DISCOVERY OF SOIL BACTERIA WHICH DISPLAY BROAD-SPECTRUM ANTIBIOTIC ACTIVITY

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Abstract

Antibiotic-resistant ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species*) are the leading cause of hospital-acquired infections, and thus, the discovery of novel antibiotics that kill them is crucial. Soil collected from Palm Beach County by undergraduates in Life Science Lab (RI:BSC1005L) contained multiple bacterial isolates with broad spectrum antibiotic activity. Soil bacteria that kill the safe relatives of the ESKAPE pathogens and safe models (*Erwinia carotovora, Bacillus subtilis and Mycobacterium smegmatis*) were characterized by microbiology assays and bioinformatics to determine their identities. Antibiotic compounds were extracted from four bacteria that kill both Gram-negative and -positive safe relatives; these bacteria were determined to be Gram-negative and 83-97% homologous to various *Pseudomonas* species.

Introduction

Antibiotic resistance is recognized as one of the most significant health threats to the human population; two million Americans suffer from bacterial infections acquired from hospitals every year (Infectious Diseases Society of America). Patients infected with multi-drug resistant (MDR) bacteria are subjected to more harmful, last-resort drugs and the cost of curing these patients amounts to over $30 billion annually (Davis et al., 2017; Infectious Diseases Society of America). Pharmaceutical companies have eluded funding new antibiotic research because antibiotics are less lucrative than long-term use drugs (Davis et al., 2017). Studies have shown the direct relationship between inappropriate antibiotic usage and new resistant bacteria (Ventola, 2015a). The overuse of antibiotics is the main cause of antibiotic resistance because treatment only kills a majority of the harmful bacterial cells and leaves the stronger cells to reproduce (Ventola, 2015a). MDR bacteria have been plaguing US healthcare and US citizens since penicillin was introduced in 1943 (Davies & Davies, 2010; Ventola, 2015a); it has become imperative that not only patients, but also doctors, understand the importance of the correct use of the appropriate antibiotics (Ventola, 2015).

New classes of antibiotics with new mechanisms of action need to be discovered to replace the current drugs that are ineffective (Sautter & Halstead, 2018). The urgency to find new antibiotics has led to the investigation into soil bacteria that produce secondary metabolites capable of killing neighboring bacteria. Soil is considered a microbe "hot spot" to find antibiotic producing isolates (Lau, van Engelen, Gordon, Renaud, & Topp, 2017; Rafiq et al., 2018). Additionally, the lack of chemical diversity of known antibiotics may be due to the limits of the microbes currently being utilized for their antibiotic compounds (Rafiq et al., 2018). While different antibiotics (e.g. erythromycin and vancomycin) are isolated from the same genus of bacteria (e.g. *Streptomyces*), soil can be seen as an infinite source of novel antibiotic producing microbial genera (Rafiq et al., 2018).

At Florida Atlantic University (FAU), the Biological Sciences Department has recently integrated Tiny Earth (TE) into its curriculum. TE "studentsources" the discovery of new antibiotics through research experience-based courses for undergraduates (Tiny Earth, 2018). At FAU, students with non-STEM (science, technology, engineering and mathematics) majors conduct TE lab experiments in an Intellectual Foundations Program course, Life Science Labs (RI:BSC 1005L). Students are given a soil collection kit and collect soil, reporting the GPS coordinates and the location description (i.e. light exposure, type of soil, etc.). Students isolate the bacteria from the soil by step-wise diluting soil in water and putting dilutions onto plates of bacterial food (media). Each student chooses one of sixteen growth conditions (including variations in food, temperature and light exposure) for their bacteria. Different growth conditions affect the bacterial biodiversity that can be cultured in the lab and the secondary metabolites (e.g. antibiotics) that the bacteria produce. Students perform antibiotic assays against the safe relatives/models. Traditional microbiology assays are performed on antibiotic-producing bacteria to classify them based on common structures, physiology and biochemistry. DNA analysis is done to determine phylogenetic relationships. The students preserve their bacteria at -80°C for future experimentation.

TE was started at Yale University in 2012 by Dr. Jo Handelsman in an effort to inspire upcoming scientists, as well as address the dwindling number of effective antibiotics by exploring her passion, soil microbes (Tiny Earth, 2018). TE was implemented to change the way students participate in science. Now, TE is comprised of over 275 different schools across 40 states and fourteen countries (Tiny Earth, 2018). Hands-on experiments with relevant work, such as the global antibiotic crisis, help shape a new appreciation for non-STEM students of research conducted in academia. Through the use of citizen science, TE has been able to attain bacterial isolates from all over the world in an effort to discover new antibiotics from its participants' own backyards. There is plenty of opportunity to discover a new antibiotic in the soil; over two thirds of known antibiotics originate from soil bacteria (Hernandez, Tsang, Bascom-Slack, & Handelsman, 2016a).

During the first four semesters of TE at FAU, hundreds of antibiotic-producing bacteria were isolated and preserved by undergraduates. For the current study, the bacteria found in the 2016-2017 school year were revived from -80°C and all of the students' experiments were repeated. Antibiotic activity assays were also repeated and each bacteria was assigned an "antibiotic activity score" based on the number of safe relatives/ models (0 – 9) that they kill. One hundred bacterial isolates with the highest antibiotic activity scores were then passed to the microbiology teaching lab students to perform additional microbiology assays. Four of these bacterial isolates, CJB7, KTG3, TMV16 and TMV21, with antibiotic activity scores of 8 or 9, were selected for additional analyses, including performing organic extractions, assaying the antibiotic activities of the different extraction layers and farther purification/ structure elucidation of antibiotic compounds.

Methods

Overview: At FAU, each TE-Life Science lab student retrieves a soil sample from Palm Beach County and isolates bacteria. These bacteria are then tested against the six safe relatives of the ESKAPE pathogens

(see Table 1).

Table 1: ESKAPE Pathogens and Their Corresponding Safe Relatives (With Abbreviations)

ESKAPE Pathogen	Safe Relative/Safe Model
Enterococcus faecium	*Enterococcus raffinosus (E. raff)*
Staphylococcus aureus	*Staphylococcus epidermidis (S. epi)*
Klebsiella pneumoniae	*Escherichia coli (E. coli)*
Acinetobacter baumannii	*Acinetobacter baylyi (A. bay)*
Pseudomonas aeruginosa	*Pseudomonas putida (P. put)*
Enterobacter species	*Enterobacter aerogenes (E. aero)*
Safe Model Organism	*Erwinia carotovora (E. caro)*
Safe Model Organism	*Bacillus subtilis (B. sub)*
Safe Model Organism	*Mycobacterium smegmatis (M. smeg)*

The students perform microbiology assays and DNA analysis to identify the genus of antibiotic-producing bacteria. In this study, frozen stocks of all antibiotic-producing bacteria found in the 2016-2017 school year were revived and grown on conditions (chosen by the Life Science students) to verify their data. Experiments by the FAU-TE research lab validated results for: antibiotic production (assigning antibiotic scores, see the introduction), Gram staining, MacConkey agar assays, catalase production, sulfide production, indole production and motility. One hundred bacterial isolates with the highest antibiotic activity scores were given to the undergraduate microbiology teaching lab students enrolled in MCB 3020L to perform the phenylethanol plate, Litmus milk and phenol red broth assays. The FAU-TE research lab chose four bacteria, the CJB7, KTG3, TMV16 and TMV21 bacteria, with the highest antibiotic activity scores, to perform ethyl acetate/water extractions to learn more about the bacterial compounds with antibiotic activity. All media and reagents were purchased from Fisher Scientific except where noted. Protocols were performed and/or modified as previously described (Hernandez, Tsang, Bascom-Slack, & Handelsman, 2016b; Scheurle, 2016).

Growing Bacteria: The CJB7, KTG3, TMV16 and TMV21 bacteria were assigned the highest antibiotic activity scores (8 or 9) and were considered the most important to study. The growth conditions that were originally chosen by the Life Science lab students were used to duplicate bacterial growth and production of the same secondary metabolites. The CJB7 and KTG3 bacteria were grown on Potato Dextrose Agar (PDA) and incubated under continuous light exposure at 25°C. The TMV16 and TMV21 bacteria were grown on Luria Broth (LB) agar and incubated in the dark at 30°C.

Antibiotic Assay: Antibiotic activity scores were assigned conservatively based only on the activity that could be confirmed. The CJB7, KTG3, TMV16 and TMV21 bacteria were assayed against nine safe relatives/models to verify their antibiotic killing scores. On their respective media plates, 100µl of individual safe relative stationary cultures were spread using a sterile hockey stick and allowed to dry. Using a sterile toothpick, each bacterial isolate was patched onto their respective plates with dried safe relative culture. Killing of the safe relative was observed by a "zone of inhibition", an area surrounding the bacterial patch where the safe relative was killed by a compound secreted by the bacterial patch.

Dried extractions were also assayed by "zone of inhibition" detection; extraction layers were resuspended in 80µl methanol before patching 30µl onto one spot on an agar plate. Once the patch of resuspended extract was dry, top agar with safe relative was poured on the plate. Extraction antibiotic activity scores could be affected by the types of solvents used and the polarity of the extracted compounds.

Gram Stain: Gram staining is a differential method of characterizing bacterial cell walls based on composition. Bacterial cell walls contain highly cross-linked amino acids and sugars called peptidoglycans that protect cells and give cells their shape. The cell wall structure is determined with two dyes, crystal violet and safranin, which are retained according to the amount of peptidoglycan in the cell well. A Gram-negative bacterial cell wall contains little peptidoglycan, cannot retain the crystal violet dye and stains pink by the safranin. A Gram-positive bacterial cell wall with a large amount of peptidoglycan can retain the crystal violet dye and stains purple. Bacteria were patched onto 5µl of sterile deionized water atop a microscope slide. Bacteria were fixed by heating to evaporate the water leaving a smear of bacterial cells. The bacteria were flooded with crystal violet, rinsed gently with deionized water, then soaked in Gram's iodine. After a series of rinses, the bacteria were soaked in safranin, rinsed again, allowed to dry and finally examined under a compound microscope (magnification of 40x). The bacteria were Gram (-) if the cells stained pink or Gram (+) if the cells stained purple. Results were verified by MacConkey and phenylethanol plate assays described below.

MacConkey Agar Assay: MacConkey agar is a selective media that inhibits the growth of Gram (+) bacteria by disrupting the cell wall with crystal violet and bile salts. Bacteria are Gram (-) if they grow. MacConkey agar also reveals the ability of bacteria to ferment lactose by the presence of a pH indicator. Bacteria fermenting lactose release acid by-products that turn the bacterial colonies from their normal pigment to red/pink. Each bacterial isolate was streaked onto a MacConkey agar plate and incubated for 24 hours in 30°C.

Phenylethanol (PE) Plate Assay: PE plates contain selective media that allows Gram (+) bacterial growth. Phenylethanol inhibits Gram (-) bacterial growth by blocking DNA synthesis. Bacteria were streaked onto PE plates and incubated for 24 hours in their respective conditions (chosen by the Life Science lab students originally).

Litmus Milk Assay: Litmus milk is a complex media used to differentiate bacterial species by characterizing the ways in which they break down various components of the media and the subsequent by-products. Litmus milk is composed of milk, lactose, casein and litmus (pH and oxidation-reduction indicator). Each bacterial isolate was inoculated into separate litmus milk broths and incubated at their respective temperatures for 24 hours. If a bacterial species can break down the various components, a multitude of outcomes are possible (acidic, alkaline, gas formation, etc.) or no reaction if the species is incapable.

Phenol Red Broth (PRB) Method: A bacterial species that can ferment lactose, glucose and/or sucrose can break down these sugars in the absence of oxygen. As end-products, the sugar is partially oxidized and acid and gas are produced. Fermentation of these sugars is detected by a differential technique. A Durham tube was inserted in an inverted position within the PRB broth to collect any gas that is produced.

Each isolate was inoculated into each of three PRB tubes containing lactose, sucrose or glucose and incubated for 24 hours in their respective temperatures. Phenol red is a pH indicator that is red/pink at a neutral pH of 7 and yellow in acidic conditions.

Catalase Assay: Hydrogen peroxide (H_2O_2) is typically used as an antiseptic for cuts and wounds. Some bacteria have evolved to produce catalase (which breaks H_2O_2 into water and oxygen) as a defense mechanism against H_2O_2 (Hernandez et al., 2016b). Bacteria were inoculated with 3% H_2O_2 solution and catalase production was detected by the production of oxygen bubbles.

Sulfide, Indole and Motility (SIM) Assay: SIM is a differential semisolid medium that allows for the visualization of sulfide production, indole production and bacterial motility. Bacteria that can metabolize amino acids containing sulfur or inorganic sulfur compounds from the environment produce hydrogen sulfide (H_2S). The hydrogen that is released reacts with compounds in the SIM medium (iron, lead or bismuth) and turns black. Bacteria that metabolize tryptophan produce indole that is detected by adding Kovac's reagent. If indole is present, the top of the medium turns cherry red. Motility of bacteria is seen by growth extending from the bacterial inoculation line. Colonies of bacteria were inoculated into a tube with SIM media using a sterile needle and incubated for 24 hours at 30°C.

Extraction and Isolation of Organic Compounds: To determine which compounds had antibiotic activity in the bacteria, their organic compounds were extracted. Twelve plates of each isolate, CJB7, KTG3, TMV16 and TMV21 bacteria, were grown in their respective conditions for three days. Next, the plates were chopped and placed into four bottles that were frozen in an ethanol/dry ice bath to lyse bacterial cells and release their contents. Ethyl acetate/water (3:2) was added to the bottles and the bottles were shaken overnight. Upon separation, the **organic layer** (upper layer) was removed and the ethyl acetate was evaporated over three to four days under a ducted fume hood. The water was evaporated from the **aqueous layer** (lower layer) for three days under reduced pressure.

Colony PCR: To obtain a DNA sample of the 16S ribosomal RNA (rRNA), the DNA sequence was amplified using polymerase chain reaction (PCR). Sequencing the 16S rRNA gene has become a widely used technique for identifying microbial organisms (Gao, Lin, Revanna, & Dong, 2017) Colony PCR was conducted with degenerate TE-27F forward (5' AGR GTT TGA TYM TGG CTC AG 3') and TE-1492R reverse (5' GGY TAC CTT GTT ACG ACT T 3') primers (Integrated DNA Technologies) and Taq polymerase-PCR reagents/master mix (New England Biolabs). PCR cycling conditions were: 94o 10 min; three cycles of 94o 30 sec, 53o 30 sec and 72o 110 sec; twenty-seven cycles of 94o 30 sec, 58o 30 sec and 72o 110 sec; 72o 10 min. PCR products were visualized by 1% agarose gel electrophoresis using SyBr Safe on a

Syngene G:Box EF2 imager. Clean 1.4 kb PCR products were treated with Exo-SAP-IT (Affymetrix) to remove the residual primers; TE-27F primer (1.7 μM) was re-added and the reactions were sent to DNA Analysis Facility on Science Hill at Yale University for DNA sequencing. Homologous DNA sequences were found using the National Institutes of Health's Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

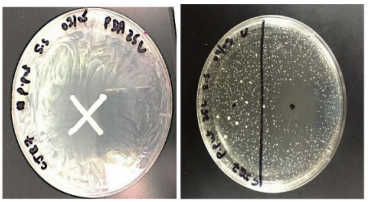
Results

Antibiotic Activity of CJB7, KTG3, TMV16 and TMV21 Bacteria, Organic and Aqueous Layers: Table 2 displays the antibiotic activity results of the organic and aqueous layers of the CJB7, KTG3, TMV16 and TMV21 bacterial extractions against nine safe relatives/models. Organic and aqueous layers were collected as described in Methods. The antibiotic activity of the aqueous extracts from the CJB7 and KTG3 bacteria could not be determined due to contamination/degradation during the drying process.

Table 2. Antibiotic Activity of CJB7, KTG3, TMV16 and TMV21 Bacteria, Organic and Aqueous Layers. Killing is denoted by "Y", minimal killing is denoted by "Y*", no killing is denoted by "N", and results that were not determined (see text) are denoted by "ND". Yellow boxes signify Gram (-) strains.

Isolate	CJB7 Bacteria	CJB7 Organic	KTG3 Bacteria	KTG3 Organic	TMV16 Bacteria	TMV16 Organic	TMV16 Aqueous	TMV21 Bacteria	TMV21 Organic	TMV21 Aqueous
Kills *S. epi*	Y	Y*	Y	Y	Y	Y	Y	Y	Y	N
Kills *E. coli*	Y	N	Y	Y	Y	ND	ND	Y	Y	ND
Kills *E. caro*	Y	N	Y	N	Y	Y*	ND	N	Y*	N
Kills *E. raff*	Y	N	Y	Y	Y	Y*	N	Y	N	N
Kills *A. bay*	Y	N	Y	Y	Y	Y	ND	Y	N	Y*
Kills *B. sub*	Y	Y*	Y	Y	Y	Y	Y*	Y	Y	Y
Kills *E. aero*	Y	Y*	Y	Y*	Y	Y	Y	Y	ND	N
Kills *M. smeg*	Y	N	Y	N	Y	Y*	Y	Y	N	N
Kills *P. put*	Y	Y	Y	Y	Y	Y	Y*	Y	Y	Y*
Antibiotic Activity Score	9	4	9	7	9	8	5	8	5	3

Bacterial Isolate CJB7: The CJB7 bacteria killed all nine safe relatives (Table 2). An example of killing by the CJB7 bacteria can be seen in Figure 1. Organic compounds responsible for killing four of the nine safe relatives were successfully extracted and an example of killing by the organic extraction layer can be seen in Figure 2. The CJB7 bacterial 16S rRNA gene has a 97% homology to that of *Pseudomonas plecoglossicida* (Table 3) and are a Gram (-) species as indicated by three assays (Table 4). The CJB7 bacteria do not ferment lactose or sucrose, glucose has not been determined (ND), metabolize casein, are motile, and produce catalase, but not sulfide or indole (Table 4).



Figures 1 and 2. CJB Bacterial and Organic Extract Antibiotic Activity Against *P. putida*. Figure 1 (left) is the antibiotic assay showing a zone of inhibition around the CJB7 bacteria (patched as an "X") and Figure 2 (right) is the antibiotic assay of the organic extract (patched on the right black dot). Left black spot on the plate represents the methanol (solvent) negative control.

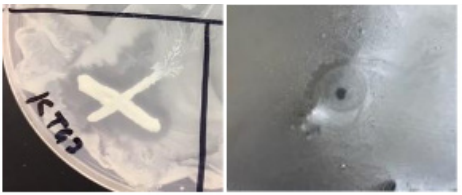
Table 3. Bacterial Identity Based on BLAST of 16S Ribosomal RNA Gene Sequence

ISOLATE	CJB7	KTG3	TMV16	TMV21
% Identity	97%	83%	97%	95%
Closest Match to DNA Sequence	*Pseudomonas plecoglossicida*	*Pseudomonas sp.* DR 5-09	*Pseudomonas mosselii* strain DJ15	*Pseudomonas plecoglossicida* strain NBAII BA-11 D-1
GenBank Accession Number	AM711588	CP011566	KY817593	HM439960

Table 4. Results of Microbiology Assays. "ND" denotes not determined due to various discrepancies.

ISOLATE	CJB7	KTG3	TMV16	TMV21
Gram Stain	Gram negative	Gram positive	Gram negative	Gram negative
MacConkey Agar Growth	Gram negative	Gram negative	Gram negative	Gram negative
PE Plate	Gram negative	Gram negative	Gram negative	Gram negative
Lactose Fermentation (on MacConkey agar)	NO	NO	NO	NO
Lactose Fermentation (on PRB)	NO	ND	NO	ND
Glucose Fermentation (on PRB)	ND	ND	NO	Yes
Sucrose Fermentation (on PRB)	NO	ND	NO	ND
Litmus Milk	Casein Metabolism	NO	NO	NO
Motility	YES	YES	YES	NO
Sulfide Production	NO	NO	NO	NO
Indole Production	NO	NO	NO	NO
Catalase	YES	NO	YES	YES

Bacterial Isolate KTG3: The KTG3 bacteria killed nine safe relatives and the active compounds for killing seven safe relatives were successfully extracted in the organic layer (Table 2, Figures 3 and 4). The KTG3 bacterial 16S rRNA gene is 83% homologous to a *Pseudomonas sp.* (Table 3) and KTG3 bacteria are a Gram (-) species although Gram staining did not confirm this finding (Table 4). The KTG3 bacteria do not ferment lactose, glucose and sucrose ND, are motile, and do not produce sulfide, indole or catalase (Table 4). The extractions of the KTG3 bacteria were sent to Dr. Lyndon West's laboratory in FAU's Chemistry and Biochemistry Department for spectroscopic analysis. Interestingly, they found a new cyclic depsipeptide (unpublished data) related to the newly discovered bananamides isolated from *Pseudomonas fluorescens* (D. Nguyen et al., 2016).



Figures 3 and 4. KTG3 Bacterial and Organic Extract Antibiotic Activity Against *A. baylyi. Figure 3* (left) is the antibiotic assay showing a zone of inhibition around the KTG3 bacteria patched as an "X". Figure 4 (right) is the antibiotic assay showing the zone of inhibition of the organic extract (patched on the black dot).

Bacterial Isolate TMV16: The TMV16 bacteria kill nine safe relatives and its active compounds are found in both the organic and aqueous extraction layers (Table 2). The organic extract killed eight of the safe relatives; killing of *E. coli* was not determined because the safe relative did not grow. Killing of *B. sub* by the organic extract is shown in Figure 5. The aqueous extract was able to kill five of the safe relatives, while the killing of three was not determined due to the irregular growth patterns of the safe relatives (Table 2). Killing of *E. aero* by the aqueous extract from TMV16 is illustrated in Figure 6. The TMV16 bacterial 16S rRNA gene has 97% homology to a strain of *Pseudomonas mosselii* (Table 3) and the TMV16 bacteria are a Gram (-) species (Table 4). The TMV16 bacteria do not ferment lactose, sucrose, and glucose ND, are motile, and do not produce sulfide or indole, but produce catalase (Table 4). Through extensive spectroscopic analysis, the TMV16 bacteria was found to produce xantholysin, a known antibiotic (unpublished data).

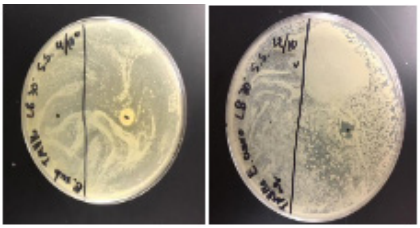
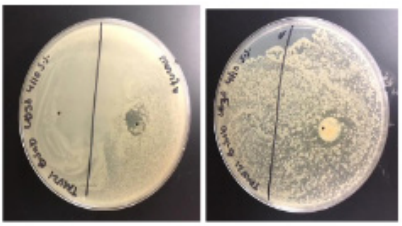


Figure 5 (left) shows the antibiotic activity as a zone of the inhibition around the TMV16 organic extract (patched on the right black dot) against *B. subtilis*. The appearance of the spot is due to the cell debris, pigments, and other organic matter.

Figure 6 (right) shows the antibiotic activity as a zone of the inhibition around the TMV16 aqueous extract (patched on the right black dot) against *E. aerogenes*. Left black spots on both plates represent the methanol (solvent) negative controls.

Bacterial Isolate TMV21: The TMV21 bacteria kills nine safe relatives and the organic extract successfully killed five; activity against *E. aero* was not determined due to safe relative growth irregularities (Table 2). The killing of *B. sub* by the organic extract from TMV21 is shown in Figure 7. The aqueous extract killed three safe relatives, including *A. bay,* which could not be killed with the organic extract and killing of *E. coli* by the aqueous extract was not determined (Table 2). The killing of *B. sub* by the aqueous extract is shown in Figure 8. The TMV21 bacterial 16S rRNA gene is 95% homologous to that of *Pseudomonas plecoglossicida* (Table 3). The TMV21 bacteria are a Gram (-) species, cannot ferment lactose according to MacConkey and Litmus, or sucrose, but can ferment glucose, are not motile, and do not produce sulfide or indole, but produce catalase (Table 4).



Figures 7 and 8. Antibiotic Activity of the TMV21 Organic and Aqueous Extracts Against *B. subtilis.* Figure 7 (left) is the antibiotic assay showing a zone of inhibition around the organic extract isolated from the TMV21 bacteria on the right black spot. Figure 8 (right) shows a zone of inhibition around the aqueous extract isolated from TMV21 bacteria on the right black spot. Left black spots on both plates represent the methanol

Discussion

Organic extracts from the CJB7 and KTG3 bacteria only killed four and seven safe relatives, respectively (Table 2), suggesting that the compounds necessary for killing the remaining safe relatives may be in the aqueous layers which could not be analyzed. The TMV16 bacteria produced the most successful results for the organic extraction; the TMV16 organic layer killed eight safe relatives (Table 2). The aqueous layer of the TMV16 bacteria killed the same five safe relatives as the organic layer (Table 2). The compound(s) necessary for killing the nine safe relatives may be found in both the organic and aqueous layers and contain polar and nonpolar components.

The organic layer of the TMV21 bacteria killed five safe relatives, while the aqueous layer killed only three; killing of *A. bay* was specific to the aqueous layer (Table 2). Even though the TMV16 and TMV21 bacteria were found in soil from the same location by one undergraduate student, they are different species based on having different antibiotic activity profiles and 16S rRNA DNA sequences (Tables 2 and 3).

Sequence analysis of the 16S rRNA genes of the CJB7, KTG3, TMV16 and TMV21 bacteria show that all had high homologies to the genus, *Pseudomonas* (Table 3). This is consistent with the varied antibiotic activities and microbiology assays in Tables 2 and 4. *Pseudomonas* species have species-specific behaviors including the metabolites they produce which explains the varied killing between bacterial extractions (D. Nguyen et al., 2016; Otton, Campos, Meneghetti, & Corcao, 2017). The diversity of *Pseudomonas* species makes them good candidates for the production of useful, novel molecules (Vásquez-Ponce, Higuera-Llantén, Pavlov, Marshall, & Olivares-Pacheco, 2018). Additionally, the CJB7, KTG3 and TMV16 bacteria are motile (Table 3). *Pseudomonas* species tend to have one flagellum and multiple pili as structures for motility (Otton et al., 2017). The Gram-negative results of the Gram stains, MacConkey plates and PE plates (with the exception of the KTG3 bacterial Gram stain) were consistent with the Gram-negative characteristic of the *Pseudomonas* genus (Table 3) (Otton et al., 2017). Species in the *Pseudomonas* genus utilize sugar but usually not by fermentation; this was true for the CJB7, KTG3 and TMV16 bacteria by the negative results on MacConkey plates, Litmus milk and PRB assays (Table 4) (Siegrist, 2007). The TMV21 bacteria, however, fermentglucose as indicated by the positive result on the glucose PRB assay (Table 4). None of the four bacteria metabolize tryptophan (as indicated by negative results on indole production, Table 4) but it has been shown that *Pseudomonas* species can utilize tryptophan through other pathways which result in end-products other than indole (Bortolotti et al., 2016). For example, the bacteria studied here may metabolize tryptophan to make anthranolite, a chemical responsible for the quorum-sensing system, or communicating system, of certain *Pseudomonas* species (Bortolotti et al., 2016). Catalase production, typical of aerobic bacteria, is observed for the CJB7, TMV16 and TMV21 bacteria, also supporting their identification as species in the *Pseudomonas* genus, which is aerobic (Iglewski, 1996). The KTG3 bacteria, on the other hand, do not produce catalase and future studies are warranted on this anomaly.

Conclusion

Results of this study demonstrate the importance of citizen science by the immense amount of data accumulated by undergraduate non-STEM students and FAU's TE research lab. Bacteria are being successfully characterized using a variety of techniques that may lead to new potential antibiotics. Four bacterial isolates that were originally isolated and characterized by undergraduate non-STEM students were selected for farther characterization in this study by FAU's TE research lab. The CJB7, KTG3, TMV16 and TMV21 bacteria have broad-spectrum antibiotic activity profiles, strong 16S rRNA gene homology to the *Pseudomonas* genus and similar taxonomic characteristics of the *Pseudomonas* genus according to classic microbiology assays. Secondary metabolites responsible for the antibiotic activities of the KTG3 and TMV16 bacteria were successfully extracted and structure elucidation is complete for the KTG3 depsipeptide and the TMV16 xantholysin (unpublished data). Future studies will explore the antibiotic activity of the pure KTG3 peptide, including its mechanism of action. Additionally, extensive spectroscopic analysis to characterize the cyclic framework of the KTG3 peptide, sequencing of KTG3 bacterial genomic DNA and cytotoxicity studies are underway in hopes of characterizing and reporting a novel antibiotic. Extraction protocols of compounds with antibiotic activity from the TMV16 and TMV21 bacteria are ongoing (data not shown). Future directions involve structure elucidation of the compounds and genomic sequencing of the CJB7, TMV16 and TMV21 bacteria. Finding new antibiotics to kill MDR bacteria is vital. The findings in this study are the beginning steps toward critical discoveries as Tiny Earth at FAU continues to isolate hundreds of potential antibiotic producers.

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