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Biogeography of Special Metabolites Produced by Ant-Associated Bacteria

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Biogeography of Special Metabolites Produced by Ant-Associated Bacteria

A Thesis Presented By

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Abstract

There is an urgent and growing need for novel antibiotics with the growing prevalence of antibiotic-resistant pathogens. Ecologically-guided discovery is a strategy that focuses on how and where specially evolved metabolites such as antibiotics are used in nature. This strategy reveals unique active molecules with potential for human use. An unexplored niche with great potential for antibiotic discovery exists with Southwestern fungus-farming *Trachymyrmex* ants and their Actinobacteria symbionts. In exchange for nutrients from the ant, the ants' bacterial symbiont produces antifungal molecules that protect the ants' fungal cultivar from invasion by pathogenic microorganisms. They also produce antibacterial molecules to protect themselves from niche competitors. Recently, new antibiotics have been isolated from *Trachymyrmex* ant symbionts collected in the southwestern U.S., such as a novel analog of the antibiotic nocamycin and an antifungal compound, ECO-0501. These findings suggest that this unique environment may yield additional novel metabolites. Reported here are discoveries from a large-scale and systematic analysis of symbiont-derived natural products from this niche to understand their distribution and discovery potential, with the goal of discovering and characterizing novel bioactive compounds. We have assembled a collection of Actinobacteria isolated from colonies of *Trachymyrmex* ants in Arizona, New Mexico, and New York. An extract library has been prepared from this collection, and antibiotic activity testing of that library indicates the presence of ecologically and clinically relevant active compounds. Metabolomics data reveals patterns with regards to ant genus and the metabolites present in crude extracts. This method has great potential for strain prioritization in future studies.

Introduction

Antibiotic Resistance

Antibiotic resistance is a rapidly worsening problem in our world. This is due to abundant overuse of antibiotics currently in clinical use. Over time, bacteria and other microorganisms build up resistance mechanisms to drugs, especially when they are used too frequently or improperly.[1,2] The CDC estimates that every year 2.8 million people acquire antibiotic-resistant infections that result in 35,000 deaths.[3] The most deadly antibiotic-resistant pathogens have been labeled the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudonmonas aeruginosa,* and *Enterobacter*). These pathogens have acquired the ability to evade antibiotics through a number of mechanisms including drug inactivation, modification of drug binding sites, changes in cell permeability, and biofilm formation.[4]

There is now widespread resistance to many antibiotics in use, but little effort is being made to find new ones. Antibiotic discovery has slowed since the 1980s and a new class of antibiotics hasn't been discovered in over 30 years.[5] There is an urgent need for new anti-bacterial and anti-fungal compounds to combat antibiotic-resistance microorganisms, but the field has been neglected.

The typical approach to antibiotic discovery is to identify active compounds that are special metabolites of bacterial or fungal species. This is because bacteria and fungus produce antibiotics in nature in order to compete with one another for resources.[6] This has often been done using a strategy called bioactivity-guided isolation in which microorganisms must first be identified as being able to inhibit the growth of other microorganisms of interest.[7] This strategy is time-consuming and in culture-dependent discovery like this it is possible that some active

compounds are missed. Another new approach to antibiotic discovery is to improve culturing conditions so that they are closer to the natural environment that a species of bacteria or fungus might live in.[8] One option for this is a co-culture where two strains are grown together with the strategy of putting competitive pressure on both strains so they produce more active compounds against each other. This takes advantage of the assumed ecological role of antibiotics as tools used by bacteria to out-compete other species that could pose a threat. This is a very niche method, however, and more efficient methods for antibiotic discovery are needed.[9]

Two culture-independent strategies are genomic mining and metabolomics. Genomic mining is a strategy where the genome of a bacteria is found, and biosynthetic gene clusters are identified. This approach is relatively new and could reveal many antibiotics that we don't yet have the tools to extract or synthesize.[10] It is already known from genomic mining that bacteria have the means of building far more antibiotics than are ever found when they are cultured and extracted.[9] Metabolomics is a comprehensive analysis of all metabolites produced by a biological specimen and moves beyond bioactivity-guided isolation.[11] This strategy can also reveal compounds that may not have been found using culture-dependent methods. Both genomic mining and metabolomics have a place in the future of antibiotic discovery.

What is needed is an efficient strategy for knowing where to find active compounds without attempting to isolate compounds from hundreds or thousands of strains with little to no yield. Ecologically guided discovery is strategy that considers factors in the environment that would result in the production of special metabolites. This could include the ecological role special metabolites would play in the ecosystem.[12] It could also mean understanding that certain environments yield certain molecules and this can be leveraged to benefit human health. A prevalent example of ecologically guided discovery is the study of marine microbes and their

metabolites. Recently, a new antibiotic capable of killing colistin-resistant *A. baumannii* was isolated from the bacterial symbionts of shipworms that boost the worms' immune systems with their secreted antibiotics in exchange for nutrients from the shipworms' diet.[12] *A. baumanii* is one of the ESKAPE pathogens, the group of deadly antibiotic-resistance prone pathogens. This is an example of very successful ecologically guided discovery based on the known symbiotic relationship of the worms and their bacteria. Multiple new antibiotics have also been mined from the genomes of sponge-associated bacterial symbionts which play crucial roles in sponge chemistry.[7] Ecologically guided discovery could be used alongside the methods like metabolomics to create a process that is not only more efficient but would result in a higher yield of more diverse compounds. One such opportunity for ecologically guided discovery lies with fungus-farming ant and their bacterial symbionts.

Fungus-Growing Ants and Their Bacterial Symbionts

About 50 million years ago, leafcutter ants domesticated a fungal cultivar. This fungal crop subsists on leaves and other plant matter brought to it by the ants. The fungal crop is grown in chambers in the colony's nest and provides the ants' primary food source.[13] Fungus farming ants include genera *Acromyrmex, Apterostigma, Atta, Cyphomymerx,* and *Trachymyrmex* among others and encompasses over 200 species.[14] These ants are found exclusively in North and South America. To farm fungal cultivar, these ants participate in a symbiotic relationship. Antibiotic-producing Actinobacteria, namely *Pseudonocardia,* live on the cuticle of ants and survive off nutrients secreted from specialized structures of the ant's exoskeleton (Figure 1).[15,16] In exchange, the bacteria produces anti-fungal compounds that stop opportunistic, pathogenic fungi such as *Escovopsis*, which feeds on cultivar and can destroy a colony's entire food source.[13,17]

Additionally, Actinobacteria must protect their own place in the symbiosis and produce antibacterial molecules to keep opportunistic bacteria from taking their place on the ant.[6] This could provide novel antibacterial or antifungal compounds to combat the problem of antibiotic resistance. Recently, it has been hypothesized that *Amycolatopsis* bacteria can also participate in this symbiotic relationship in place of *Pseudonocardia*.[18] Several recent studies have isolated *Amycolatopsis* from fungus-growing ants and several strains have been included in this study.[18,19]

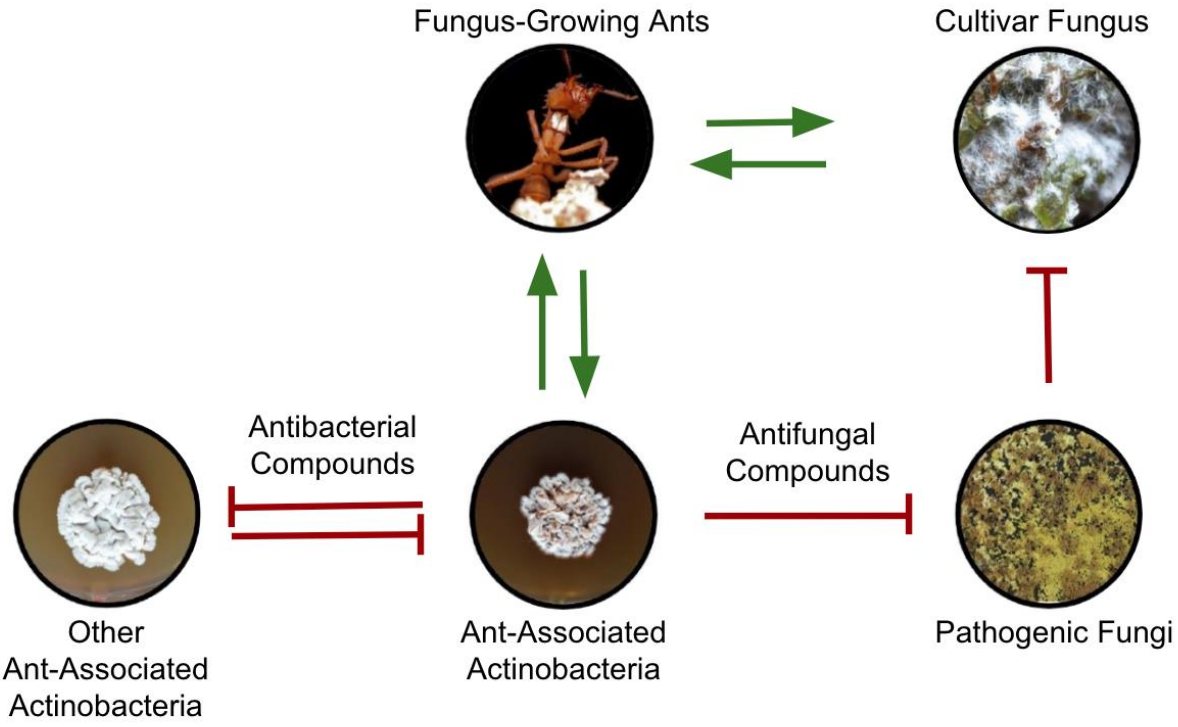


Figure 1. The symbiotic relationship between fungus-farming ants and their Actinobacteria symbionts provides protection for the ants' food source.

Many bacterial strains in this system of fungus-growing ants and their symbionts have been evaluated before for active compounds and have yielded several. These species are often found in rainforest environments in South America and have been widely studied in Panama in

particular. Dentigerumycin, an antibiotic produced by Actinobacteria associated with *Apterostigma dentigerum*, has antifungal and antibacterial activity.[20] Rebeccamycin was also isolated from a bacterial strain associated with *A. dentigerum*, but it only has antibacterial activity. Both of these strains come from Panama. *Trachymyrmex* ants reside in North America, and some can be found in the northeast. A thiopeptide named GE37468 with activity against human pathogens was extracted from ant-associated *Pseudonocardia* isolated from a *T. septentrionalis* ant collected in Centereach, NY.[21] In the American southwest, a nocamycin analog, nocamycin V, was found in ant-associated *Amycolatopsis* bacteria.[18]

Fungus-growing ants are typically a rainforest species and so this unique desert environment is expected to yield unique compounds not found with these ants in other parts of the world. This has been shown to be true with both GE37468 and nocamycin V, both found outside of the better-characterized Panama ant symbionts, and this makes the Madrean Archipelago in the desert southwest a particularly exciting system to mine for new antibiotics. Furthermore, new *Trachymyrmex* ant species and their associated strains that have not yet been studied in relation to natural products reside here. This niche in its unique ecosystem presents an opportunity to test the viability of biogeography-guided discovery of new antibiotics as specific features of the system could reveal biogeography drivers of chemical diversity.

Biogeography of the Sky Islands

The Madrean Sky Islands are a series of mountain ranges in southern Arizona, New Mexico, and northern Mexico. The mountains are referred to as “islands” in a desert “sea” due to their elevation and the isolation of species that occupy them.[22] The Sky Islands have unique biogeography because the distribution of plant, insect, and animal species varies with elevation

and temperature. Biogeography refers to the geographical distribution of species and ecosystems and how this affects species and ecosystems. The climate on the mountains varies from desert to subalpine forests as the altitude increases, and this dramatic change in climate results in incredible biodiversity over the "island." These mountains are responsible for 59% of Arizona's ant population and among the ants here are many species of *Trachymyrmex* fungus-growing ants.[23] These species include *Trachymyrmex arizonensis, Trachymyrmex carinatus, Trachymyrmex desertorum,* and *Trachymyrmex pomonae. T. arizonensis, T. carinatus* and *T. pomonae* prefer mid-elevation oak forests.[24–26] *T. desertorum* is typically found at lower elevations in true desert environments.[27]

It is expected but not confirmed that the special metabolites produced by the Actinobacteria harbored by these ants will follow biogeographical patterns based on the location they were collected from and/or their ant host. For example, secondary metabolites from *desertorum* species are expected to vary from other species due to their preference for true desert environments over the oak forests inhabited by other species as it has been shown that factors like local habitat can affect the kinds of special metabolites produced. For example, a study found that soil bacteria living in different soil environments will produce different special metabolites while bacteria in similar environments have conserved biosynthetic gene clusters and produce far more closely related metabolites.[28] Additionally, these ants encounter far different local environments with different threats than the previously studied ant species from South America. The presence or absence of certain special metabolites could be influenced by several factors including other Actinobacteria strains present in a specific geographical location and the extent to which they must compete for resources, as well as the presence or absence of different species of pathogenic fungi.[19] The distribution of the ants itself is also limited by the geography

of the sky islands as temperatures drop and ecosystems change with elevation. A better understanding of the effects of biogeography on the niche will aid antibiotic discovery efforts in the future.

The goal of this study was to understand how special metabolites are distributed throughout this niche with respect to ant host, collection location, and habitat. Additionally, metabolomics analysis such as presented here could prove to be a useful tool for biogeography guided discovery of novel antibiotics. Recently, a study mapped biogeography patterns to patterns based on phylogeny and taxonomy of microbes that produce polyketide antibiotics.[29] This study aims to map biogeography patterns to metabolomics of ant-associated strains. This data presents an opportunity for strain prioritization from mass spectrometry data. The metabolomics data combined with the activity testing data could provide a roadmap for even larger scale studies of this niche in the hunt for more antibiotics.

Experimental Design

This study is a large-scale survey of the active compounds produced by ant-associated bacteria in this desert niche. Strains were collected over three years, 2017, 2018, and 2021 as well as two states, Arizona and New Mexico (Figure 2). Active compounds from these strains were extracted and investigated in two ways. First, activity testing against a panel of clinically and ecologically relevant pathogens was performed. The panel included both Gram-negative and Gram-positive bacteria as well as fungus. Second, the rough extractions were run on a high-resolution LCMS to investigate which metabolites each strain produced. This data was analyzed using Principal Component Analysis plots, a statistical method that examines similarities and differences in a data set.

Figure 2. Map of ant collection sites for Actinobacteria isolation in New Mexico and Arizona.

Ant host appears to have the greatest effect on which metabolites are produced, especially the genus of the ant (*Amycolatopsis* versus *Pseudonocardia*). The ant species, collection location, and habitat did not appear to have much effect on the type and quantity of special metabolites produced. Perhaps most importantly, however, is the opportunity presented by the metabolomics data for strain prioritization in future large-scale studies of a similar nature.

Methods

Culturing New Strains

Actinobacteria was collected and streaked onto chitin isolation plates from *Trachymyrmex* ants by Professor Van Arnam. In 2021, 53 strains were collected. The chitin isolation plates were monitored in the lab until white, chalky *Pseudonocardia* colonies presented. In a sterile laminar flow hood using sterile technique, *Pseudonocardia* colonies on the chitin plates were gently scraped with a flame-sterilized metal loop. The metal loop was then streaked across YMEA plates (25 mL agar per 100 x 15 mm Petri dish). These YMEA plates were incubated at 30°C until the streaked bacteria grew to the point where there were clear, uncontaminated, isolated colonies on the plate. In the laminar flow hood, a clean colony was lifted from the plate with a flame-sterilized metal dental spatula. The colony was deposited in a sterile screw top vial with 100 μL of sterile water. The vial was capped and agitated to break up the colony. The contents of the vial were then pipetted onto the center of a fresh YMEA plate. A sterile L-spreader was used to spread the spore and water mixture in a 1 inch by 1 inch square in the center of the plate. These plates were incubated at 30°C until the patch grew into a 1 inch by 1 inch lawn of uncontaminated bacteria.

Spore Stock Creation for New Strains

Once grown in, 1 mL of sterile water was pipetted onto the top of the bacterial "patch" in the laminar flow hood. A sterile L-spreader was used to scrape the bacteria off the agar. The resulting spore and water solution on the YMEA plate was pipetted off the plate into a sterile screw top vial. This process was repeated once more. The mixture in the screw top vial was split between two large YMEA plates (60 mL agar per 150 x 15 mm Petri dish) by pipetting. A sterile

L-spreader was used to spread the mixture across the entire surface of the plate. The plate was incubated at 30°C until the bacterial lawn had grown in thickly. After 2 to 3 weeks, spore stock was made from the bacterial lawns on the large YMEA plates. In the laminar flow hood, 7 mL of sterile water was added to the bacterial lawn. A sterile L-spreader was used to vigorously scrape the bacterial lawn off the agar. The mixture was pipetted off the plate into a sterile 50 mL conical tube. This process was repeated up to two times on each large YMEA plate. The spore and water mixture from large YMEA plates with the same strain code were combined in the 50 mL conical tubes. The final volume in the 50 mL conical tube was made up to 25 mL with sterile water then capped and vortexed until the mixture was homogeneous throughout. In the sterile hood, the resulting suspension was slowly poured through a sterile cotton filter into a new sterile 50 mL conical tube. The cotton filter was rinsed with 5 mL of sterile water once the suspension had passed through. The 50 mL conical tube was capped and centrifuged for 10 min at maximum speed. In the sterile hood immediately following centrifugation, the supernatant was discarded, and the spore pellet was resuspended in 1 mL of sterile water and 1 mL of a 50% glycerol and sterile water solution. The spore mixture was aliquoted into 4 small screw top vials for long-term freezer storage at -80°C. The spore mixture was also streaked on a YMEA plate (25 mL agar per 100 x 15 mm Petri dish) using a flame-sterilized metal loop. The YMEA plate was incubated at 30°C for one week to monitor for non-*Pseudonocardia* contamination. From the strains collected in 2021, 30 strains were successfully cultured and stored for later use.

Growing Strains for Extractions

Actinobacteria strains collected in 2017, 2018, and 2021 by the Van Arnam lab were plated for extractions. In the sterile laminar flow hood, 70 μL of sterile water was pipetted onto

the center of a YMEA plate (25 mL agar per 100 x 15 mm Petri dish). 5 μL of spore stock was pipetted onto the sterile water spot. A sterile L-spreader was used to spread the water and spore mixture across the entire surface of the plate. The plates were left open to dry in the hood for five minutes before being incubated at 30°C. Each strain was plated in triplicate. Overall, 5 batches were plated with 10-20 unique strains in each batch. The bacteria were grown in batches due to limitations in the number of extractions that could be done in a single day. The plating of batches was staggered to be on different days. A media-only control and a control of *Pseudonocardia* strain GA6-1—a strain well-studied by the lab—were plated in triplicate with each batch.

Extractions

Plated strains were grown at 30°C for 12 days before being removed from the incubator for extraction. A representative plate from each strain was photographed to show the progression of bacterial growth (Figure 12 in the Appendix). A metal spatula was cleaned with ethyl acetate and then used to cut up the agar with the bacterial lawn for each plate into 1 cm by 1 cm squares. The metal spatula was used to transfer the squares for each plate into 50 mL conical tubes. The metal spatula was cleaned with ethyl acetate between each plate. Using a 10 mL graduated cylinder, 20 mL of ethyl acetate was added to each tube containing agar chunks. The tubes were closed and left to soak at room temperature. After 24 hours, a plastic funnel was cleaned with ethyl acetate. A clean coffee filter was placed in the funnel and a 20 mL scintillation vial was placed beneath the funnel. The contents of one 50 mL tube containing agar and ethyl acetate was poured through the coffee filter and funnel set up into each corresponding 20 mL scintillation vial. The agar was left to filter for 1 minute for each tube. Between every tube, the coffee filter was changed, and the funnel was rinsed with ethyl acetate. Vials were then uncapped and placed

in the SpeedVac. The SpeedVac was set to manual run with a room temperature centrifuge chamber and left to run overnight. The following morning once the ethyl acetate had evaporated off the extracts, the scintillation vials were capped and stored at -20°C.

Redissolving Extracts

To redissolve the extracts for further assays, 500 μL of Liquid Chromatography Mass Spectrometry (LCMS)-grade methanol was added to each 20 mL scintillation vial with dried extract. The vials were sonicated for 5 seconds each and then left to sit at room temperature for 30 minutes. 200 μL of the redissolved extract was aliquoted into glass HPLC vials to be dried and stored as an archive sample. These vials were uncapped and left in the SpeedVac overnight. 75 μL of the extract was aliquoted into labeled 1.5 mL microcentrifuge tubes for LCMS prep. The remaining 150 μL of extract was left in the tightly capped 20 mL scintillation vials and stored at -20°C for future activity testing.

Liquid Chromatography Mass Spectrometry Sample Preparation

LCMS samples were prepared from the extracts redissolved in LCMS-grade methanol. The 75 μL aliquoted into microcentrifuge tubes was centrifuged in a microcentrifuge for 1 min at 1300 rpm. 50 μL of the centrifuged extract were pipetted into glass inserts in LCMS autosampler vials. An additional 150 μL of LCMS-grade methanol were added to the inserts. The solution in the inserts was gently mixed by pipetting up and down once. The LCMS vials were capped and stored in the -20°C freezer until needed the following day.

Liquid Chromatography Mass Spectrometry Mass Spec I

Crude extracts were run on a Waters Xevo G2-XS ESI qTof mass spectrometer in resolution mode coupled to a Waters Acquity H-class UPLC (Waters Acquity BEH C18 1.7 μm 2.1 mm x 50 mm column, 0.3 mL/min). A ten-minute method in positive mode collecting MS1 data was used. Extracts were placed in a randomized order. All prepared samples were subjected to LCMS analysis. The UPLC method was 5% acetonitrile in water (both MS grade) with 0.1% formic acid for 1 min, then 5% acetonitrile in 95% water with 0.1% formic acid over 5 min, followed by 95% acetonitrile in water with 0.1% formic acid for 2 min. MS parameters were as follows: capillary voltage of 0.50 kV, cone voltage of 30, and a source temperature of 100 °C.

Drying and Redissolving Extracts for LCMS

It was necessary to repeat the MS1 run, so extracts prepared for LCMS that had been stored in LCMS vials in the -20°C freezer were dried down on the SpeedVac. After being dried down completely, 100 μL of LCMS-grade methanol was added to each vial and pipetted up and down twice to mix. The extracts were left at room temperature overnight so the compounds would fully redissolve. Another MS1 run was completed as described above.

MzMine Data Processing

The raw Waters LCMS data files were converted into .mzXML files using Proteowizard. The converted files were uploaded into MzMine version 2.53. Mass detection was run in centroid mode with a noise level of 1,000. After mass detection, chromatograms were created using the ADAP chromatogram builder. For this, parameters were as follows: the retention time was set from 0.00 to 10.01 minutes, the MS level was 1, the polarity was set to positive, the minimum

group size in number of scans was 5, the group intensity threshold was 100, the minimum highest intensity was 1,000 and the m/z tolerance was set to 7.0 ppm. Chromatograms were then deconvoluted using the baseline cutoff algorithm. And the MEDIAN m/z center calculation. The minimum peak height was set to 10,000, the peak duration was 0.00-0.80, and the baseline level was 1,000. Finally, an aligned feature list was made with the deconvoluted chromatograms. The m/z tolerance was set to 7.0 ppm, the weight for m/z was 10, the retention time tolerance was 0.5 absolute min, and the weight for retention time was 10.

Activity Testing

Preparing Inoculum: Escovopsis sp.

In a tissue culture hood using sterile technique, a small flame-sterilized metal spatula was used to scrape a 1 cm x 0.5 cm patch of *Escovopsis* off a growth plate. The fungus was added to a sterile 1.5 mL microcentrifuge tube. A sterile pestle was used to grind the fungus in the tube until it was homogenous. 500 μL of sterile water were added to the tube. This process was repeated six times. All six tubes were then vortexed until the fungus and water mixtures were homogeneous. The mixtures from each tube were added to sterile culture tubes (13 mL snap top). 10 mL of molten YMEA top agar (55°C, 0.75% agar) were pipetted into the sterile culture tubes. The tubes were capped and inverted once to mix, then the contents were poured over the top of YMEA plates (60 mL agar per 150 x 15 mm Petri dishes). The plates were gently swirled until the mixture was a smooth, even layer across the surface of the plate.

Preparing Inoculum: Staphylococcus aureus and Escherichia coli

Under a flame on a sterilized bench top, a flame-sterilized metal loop was used to streak *S. aureus* onto an LB plate (25 mL agar per 100 x 15 mm Petri dish) from a frozen stock. The plate was incubated at 37°C overnight. The following day under a flame on a sterilized bench top, 5 mL of MH media was pipetted into a sterile culture tube. A flame-sterilized metal loop was used to pick up an *S. aureus* colony. The colony was swirled into the media using the loop. The culture tube was capped and incubated in a shaking incubator at 37°C for 48 hours. On the sterilized bench top under a flame, 100 μL of the liquid culture was added to 15 sterile culture tubes. To each tube one at a time, 10 mL of molten LB top agar (55°C, 0.75% agar) were pipetted in, then pipetted up and down twice to mix, then pipetted onto an LB plate (60 mL agar per 150 mm x 15 mm Petri dish). The large LB plates were gently swirled until the top agar mixture created a smooth, even layer across the entire surface of the plate. This entire process was repeated identically with *E. coli.*

Preparing Inoculum: Amycolatopsis 17SM-2A and Pseudonocardia 17SM-1 and 18PO-3

In a sterile laminar flow hood using sterile technique, 10 μL of 17SM-2A was added to 15 sterile culture tubes. One tube at a time, 10 mL of molten YMEA top agar (55°C, 0.75% agar) was pipetted into each culture tube, pipetted up and down twice to mix, then pipetted onto the surface of large YMEA plates (60 mL agar per 150 mm x 15 mm Petri dish). The large YMEA plates were swirled gently until the top agar mixture created a smooth, even layer across the entire surface of the plate. This process was repeated for *Pseudonocardia* 17SM-1 and 18PO-3.

Preparing Inoculum: Candida albicans

In a tissue culture hood, a flame-sterilized metal loop was used to streak *C. albicans* onto a YPD plate (25 mL agar per 100 x 15 mm Petri dish) from a frozen stock. The plate was sealed with parafilm and incubated at 30°C for 48 hours. Two days later in the tissue culture hood, 5 mL of YPD media was pipetted into a sterile culture tube. A flame-sterilized metal loop was used to pick up a *C. albicans* colony. The colony was swirled into the media using the loop. The culture tube was capped and incubated in a shaking incubator at 30°C for 48 hours. In the tissue culture hood, 100 μL of the liquid culture was added to 6 sterile culture tubes. One tube at a time, 10 mL of molten YPD top agar (55°C, 0.75% agar) was pipetted into each culture tube, pipetted up and down twice to mix, then pipetted onto the surface of large YPD plates (60 mL agar per 150 mm x 15 mm Petri dish). The large YPD plates were swirled gently until the top agar mixture created a smooth, even layer across the entire surface of the plate.

Activity Testing: Fungus

Flame-sterilized metal tweezers were used to place 25 sterile paper dots at even intervals across the surface of the top agar layer. 10 μL of each extract was pipetted onto the appropriate dot. An antifungal control of nystatin (50 μg) was included on one dot. Plates were sealed with parafilm and incubated at 30°C. The diameter of zones of inhibition from the extracts were measured after 24 hours.

Activity Testing: Bacteria

Flame-sterilized metal tweezers were used to place 15 sterile paper dots at even intervals across the surface of the top agar. 5 μL of each extract was pipetted onto the appropriate paper

dot. An antibiotic control of penicillin G (10 μg) for Gram-positive bacteria or Streptomycin (10 μg) for Gram-negative bacteria was included on one dot. The plates were incubated at 30°C overnight. The diameter of zones of inhibition were measured after 7 days.

Results and Discussion

To get an initial picture of the activity of the collected strains, the crude extracts from 54 strains collected in New Mexico, Arizona, and New York were used for activity testing against seven representative microorganisms: *S. aureus, E. coli, Pseudonocardia* 17SM-1, *Pseudonocardia* 18PO-3, *Amycolatopsis* 17SM-2A, *Escovopsis*, and *C. albicans*. *S. aureus* and *E. coli* are both clinically relevant strains. The first is Gram-positive and the latter Gram-negative. The *Pseudonocardia* and *Amycolatopsis* strains are also Gram-positive, but these strains are ecologically relevant. 18PO-3 was collected in Arizona while 17SM-1 and 17SM-2A were collected in New Mexico. *Escovopsis* and *C. albicans* are fungi. *Escovopsis* is the canonical pathogenic fungus that can threaten the ants' cultivar fungus. *C. albicans* is a clinically relevant pathogenic yeast. Extracts were spotted on discs placed on a lawn of the microorganism and the diameter of the zone of inhibition around the disc was measured after a few days.

Table 1. Relative activity of extracts against clinical and ecologically relevant microorganisms of interest. Activity was measured as the diameter of the zone of inhibition. The star indicates the strain is an *Amycolatopsis* strain. The square indicates the growth of the extracted strain was sparse. GA6-1 is included multiple times as it was grown with each batch.

Strain	Host Species	Collection Location	*Escherichia coli*	*Staphylococcus aureus*	*Amycolatopsis* 17SM-2A	*Pseudonocardia* 17SM-1	*Pseudonocardia* 18PO-3
17SM-1	*T. smithi*	Las Cruces, NM					
17SM-2A*	*T. smithi*	Las Cruces, NM					
17SM-3	*T. smithi*	Las Cruces, NM					
17SM-6	*T. smithi*	Las Cruces, NM					
17SM-8*	*T. smithi*	Las Cruces, NM					
17SM-14	*T. smithi*	Las Cruces, NM					
17SM-15	*T. smithi*	Las Cruces, NM					
18SM-1*⋄	*T. smithi*	Las Cruces, NM					
18SM-2A*⋄	*T. smithi*	Las Cruces, NM					
18SM-3A*	*T. smithi*	Las Cruces, NM					
18SM-5A	*T. smithi*	Las Cruces, NM					
18SM-6	*T. smithi*	Las Cruces, NM					
18SM-7A	*T. smithi*	Las Cruces, NM					
18PO-1a	*T. pomonae*	Chiricahua Mtns, AZ					
18PO-2a	*T. pomonae*	Chiricahua Mtns, AZ					
18PO-4a	*T. pomonae*	Chiricahua Mtns, AZ					
21PO-1⋄	*T. pomonae*	Chiricahua Mtns, AZ					
21PO-2	*T. pomonae*	Chiricahua Mtns, AZ					
21DE-1	*T. desertorum*	Santa Catalina Mtns, AZ					
21DE-1X	*T. desertorum*	Santa Catalina Mtns, AZ					
21DE-2	*T. desertorum*	Santa Catalina Mtns, AZ					
21DE-6X*	*T. desertorum*	Santa Catalina Mtns, AZ					
21DE-8	*T. desertorum*	Santa Catalina Mtns, AZ					
21DE-11⋄	*T. desertorum*	Santa Catalina Mtns, AZ					
18AZ-1a	*T. arizonensis*	Chiricahua Mtns, AZ					
18AZ-4a	*T. arizonensis*	Chiricahua Mtns, AZ					
18AZ-5a	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-3	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-4	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-5	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-6	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-7	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-8	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-9	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-11	*T. arizonensis*	Chiricahua Mtns, AZ					
21AZ-17	*T. arizonensis*	Sierra Ancha Mtns, AZ					
21AZ-18	*T. arizonensis*	Sierra Ancha Mtns, AZ					
21AZ-19⋄	*T. arizonensis*	Sierra Ancha Mtns, AZ					
21CA-1	*T. carinatus*	Dragoon Mtns, AZ					
21CA-4	*T. carinatus*	Dragoon Mtns, AZ					
21CA-5	*T. carinatus*	Dragoon Mtns, AZ					
21CA-10	*T. carinatus*	Dragoon Mtns, AZ					
21CA-13	*T. carinatus*	Sierra Ancha Mtns, AZ					
21CA-15	*T. carinatus*	Sierra Ancha Mtns, AZ					
17SE-3	*T. septentrionalis*	Centereach, NY					
17SE-9	*T. septentrionalis*	Centereach, NY					
GA6-1 (1)	*A. dentigerum*	Panama					
GA6-1 (4)	*A. dentigerum*	Panama					
GA6-1 (5)	*A. dentigerum*	Panama					
Penicillin G	N/A	N/A					
Streptomycin	N/A	N/A					
media	N/A	N/A					

**Amycolatopsis*
⋄ Sparse growth

Of the 47 crude extracts tested, 17 had activity (Table 1). Extracts of *T. pomonae* strains and *T. carinatus* strains had little to no activity. The extracts with the most activity came from *T. arizonensis* and *T. smithi* strains. In general, active extracts tended to show ecologically relevant activity against Gram-positive bacterial strains. 3 of the 47 extracts showed some weak activity against the Gram-negative *E. coli* strain and 2 extracts had activity against the Gram-positive *S. aureus* strain. This means these extracts have clinically relevant activity. Strains 18SM-2A, 18SM-3, 21AZ-7, and 21AZ-9 had the strongest activity against other ant-associated strains of *Pseudonocardia.* Surprisingly, a number of strains had activity against *Amycolatopsis* 17SM-2A, a strain that produces the antibiotic nocamycin V and that has been shown to be relatively resistant to ant-associated bacterial compounds.[18] Interestingly, some of these extracts performed better than the clinical antibiotics used as controls. Penicillin G has activity against Gram-positive bacteria, but it was found to have no activity against the ant-associated *Pseudonocardia* and *Amycolatopsis* strains (Table 1 and Appendix Table 3). This makes 18AZ-1a an even more intriguing target for isolating and characterizing the active molecule(s) responsible for this result as it has broader activity than Penicillin G.

None of the extracts had activity against fungus which was surprising based on previous studies which have shown that 17SM-2A and 17SM-8 produce a molecule called ECO-0501, with antifungal activity and the Panama strain GA6-1 produces a molecule called dentigerumycin with antifungal activity.[19,20] This could indicate that the methods used for extractions and activity testing should be updated for antifungal searches. It could also mean that the concentration of the antifungal molecules in the extracts was too small for activity to be seen. This would further suggest that not all possible activity can be seen when the crude extracts are tested in this way.

In terms of biogeography, the activity seen in this study is mostly limited to ants collected in the Chiricahua Mountains, AZ and Las Cruces, NM with only a few active strains from the Sierra Ancha Mountains, AZ. This could be due to the fact that the majority of the strains originate in these locations. As stated above, ant host also appeared to have an effect on the presence or absence of activity as *arizonensis* and *smithi* strains had the greatest prevalence and strongest activity. This could again be due to their disproportionately large inclusion in the study.

In order to better understand patterns and correlations in the chemical makeups of the extracts and their biogeographical information, metabolomics was done. The crude extracts were shot on a high-resolution Liquid Chromatography Mass Spectrometer (LCMS). The chromatograms were exported and analyzed in MzMine 2.53 and PCA plots were generated from a list of aligned features. Features are determined based on retention time and m/z tolerance. PCA plots show clusters of samples based on their similarities which are determined by aligned feature lists.[30] For LCMS data, this means using feature abundance levels across runs to determine the axes of the plot and the clustering of samples.[31] PCA plots are a common method for analyzing biogeographical trends in special metabolites and have been used in many other studies looking at other natural products sources of special metabolites.[28,32,33] In this experiment, PCA plots were used to understand if collection location or ant host influenced the chemical makeup of the extracts.

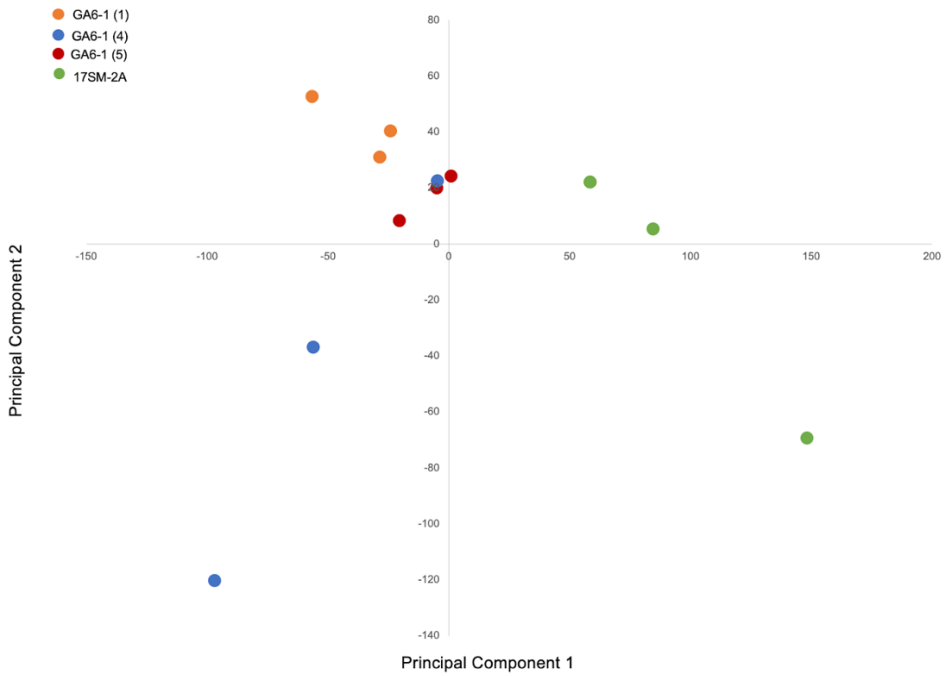


Figure 3. PCA plot showing the batch dependence and sample to sample variability of the extracts. Pictured here are triplicates from batches 1, 4, and 5. Also included is a strain of *Amycolatopsis*, 17SM-2A.

First, the effects of batch dependence and sample to sample variability had to be understood (Figure 3). GA6-1, an ant-associated bacterial strain from Panama that has been well-characterized, was grown in triplicate along with each of the five extraction batches. For batches 2 and 3, no bacterial growth was seen and so extracts were not made and do not appear here. Batches 1, 4, and 5 were plotted together with an *Amycolatopsis* strain to see if there were any batch effects and how large the sample-to-sample variability was. In general, the GA6-1 samples cluster together while the *Amycolatopsis* strain is separate as expected. Interestingly, the GA6-1 from batch 4 appears to have larger sample-to-sample variability indicated by the larger spread of these points on the PCA plots. Ideally, the variability would be quantified using scree plots and factor loading plots. Scree plots visualize how much variance each component explains and factor loading plots show how much a variable correlates with a component.[34] With these plots, the factors behind the variance in these plots could be better understood, but due to limitations

with the available software, they are not included here. PCA plot trends will instead be described qualitatively. For the same reasons, only the first two principal components are plotted, but these components should contain the greatest variability, so this is most likely the most valuable information regardless.

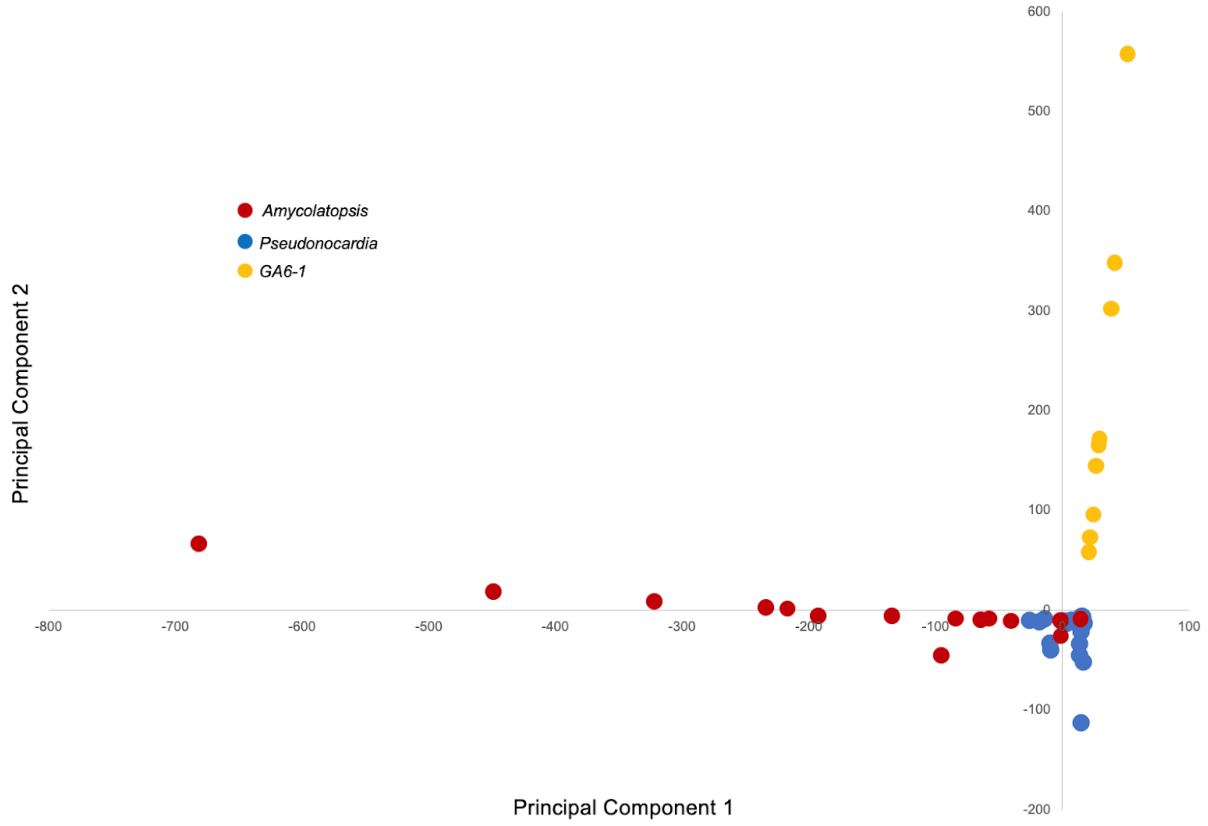


Figure 4. The similarities and differences in the *Amycolatopsis* (red) and *Pseudonocardia* (blue) strains. The GA6-1 strains in yellow are also *Pseudonocardia* strains, but they were collected in Panama and used as a control. Triplicates plotted.

To see the similarities and differences in the extracts, a PCA plot was generated with all extracts (Figure 4). As expected, the *Amycolatopsis* strains separated from the *Pseudonocardia* strains. Comparing the *Amycolatopsis* strains to the *Pseudonocardia* strains shows that the *Pseudonocardia* strains tend to cluster more tightly while there is more variation in the

Amycolatopsis strains. This suggests that *Pseudonocardia* strains may produce similar special metabolites while *Amycolatopsis* strains vary more in the metabolites they produce.

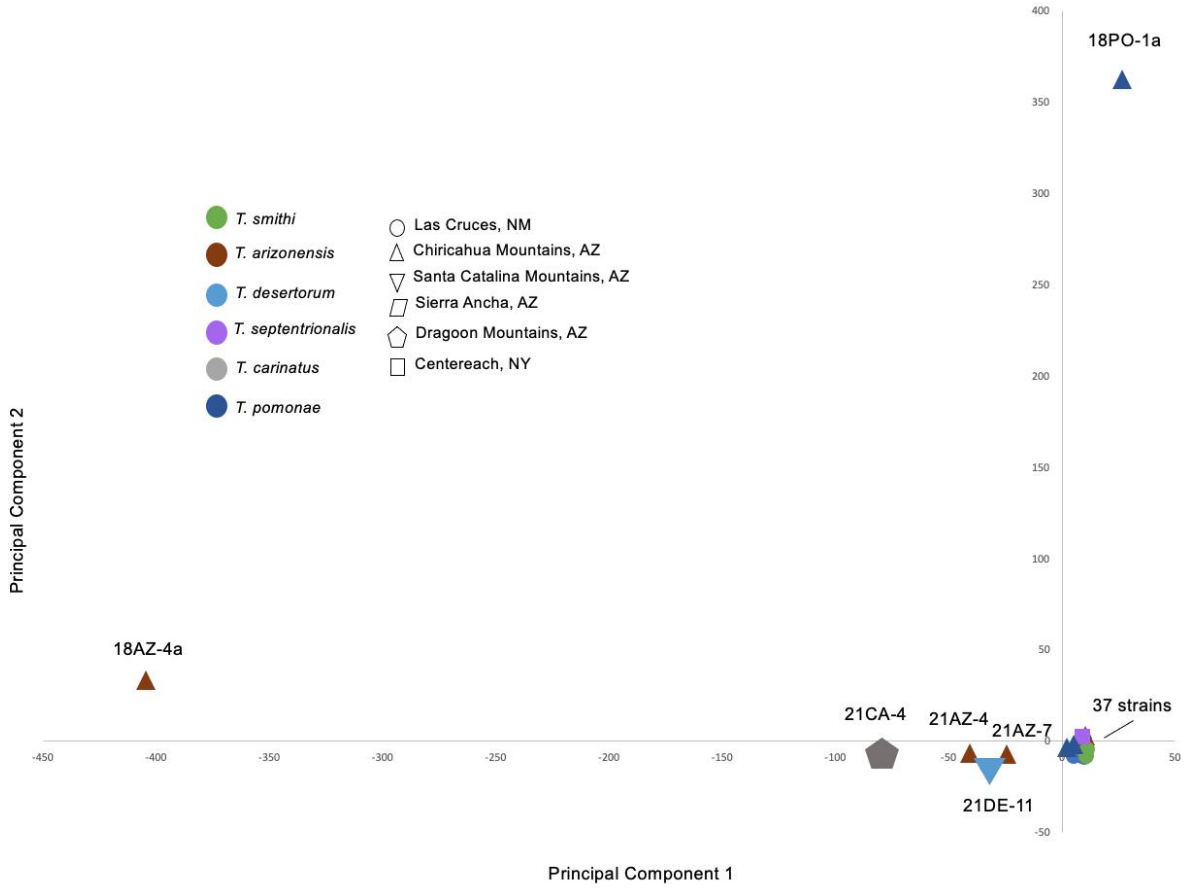


Figure 5. Ant hosts and collection locations for the *Pseudonocardia* strains. The ant hosts are represented by different colors while the collection locations are represented by different shapes. One replicate is plotted.

Similarities and differences in the extracts based on ant collection location and ant host were also analyzed. Because all but one of the *Amycolatopsis* strains were from Las Cruces, NM, and the *Pseudonocardia* strains had been collected from a variety of locations, the focus was placed on the *Pseudonocardia* strains to understand how collection location and ant species affect the metabolites produced by the strains. Strains collected from the Chiricahua Mountains—indicated by the triangles—tended to be more variable (Figure 5). This included

both *pomonae* strains and *arizonensis* strains. The distance of these points from others indicates that these strains likely produce distinct metabolites. As the primary metabolites for these strains are expected to be extremely similar, the distinct metabolites are likely secondary metabolites that should be more unique to individual strains. Overall, there are no substantial conclusions that can be made about the influence of ant host or collection location on the metabolic profile of the strains.

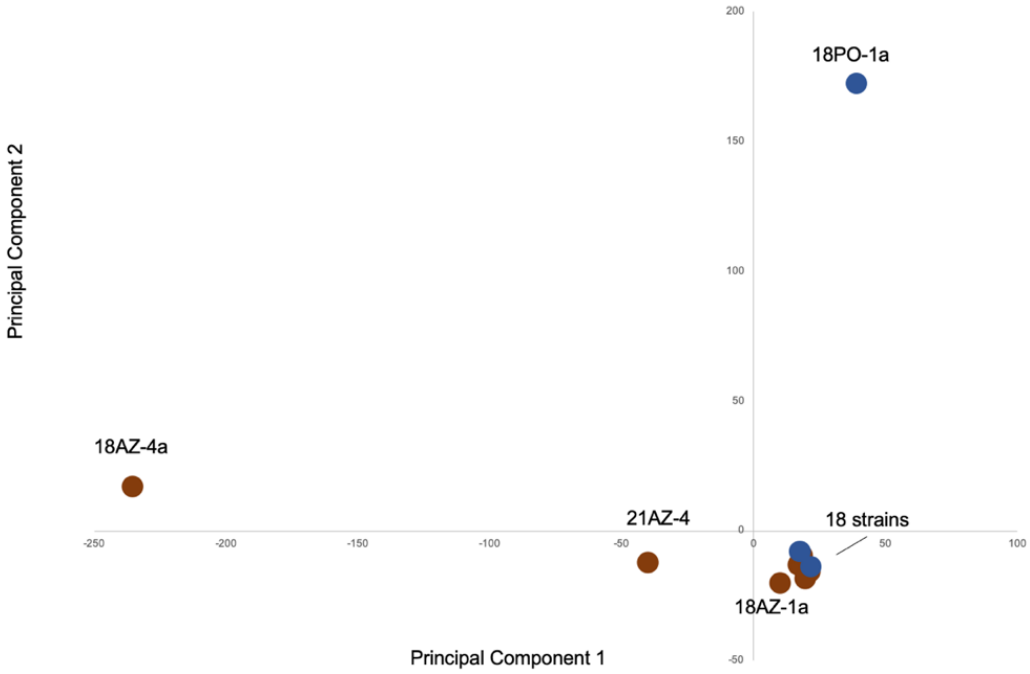
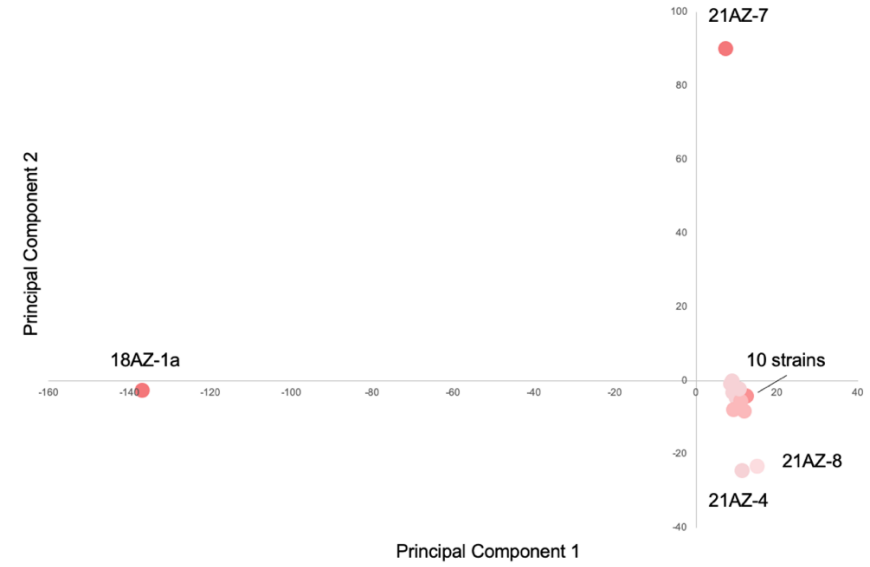


Figure 6. Strains collected from the Chiricahua Mountains. The *arizonensis* strains are red and the *pomonae* strains are blue.

The strains from the Chiricahua mountains had more variability when compared to the rest of the *Pseudonocardia* strains—although this could also be due to the fact that there are more strains from here than the other locations—so these extracts were plotted (Figure 6). These strains somewhat separate based on ant host, but any connections between ant host or collection

location and active compounds cannot be confidently confirmed when multiple species are plotted together as the majority of the extracts cluster near the origin. This shows that ant species must be considered individually when studying trends in activity.

A



B

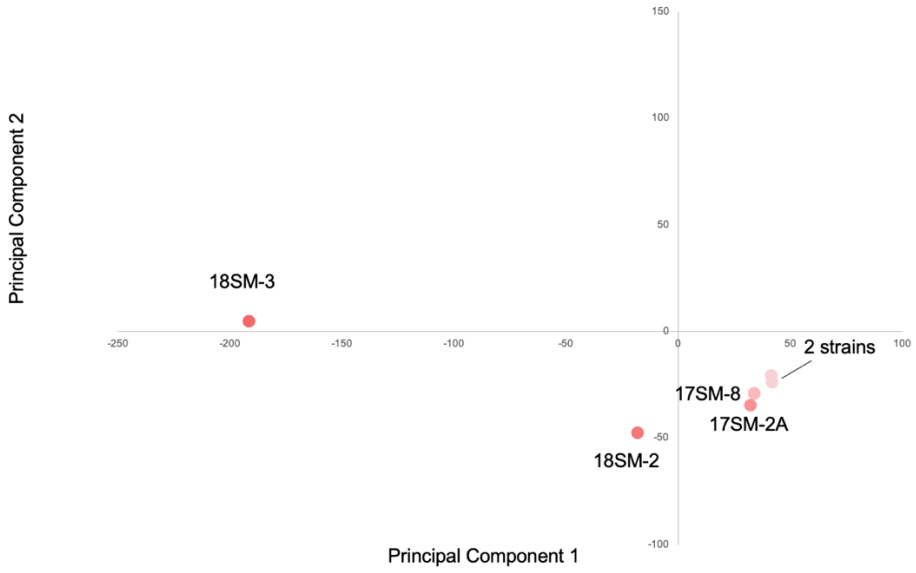


Figure 7. A. PCA plot showing relative activity of the *Pseudonocardia T. arizonensis* strains. Darker pink indicates greater total activity. Total activity was found by summing the centimeters of the zones of inhibition against all strains and using Excel conditional formatting to assign shades of pink to values. One replicate plotted. **B.** PCA plot showing relative activity of the *Amycolatopsis T. smithi* strains. Darker pink indicates greater activity. One replicate plotted.

When strains collected from one ant species are considered, active strains clearly separate from inactive strains suggesting the presence of unique compounds. This can be seen in Figure 7 where darker points represent more active strains. PCA plots such as these could be useful for strain prioritization if more strains are collected and analyzed in the future. It is important to note that not all strains that separated in PCA plots had measured activity. In Figures 5 and 6, a *pomonae* strain separates from other *Pseudonocardia* strains despite the activity data in Table 1 showing that this extract did not inhibit any strains of interest. This could be a result of noncomprehensive activity testing meaning that this strain produces unique metabolites that don't have activity against the panel of bacteria and fungus included in activity testing, but may have activity against other, more relevant strains. This could include strains from locations closer to the collection location for this particular *pomonae* strain.

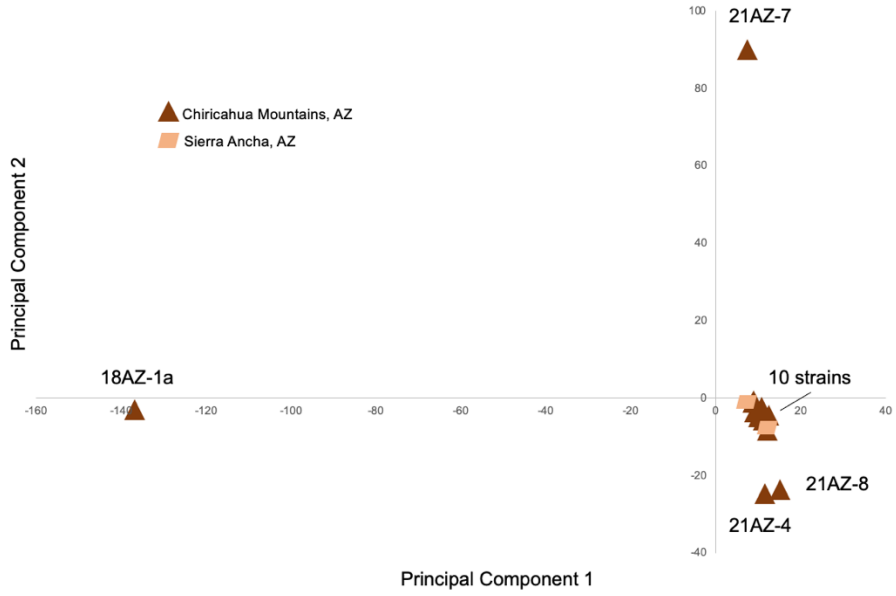


Figure 8. All *T. arizonensis* strains marked by location. Dark red triangles are strains from the Chiricahua Mountains and orange rhombuses are strains from Sierra Ancha.

Plotting all *arizonensis* strains to see trends in the collection location reveals that active strains (18AZ-1a and 21AZ-7) stand out, but that there does not appear to be any relationship between the metabolites present and the collection location of the strain (Figure 8).

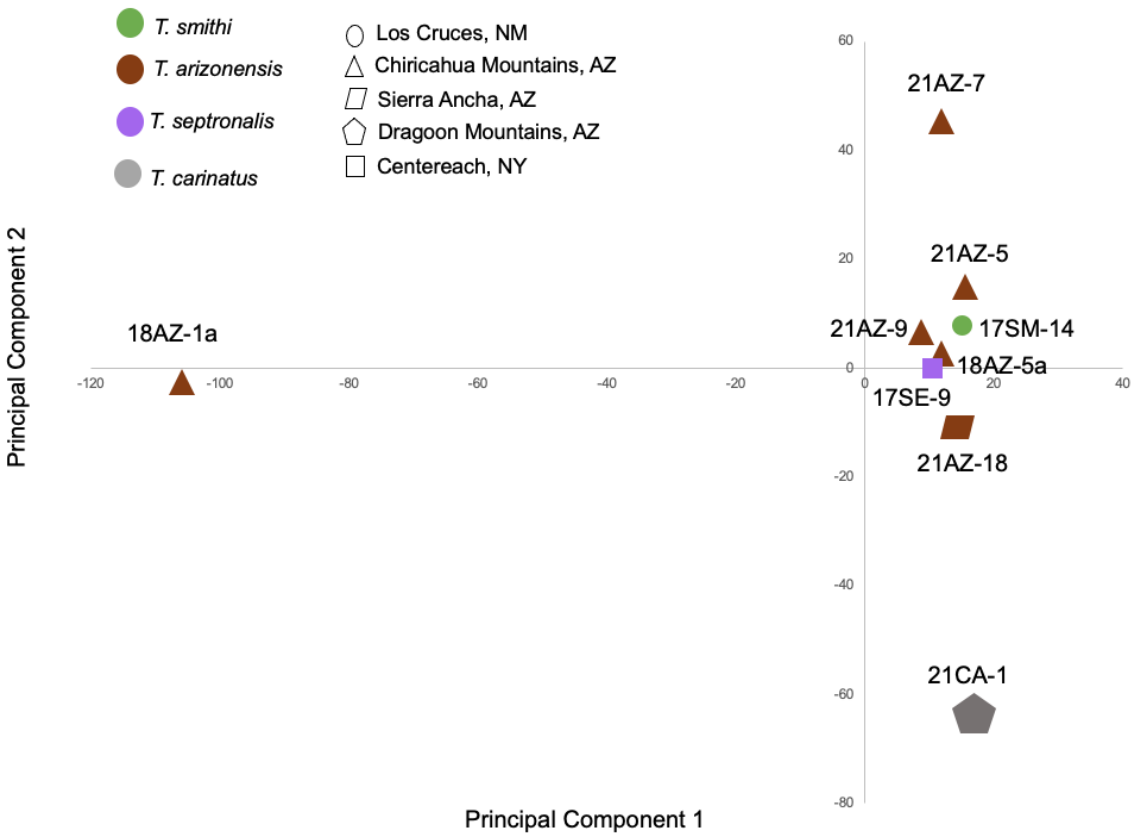


Figure 9. All *Pseudonocardia* strains with activity against any of the microorganisms of interest. As above, shape corresponds to location and color corresponds to ant species.

In looking more closely at strains with activity to see if they cluster based on ant host or collection location, strains do not appear to cluster based on either of these factors, but rather based on activity profile (Figure 9). The strains on the right all had activity against ecologically relevant strains 18PO-3 and 17SM-1 while the one strain on the far left, 18AZ-1a, also had

clinical activity. With this in mind, the active strains were separated into activity profiles: strains with Gram-positive clinical activity, strains with Gram-negative activity, strains that only had activity against *Pseudonocardia* strains, and strains that had activity against *Amycolatopsis* as well as *Pseudonocardia.*

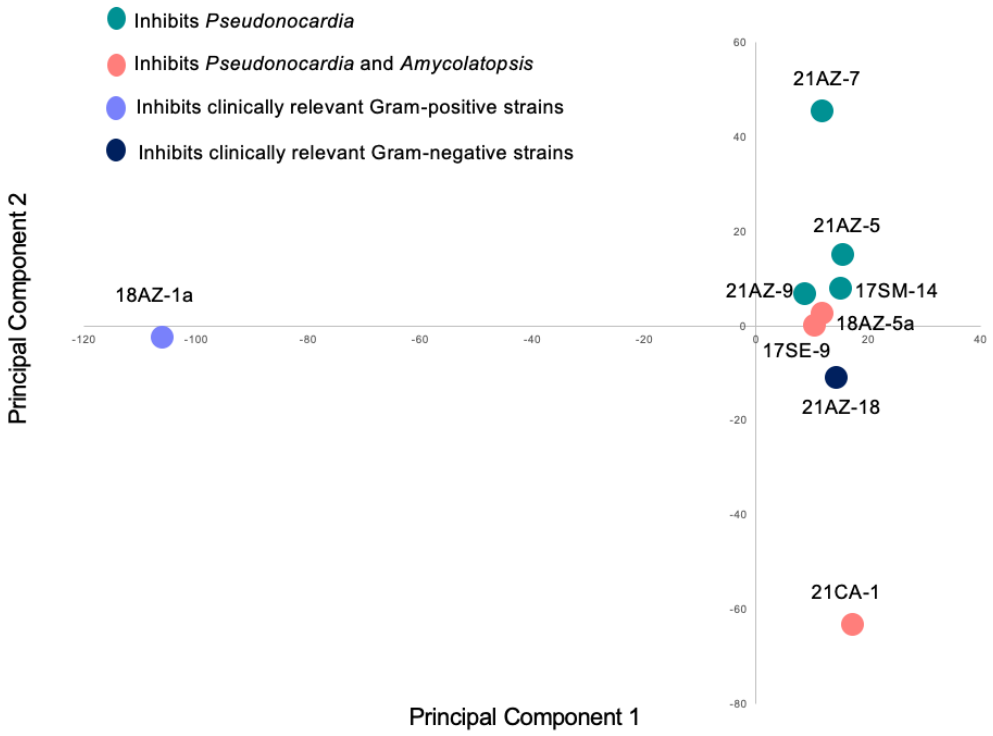


Figure 10. PCA plot of all active *Pseudonocardia* strains marked by activity profile. Activity profiles were chosen based on activity results in Table 1.

All active *Pseudonocardia* strains were plotted again but marked by activity profile in order to visualize how similar the chemical makeups of activity profiles are. (Figure 10). Strains with the same activity profiles tended to cluster, indicating that the same compounds may be present in the extracts and responsible for the activity seen. Importantly, 18AZ-1a separates from the rest of the extracts. This strain was the only strain with clinically relevant activity against *S.*

aureus, and this result suggests that the compound or compounds in the extract responsible for the activity seen are quite different than the compound(s) responsible for the ecologically relevant activity. The strain 21AZ-18 had weak activity against *E. coli*, but stronger activity against *Pseudonocardia* and *Amycolatopsis*, possible explaining why the extract clusters more closely with extracts that inhibit those strains (Table 1). This result is very important for strain prioritization as it shows that chemical characterization may be redundant for strains sharing an activity profile. The process of isolating active compounds is labor intensive and making this process as efficient as possible will help future studies focused on specific active metabolites.

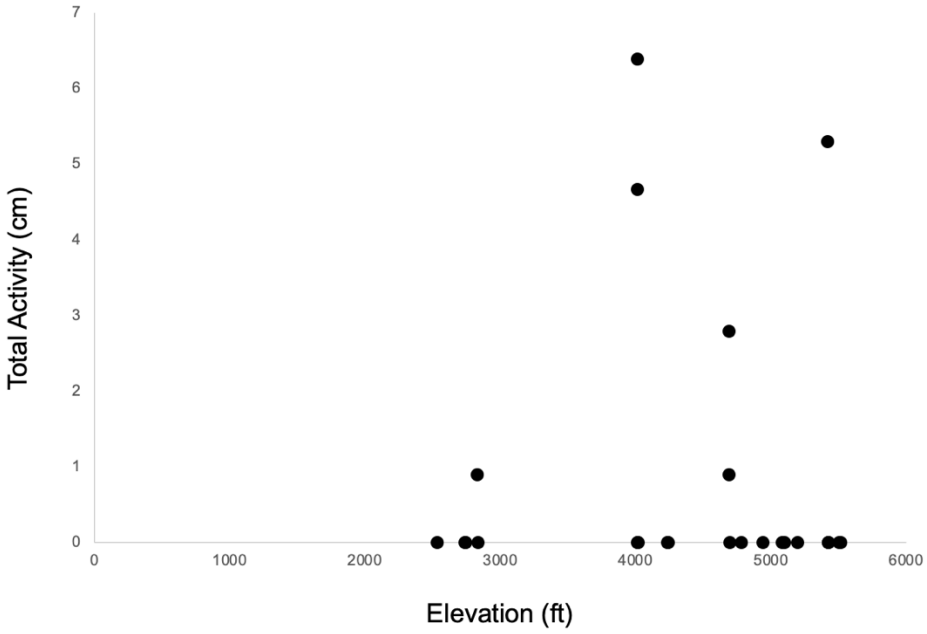


Figure 11. Linear regression plot of total activity versus elevation of collection location for all strains.

Finally, the relationship between the habitat of the collection location and the activity of the strains collected there was examined using a linear regression plot (Figure 10). The elevation of the collection location was plotted against the total activity of the strain (a sum of all the

activity a strain had). The elevation is a good measure of what the local habitat is like because of the unique area these ants live in. For example, over 4,500 feet, the ants live in a mixed oak forest habitat rather than a desert habitat.[23] There was no correlation between the amount of activity a strain had and the elevation at which it was collected, suggesting that there is no relationship between the local habitat and the amount of activity strains in that area have. It is possible that the type of active molecules present in different habitats varies, but further studies would need to be completed to understand if this is true.

Significance

This large-scale search for active molecules and analysis of the biogeography of these active molecules revealed the discovery potential for this under-explored desert niche. Several active extracts were found through activity testing including extracts with ecologically relevant activity, clinically relevant activity, or both. While currently the majority of these molecules remain uncharacterized, the unique desert environment suggests that these compounds will also be novel molecules or compounds not found in ant-associated bacteria before. Activity-guided fractionation using an HPLC can be used to purify active compounds from the extracts and then NMR can be used to elucidate their structures.

The discovery of active compounds that have clinically relevant activity is important as these molecules have the potential for use against antibiotic-resistant bacteria if they are novel. If they are not novel compounds, but instead existing antibiotics, this will provide a novel ecological role for these compounds. Comparing the clinical activity to the ecological activity of compounds provides a better understanding of the compound itself and how it may function as an antibiotic.

Further analysis is needed to completely understand the biogeography of these compounds. For example, a detailed analysis of the presence or absence of known compounds through the niche will be necessary for understanding what factors influence the production of certain molecules. It will also be important to perform genomics with as many active strains as possible to understand the phylogenetic history of these bacteria and their relationships to one another. From this data, it also seems that another large-scale collection trip would help answer

lingering questions about the effect of biogeography. More ants from fewer locations or the same ant species from several locations would be two ways to approach this.

With the data presented here, it is possible to say that the ant host, specifically the ant genus, has an effect on what kinds of molecules are produced. Further study of the LCMS data is needed to accurately parse all the information contained in the PCA plots generated, however, the PCA plots have proven to be a useful tool for predicting the presence or absence of active compounds for bacteria from future collection trips. From the activity plots, it can be seen that strains with active compounds tend to separate from inactive strains. Strains also tend to cluster based on activity profile. This metabolomics approach could inform strain prioritization in future studies.

Overall, this study has identified strains with active compounds that need to be characterized, for example: 18AZ-1a, the strain with clinical and ecological activity. Genomics analysis to search for biosynthetic gene clusters and chemical characterization are the next step for active strains.

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Appendix

Table 3. Information about ant host species and collection location for all bacterial strains included in the study.

Strain	Host Species	Bacterial Genus	Collection Location
17SM-1	*smithi*	*Pseudonocardia*	Las Cruces
17SM-2A	*smithi*	*Amycolatopsis*	Las Cruces
17SM-3	*smithi*	*Pseudonocardia*	Las Cruces
17SM-6	*smithi*	*Pseudonocardia*	Las Cruces
17SM-8	*smithi*	*Amycolatopsis*	Las Cruces
17SM-8B	*smithi*	*Amycolatopsis*	Las Cruces
17SM-14	*smithi*	*Pseudonocardia*	Las Cruces
17SM-15	*smithi*	*Pseudonocardia*	Las Cruces
18SM-1	*smithi*	*Amycolatopsis*	Las Cruces
18SM-2A	*smithi*	*Amycolatopsis*	Las Cruces
18SM-3A	*smithi*	*Amycolatopsis*	Las Cruces
18SM-3B	*smithi*	*Amycolatopsis*	Las Cruces
18SM-3C	*smithi*	*Amycolatopsis*	Las Cruces
18SM-5A	*smithi*	*Pseudonocardia*	Las Cruces
18SM-5B	*smithi*	*Pseudonocardia*	Las Cruces
18SM-6	*smithi*	*Pseudonocardia*	Las Cruces
18SM-7A	*smithi*	*Pseudonocardia*	Las Cruces
18SM-7B	*smithi*	*Pseudonocardia*	Las Cruces
18SM-7C	*smithi*	*Pseudonocardia*	Las Cruces
17SE-3	*septronalis*	*Pseudonocardia*	Centereach
17SE-9	*septronalis*	*Pseudonocardia*	Centereach
18PO-1a	*pomonae*	*Pseudonocardia*	Chiricahua Mtns
18PO-2a	*pomonae*	*Pseudonocardia*	Chiricahua Mtns
18PO-4a	*pomonae*	*Pseudonocardia*	Chiricahua Mtns

21PO-1	*pomonae*	*Pseudonocardia*	Chiricahua Mtns
21PO-2	*pomonae*	*Pseudonocardia*	Chiricahua Mtns
21DE-1	*desertorum*	*Pseudonocardia*	Santa Catalina Mtns
21DE-1X	*desertorum*	*unknown*	Santa Catalina Mtns
21DE-2	*desertorum*	*Pseudonocardia*	Santa Catalina Mtns
21DE-6X	*desertorum*	*Amycolatopsis*	Sierra Ancha Mtns
21DE-8	*desertorum*	*Pseudonocardia*	Santa Catalina Mtns
21DE-11	*desertorum*	*Pseudonocardia*	Santa Catalina Mtns
18AZ-1a	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
18AZ-4a	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
18AZ-5a	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-3	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-4	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-5	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-6	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-7	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-8	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-9	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-11	*arizonensis*	*Pseudonocardia*	Chiricahua Mtns
21AZ-17	*arizonensis*	*Pseudonocardia*	Sierra Ancha Mtns
21AZ-18	*arizonensis*	*Pseudonocardia*	Sierra Ancha Mtns
21AZ-19	*arizonensis*	*Pseudonocardia*	Sierra Ancha Mtns
21CA-1	*carinatus*	*Pseudonocardia*	Dragoon Mtns
21CA-4	*carinatus*	*Pseudonocardia*	Dragoon Mtns
21CA-5	*carinatus*	*Pseudonocardia*	Dragoon Mtns
21CA-10	*carinatus*	*Pseudonocardia*	Dragoon Mtns
21CA-13	*carinatus*	*Pseudonocardia*	Sierra Ancha Mtns
21CA-15	*carinatus*	*Pseudonocardia*	Sierra Ancha Mtns
GA6-1 (1)	*dentigerum*	*Pseudonocardia*	Panama
GA6-1 (4)	*dentigerum*	*Pseudonocardia*	Panama
GA6-1 (5)	*dentigerum*	*Pseudonocardia*	Panama

Table 3. Zones of inhibition for antibiotic controls. The diameter of the zone was measured.

Antibiotic	Amount (μg)	Microorganism	Zone of Inhibition (cm)
Penicillin G	10	*S. aureus*	4.0
Penicillin G	10	*Pseudonocardia* 17SM-1	0.0
Penicillin G	10	*Pseudonocardia* 17SM-1	0.0
Penicillin G	10	*Amycolatopsis* 17SM-2A	0.0
Streptomycin	10	*E. coli*	0.0
Nystatin	50	*C. albicans*	2.2
Nystatin	50	*Escovopsis sp.*	4.2

Figure 12. All strains grown for extractions. Order is reflected in Table 2. Seemingly larger plates have no significance, they are the same size, but those photos were taken by a different lab member.

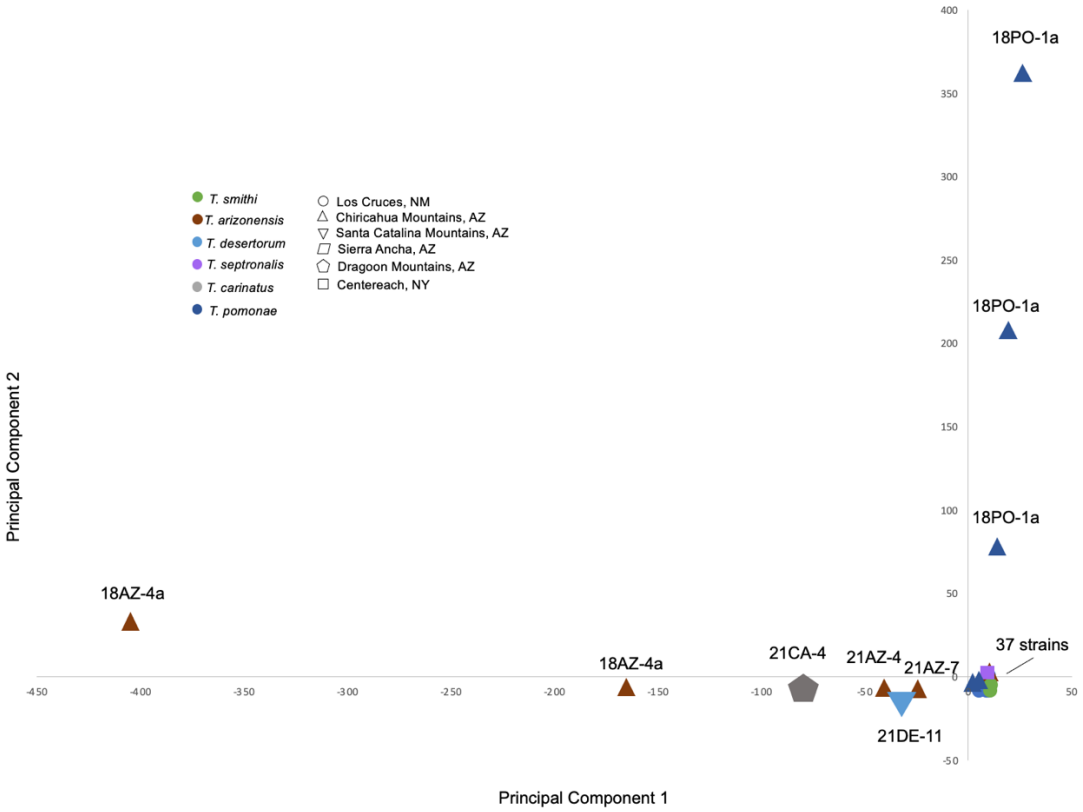

Figure 13. PCA plot of all *Pseudonocardia* strains with triplicates included for comparison with the *Pseudonocardia* plot in Results/Discussion.