent of earlier trial (Figure 5). The four bands all travelled to the area of the gel corresponding to approximately 1500 bps, indicating success of PCR in preparation for 16S rRNA sequencing. Eurofins sanger sequencing produced electropherograms from all four PCR samples using 27F, 590R, and 906F primers for a total of twelve sequence results. A combination of fourteen sequences, with data from both the initial and second round of sequencing, were considered successful enough to be used in the assembly of a revised consensus sequence. An example of a successful electropherogram is provided in Figure 7.

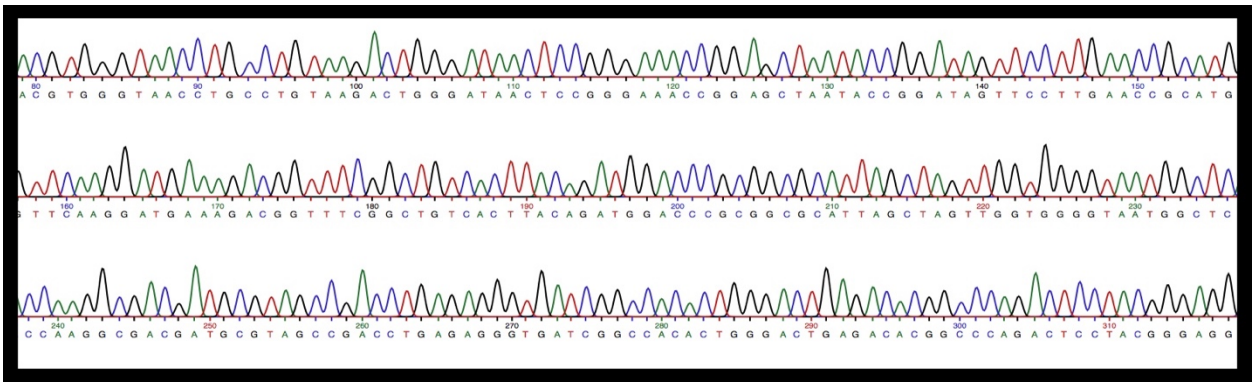

Figure 7. An example of successful electropherogram data from sanger sequencing using the 27F primer. Clear and smooth peaks are indicative of successful sanger sequencing. Colors correspond to nucleotides, with guanine being represented by black, thymine represented with red, adenine represented with green and cytosine represented with blue.

The revised consensus sequence was aligned using the bioinformatics tool Geneious Prime MAFFT plugin, with default settings and automatic algorithm selection. The results show 99.5% pairwise percent identity to the species *Bacillus pumilus*, with high alignment to multiple samples labelled as "Uncultured *Bacillus*" as well. This alignment is shown in Figure 8.

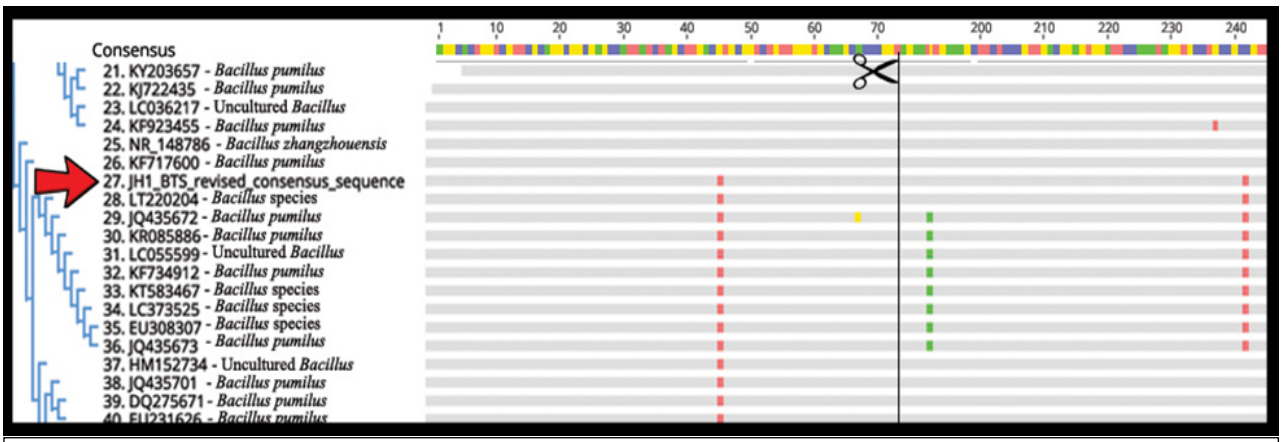

Figure 8. Screenshot of analysis using the bioinformatics tool Geneious Prime showing alignment of revised consensus sequence to known bacterial sequences in the NCBI database. Scissors indicate the area of sequence where all samples aligned that was removed for demonstration purposes. Colored markers indicate variations in sequence.

DISCUSSION

Visualization under the microscope clearly displays the bacillus shape of JH1 cells, making it likely that the sequence belongs to the genus *Bacillus.* The result of pink cells after gram staining indicate JH1 as a Gram-negative bacteria. While most species within this genus are Gram-positive, Gram-variable *Bacillus* have been reported (Long, Prober, & Fischer, 2018) as well as Gram-differentiation through the aging process. The presence of green stained cells after treatment with malachite green indicates endospores, identifying JH1 as an endospore forming bacteria. Many *Bacillus* species can be identified as endospore-forming, further supporting our isolated bacterium as a species of *Bacillus* (Turnbull, 1996).

JH1 displayed variable antagonistic ability when streaked in a thin line against SR strains. A closer look at these plates reveals that zones of inhibition are present when the streak of JH1 is thicker. This hypothesis was tested by streaking JH1 in purposefully thicker lines against pathogenic strains. Thicker streaks displayed much higher incidence of antagonistic activity, as all plates exhibit at least some zone of inhibition with this method. These results indicate that the number of JH1 cells leading to a higher concentration of antibiotic production may be crucial in its antagonistic abilities, especially against specific strains of bacteria such as *E. coli.*

The first round of sanger sequencing provided multiple successful sequences for assembly and alignment. However, using only this data for nucleotide BLAST, the sequence aligned with multiple *Bacillus* species. This is why custom oligo design to obtain more of the sequence was necessary. The resulting consensus sequence using both the initial sequencing data as well as the

data from using custom primers showed much more specific alignment after nucleotide BLAST. The JH1 sequence was most similar to *Bacillus pumilus*, with an aligned percent similarity of 99.5%, and was highly similar to sequences referred to as '*Bacillus* species' and 'Uncultured *Bacillus*'. These are likely representative of unspecific *Bacillus* cultures that may be *B. pumilus*. However, due to the high genetic homogeneity within *Bacillus* subgroups, it is often problematic to differentiate between species using solely 16S rRNA sequencing. This is especially true for the clade that *B. pumilus* belongs to known as the *B. subtilis* group (Liu et al. 2013).

While *B. pumilus* is ubiquitously found in soil, it ha also been found in marine environments. Although the microbial contents of aquatic environments remain an area for continuous study, preliminary studies on the content of coastal environments in China have found *B. pumilus* to be the predominant *Bacillus* species present (Parvanthi et al. 2008). With this in mind, it is possible the *B. pumilus* found on the main door of the science building on the first floor near the atrium may have originated from the aquatic environment of the turtle tank located in the greenhouse that is also on the first floor of the building. Otherwise, it may have originated from local soil and travelled on someone's hands into the building.

Recent studies have identified *Bacillus* species that produce antimicrobial substances in the form of proteins or lipoproteins (Abriouel et al. 2011). *Bacillus pumilus*, while new to the literature on antibiotics, has been found to produce fermentation fluids containing antibiotic compounds. In 2019, three antibiotic compounds were identified using silica gel column, liquid chromatography, mass spectrometry and nuclear magnetic resonance. These compounds include the fatty alcohols 3,4-dipentylhexane-2,5-diol, 1,1'-(4,5-dibutylcyclohexane-1,2-diyl)bis(ethan-

1-ol), and 1,1'-(4,5-dibutyl-3,6-diethylcyclohexane-1,2-diyl)bis(ethan-1-one). All three of these compounds have broad spectrum activity and when tested were effective against eight different pathogenic bacteria. This suggests *B. pumilis* could produce a "new generation" of antibiotic compounds that are not protein-based (Chu et al. 2019).

Future directions for this project include the continued sampling of the environment and assessment for inhibition of growth indicative of antimicrobial compounds, as well as continued research into the antibiotic activity of *B. pumilus*. With the threat of an antibiotic crisis hanging overhead, it is essential that effort is put into the study of antibiotic compounds, both naturally and synthetically derived. As shown through this study which identified a local antibiotic producer, programs such as the Small World Initiative and Tiny Earth are models for antibiotic discovery.

ACKNOWLEDGMENTS

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