COVER SHEET

TITLE: Phenylacetic acid degradation regulation in Acromyrmex ant-associated and non-ant

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ABSTRACT

Phenylacetic acid degradation regulation in *Acromyrmex* ant-associated and non-ant associated *Pseudonocardia* strains in the context of pathogen co-cultivation

The *Acromyrmex* ant agriculture system is a highly evolved symbiosis that involves four organisms; the Attine ants, the fungal cultivars, the bacterium *Pseudonocardia,* and the fungal pathogen *Escovopsis.* The bacterium *Pseudonocardia* is found on the surface of the *Acromyrmex* ants' exoskeleton and is known to produce antimicrobial compounds against the parasitic fungus *Escovopsis.* Phenylacetic acid (PAA) is a compound of interest because Attine ants produce it, *Pseudonocardia* strains have a conserved PAA degradation gene cluster, and it has showed greater growth suppression of *Escovopsis* than when compared to other pathogens. I planned analyze differential regulation of PAA catabolism expression in *Pseudonocardia* strains isolated from ant colonies and strains isolated from non-ant sources in the context of co-cultivation with *Escovopsis*, a generalist fungal pathogen *Trichoderma*, or no pathogen.

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Phenylacetic acid degradation regulation in *Acromyrmex* ant-associated and non-ant associated *Pseudonocardia* strains in the context of pathogen co-cultivation

Abstract

The *Acromyrmex* ant agriculture system is a highly evolved symbiosis which involves four organisms; the Attine ants, the fungal cultivars, the bacterium *Pseudonocardia,* and the fungal pathogen *Escovopsis*[1]. The bacterium *Pseudonocardia* is found on the surface of the *Acromyrmex* ants' exoskeleton and is known to produce antimicrobial compounds against the parasitic fungus *Escovopsis*[1,2]. Some strains of *Pseudonocardia,* however, are not found in association with Attine ants[3]. Phenylacetic acid (PAA) is a compound of interest because Attine ants produce it, *Pseudonocardia* strains have a conserved PAA degradation gene cluster, and it has showed greater growth suppression of *Escovopsis* than when compared to other pathogens[4,5]. By introducing *Escovopsis*, a pathogen specific to the Attine ant agricultural system, to *Pseudonocardia,* I expected that the PAA degradation pathway would be upregulated as compared to inoculation with *Trichoderma*, a generalist fungal pathogen. I also planned to compare this pathway's expression in *Pseudonocardia* strains isolated from ant colonies and strains isolated from non-ant sources. I planned to use Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) technology to compare the regulation of PAA degradation between treatments and between strains. By analyzing the threshold cycle (C_T) values generated from the RT-qPCR tests, I would be able to determine whether the expression of PAA degradation differs significantly when *Pseudonocardia* is grown with *Escovopsis, Trichoderma*, or without a pathogen and if PAA degradation regulation is consistent among ant-associated and non-ant-associated strains.

Introduction

The Currie Lab's extensive research into the Attine ant agriculture system offers an ideal study system to examine how chemical compounds are used in a complex symbiosis. Attine ants, commonly called leaf-cutter ants, are a tribe of ants that have evolved to cultivate fungal gardens for food[6]. The Attine leaf-cutter ants have evolved adaptations for pathogen defense of their fungal cultivars through individual and social hygiene behaviors as well as a mutualistic relationship with the antibiotic-producing bacterium *Pseudonocardia* [7].

Atta ants are a genus of fungus-farming ants that do not have the symbiotic relationship with *Pseudonocardia,* and logically it is expected *Atta* ants evolved some other form of disease control[7]. It is thought that *Atta* ants defend their fungal garden by producing chemicals such as phenylacetic acid (PAA) in their metapleural glands[7]. Because *Atta* ants produce PAA, Fernández-Marín et al. conducted bioassays which provided evidence that PAA had greater inhibitory effects against the fungal pathogen specific to the ant agricultural system, *Escovopsis*, as opposed to the generalist fungal pathogen *Trichoderma* [5,6,8]. Not only did this study suggest that PAA has application as a prophylactic, but that PAA has differential inhibition depending on the pathogen. Another study, by Mendoça et al., showed that PAA extracted from *Atta* ants has antimicrobial effects against the pathogens *Escherichia coli* and *Staphylococcus aureus*[9].

Unlike the *Atta* ant agricultural system, other Attine ants in the genus *Acromyrmex* use the antibiotic-producing bacteria, *Pseudonocardia,* to protect their fungal gardens from the fungal pathogen *Escovopsis* [6,8,]. Although chemical analyses of the compounds produced by ants associated with *Pseudonocardia* show that these ants do not produce PAA in their metapleural glands, studies have identified that *Pseudonocardia* has a conserved PAA catabolic pathway [4,9,10] The process of PAA degradation by *Pseudonocardia* may also be a defense mechanism because certain intermediates of PAA catabolism are toxic[4]. Preliminary data shows that *Pseudonocardia* appears to upregulate its PAA degradation cluster in the presence of *Escovopsis* relative to growth without a pathogen.

For my thesis, I attempted to test the expression of the PAA pathway across several *Pseudonocardia* strains associated with *Acromyrmex* ants and several *Pseudonocardia* strains not associated with ants in co-cultivation with either *Escovopsis* or *Trichoderma* to gain a better understanding of the role PAA plays within the Attine ant agricultural system. This comparison will bring a new perspective to the theorized co-evolution of *Pseudonocardia* and *Acromyrmex* ants because if ant-associated *Pseudonocardia* strains show an increased upregulation of PAA degradation in response to *Escovopsis,* it would suggest that ant-associated strains of *Pseudonocardia* have evolved better pathogen recognition and defense responses to pathogenic threats specific to the *Acromyrmex* ant agriculture system than those strains found outside of the system.

Furthermore, measuring gene expression is a method used to understand the synthesis or breakdown of a compound by an organism. There is a correlated increase in functional activity when a gene cluster is upregulated or transcribed more frequently. Although it is difficult to quantify the amount of actual chemical produced, Real-Time Quantification Polymerase Chain Reaction (RT-qPCR) technology can measure the expression of the PAA degradation pathway. Essentially, quantifying the activation of this pathway can be used as a proxy for measuring the amount of PAA degradation or synthesis activity that is occurring. I expect that although the PAA catabolism gene cluster is conserved, the regulation of the cluster will be ecologically dependent on the context in which the *Pseudonocardia* strain evolved. Thus, I expect that the ant-associated strain would have a greater upregulation of the PAA catabolic pathway in response to *Escovopsis* than non-ant-associated *Pseudonocardia* strains. I expect that because *Trichoderma* is not ecologically specific, the PAA degradation gene cluster would have the same regulation in all *Pseudonocardia* strains. Investigating the expression of this gene region in different strains and in different co-cultivation conditions, I hoped to provide insight into the function of this conserved gene cluster.

Methods

Creating a Phylogenetic Tree of the *Pseudonocardia* Strains

For my study, I used eight *Pseudonocardia* strains. Four of the *Pseudonocardia* strains were isolated from ants. These strains were: ICBG1042-Acromyrmex, ICBG1052-Cypho, ICBG1146-Trachy, and CC031209-02. The four non-ant associated *Pseudonocardia* strains were: *Pseudonocardia spinosispora*, *P. saturnea*, *P. nitrificans*, and *P. antarctica*. I created a phylogenetic tree for six of the eight strains using 16s rRNA genes sequenced by other members of my lab. I aligned the 16s sequences using MAFFT software and used FastTree software to make the tree[11,12]. I used the Interactive Tree of Life (iTOL) website to visualize the phylogenetic tree and add colors to denote if the strains were ant-associated or non-ant associated[13].

Inoculation of *Pseudonocardia* for Bioassays

Before beginning the pathogen inoculations, I cultivated the eight previously isolated *Pseudonocardia* strains at room temperature on Yeast Malt Extract Agar (YMEA) plates. I began the pathogen inoculations two weeks into growing the *Pseudonocardia* strains. Under sterile conditions, I inoculated five plates of *Pseudonocardia* with *Escovopsis* and five plates with *Trichoderma* for each strain. As my negative control, I inoculated five plates of each strain with a pathogen. The *Pseudonocardia* was grown in the center of the plate and I streaked the pathogen around the outer circumference of the plate as seen in **Figure 2**.

Zone of Inhibition Measurements and Isolation of RNA

After four days of cultivating the *Pseudonocardia* strains with *Trichoderma,* I measured the zone of inhibition by taking a ruler and measuring, in centimeters, the distance between the edge of *Pseudonocardia* to the edge of the *Trichoderma* growth. Then, I scraped a sample from each biological replicate into an Eppendorf tube. Because *Trichoderma* grows faster than *Escovopsis,* I co-cultivated the *Pseudonocardia* strains with *Escovopsis* for seven days and then measured the zones of inhibition and collected the *Pseudonocardia* samples into Eppendorf tubes for RNA isolation. If I found that the plate was contaminated during the collection of *Pseudonocardia,* I would disregard the zone of inhibition measurement, discard the plate, and not collect the *Pseudonocardia* sample. I stored the *Pseudonocardia* samples in an -80 degree freezer to preserve the samples before RNA extraction. I used a phenol:chloroform RNA extraction method that I modified to include two additional mechanical lysing steps utilizing a bead beater and sterilized pestle. The full RNA extraction procedure is attached for future use. **Figure 3** shows the results from a pilot test RNA extraction using this modified procedure. I broke up the samples into RNA extraction groups of approximately 16, careful to distribute the treatments and samples evenly throughout the treatment groups. After the extraction, I tested the quantity and quality of the RNA using Nanodrop.

Transcription of cDNA and qPCR

After the isolation of the RNA, I planned to synthesize cDNA from the RNA samples using Epicentre's MonsterScript^{TM} 1st Strand cDNA Synthesis Kit[14]. Using two primers (gyrA and rpoD) for housekeeping genes as the control and a paaA primer specific to the PAA degradation gene in *Pseudonocardia,* I planned to conduct Real Time Quantitative Polymerase

Chain Reaction (RT-qPCR)[15]. I used the following thermocycler conditions for the PCR: 1) 96.0°C for 2 minutes. 2) 94.0°C for 30 seconds. 3) 59.3°C for 30 seconds. 4) 72.0°C for 1 minute. 5) Go to step 2, repeat 35 times. 6) 72.0°C for 10 minutes. 7) 4.0°C forever.

Data Analysis

I intended to use the threshold cycle (C_T) results of my RT-qPCR test to compare the quantity of the PAA degradation genes transcribed between treatments. Once I attained the threshold cycle values, I would have run a moderate t-test to determine if the C_T values were indicative of a significant upregulation of the PAA degradation genes when *Pseudonocardia* is in the presence of *Escovopsis* or *Trichoderma.* I would have then compare the RT-qPCR results between the *Pseudonocardia* strains associated with the Attine ants and the *Pseudonocardia* strains isolated from soil and plant samples. My analysis would have followed the MIQE guidelines so that my research would be consistent and comparable to other research publications using qPCR [16].

Results

The phylogenetic tree shown in **Figure 1** includes six of the eight *Pseudonocardia* strains. The ant-associated strains are highlighted in yellow and the non-ant associated strains are highlighted in blue. The other two strains are excluded because their 16S sequences were not available at the time I made the tree. The tree shows that *P. antarctica,* a non-ant associated strain, and ICBG1146-Trachy, an ant associated strain, are closely related.

During the first round of pathogen inoculations, many of the plates of *P. saturnea, P. nitrificans*, and *P. antarctica,* and Strain CC031209-02 were contaminated. In response to the contamination, I replicated the treatments of those strains. The three different treatment plates of Strain CC031209-02 are shown in **Figure 2** for reference of the inoculations.

The results of the zone of inhibition measurements are shown in **Figure 3**. The graph to the left depicts the average zone of inhibition measurements for each strain in the *Escovopsis* treatment, whereas the graph to the right shows the measurements in the *Trichoderma* treatment. The ant-associated strains are denoted by the underline. The average zone of inhibition for the *Escovopsis* treatment is 2.79cm with a standard deviation of 0.68cm, whereas the average zone of inhibition for the *Trichoderma* treatment is 0.33cm with a standard deviation of 0.32cm. Using a two-sample Student's t-test with unequal variance and 53 degrees of freedom, the means of the two treatments were found to be significantly different (**p <.001**) with a confidence interval of 99%[17].

In a Technical Bulletin about NanoDrop Spectrometer results, ThermoScientific notes that a 260/280 nanometer absorbance "ratio of ~2.0 is generally accepted as "pure" for RNA" and the secondary measure, 260/230 nm ratio, should fall within the range of 2.0-2.2[18]. The results of my NanoDrop analysis for the RNA extractions averaged 1.95 for the 260/280 ratio and 2.05 for the 260/230 ratio, with an average quantity of 1280 ng/uL. I recorded the labels according to treatment and *Pseudonocardia* strain, as well as quantity and quality of the RNA samples in an excel sheet titled "RNA Extraction Groups" for the next student to use. **Figure 4** shows the results of the Electrophoresis Summary obtained from the Bioanalyzer, providing evidence that the modified RNA extraction procedure is effective as the bands are consistent around the 3000-nucleotide mark, as would be expected given the primer length.

In October, I tested primers paaA, rpoD, and gyrA using a *Pseudonocardia* cDNA template synthesized from a previous semester to confirm that 59.3°C is an effective annealing temperature for all three primers. The results of that PCR trial run are seen in **Figure 5**; the fluorescent yellow bars in cells 6-9 indicate that all three primers amplified the template properly. In late March, I ran a pilot run of the cDNA synthesis and PCR primer testing using *Pseudonocardia* templates isolated from this experiment. The results of the March PCR test, pictured in **Figure 6**, show that although paaA amplified the cDNA template, the housekeeping primers, rpoD and gyrA, did not. My intention for the pilot run was to find the best dilution measure for the cDNA. The results of the PCR test, shown in **Figure 6**, found that only the paaA primer amplified the samples from non-diluted 39A and 10x dilution of 39A, but not the 100x or 1000x dilutions. Multiple attempts at replicating the cDNA synthesis and PCR tests continued to result in the housekeeping primers, gyrA and rpoD, yielding no amplified product. After replicating the PCR with new stock primers of gyrA and rpoD, the gel picture in **Figure 7** demonstrates that, although the housekeeping primer gyrA now amplified the sample, the other control primer, rpoD, did not amplify the sample properly, nor did the experimental primer, paaA, which previously worked.

Due to the time spent on troubleshooting the primers, I have not yet conducted cDNA synthesis on all of my samples yet. Consequently, I have not been able to run the RT-qPCR and so I do not have the differential transcription results yet for analysis.

Discussion and Future Directions

By looking at the evolutionary relationship between the 16s sequences for the strains on the phylogenetic tree, I found that *P. antarctica,* a non-ant associated strain, and ICBG1146-Trachy, an ant associated strain, are closely related. This observation indicates that ant-association relationship may not be predictive of evolutionary relatedness. This insight will add context when analyzing the paaA catabolism regulation data. If PAA catabolism regulation is consistent among ant-association relationships, this is supportive that PAA catabolism is an adaptation useful in the Attine ant agricultural system. If the regulation is inconsistent amongst the ant-associated strains, this evolutionary history provides an alternative explanation to the evolution of PAA catabolism regulation since *Pseudonocardia* strain relatedness is not specifically tied to ant-association.

Because I started collecting the measurements for zone of inhibition midway into the experiment, I do not have measurements for both pathogen treatments for all of the strains. If a student were to further my experiment, I would encourage the student to expand the pathogen co-cultivation sample size to more extensively collect the measurements of zone of inhibition for the strains in each treatment. Although the treatments resulted in significantly different zones of inhibitions, this could be influenced not only by the defense chemicals *Pseudonocardia* produces, but also by the fungal pathogens' growth rates. *Trichoderma* is a faster growing pathogen than *Escovopsis,* as noted in the different times of co-cultivation, so replicating this experiment with an increased number of fungal pathogens, while accounting for each pathogen's rate of growth, could control for this confounding variable.

Since both ratio measurements meet the NanoDrop standards, the quality of the RNA samples is sufficient for RT-qPCR[16,18]. Although the PCR primer conditions originally worked on all three primers in October, pictured in **Figure 5**, the housekeeping primers failed to amplify the template when I replicated the PCR in March. Two subsequent attempts to replicate the PCR were unsuccessful due to technical malfunctions with the PCR thermocycler machine. In the first malfunction, the air conditioning of the thermocycler failed, so the exact temperatures were not adhered to and the amplification failed. The second malfunction occurred when using a new thermocycler machine, the lid was not closed properly and again, the procedure failed.

After the third attempt at replication shows unchanged results to the March test in **Figure 6**, I began to troubleshoot specific steps in the PCR procedure. I considered that because all three primers worked properly in October, yet failed in March, the primers might have expired. Therefore, I ordered new stock samples of gyrA and rpoD and repeated the PCR test with the new primers. The results, shown in **Figure 7**, indicated that while the expiration explanation may be the case for gyrA, since gyrA did amplify the template in the most recent PCR test, it is likely not the case for rpoD, which is again unsuccessful at amplifying the template. Unexpectedly, the paaA primer also fails to amplify the template in this PCR run. Although the annealing temperature 59.3°C was thought to work across all three primers, given the results of the October PCR test, it is worth running a temperature gradient with all three primers, paaA, gyrA, and rpoD, to determine the most effective annealing temperature. Additionally, I would encourage a future student to modify the PCR procedure to test if increasing the amount of template used for the PCR increases the amplification results. Similarly, the student could also try altering the amount of primer used to see if this alters the PCR results. Once all three primers are amplifying the sample properly, the student is able to continue the experiment by synthesizing cDNA from the RNA samples and carry on the qPCR test and threshold value analysis.

Because the RT-qPCR portion of the experiment has not been conducted yet, I still do not know if PAA catabolism regulation varies significantly depending on pathogen co-cultivation and ant-association relationship. In the future, the regulation of PAA catabolism by *Pseudonocardia* should be compared to the zone of inhibition data to determine if there is a correlation between upregulation of the paaA pathway and pathogen growth inhibition. This information can be used to better understand the role and evolution of PAA in Attine leaf-cutter ant defense mechanisms.

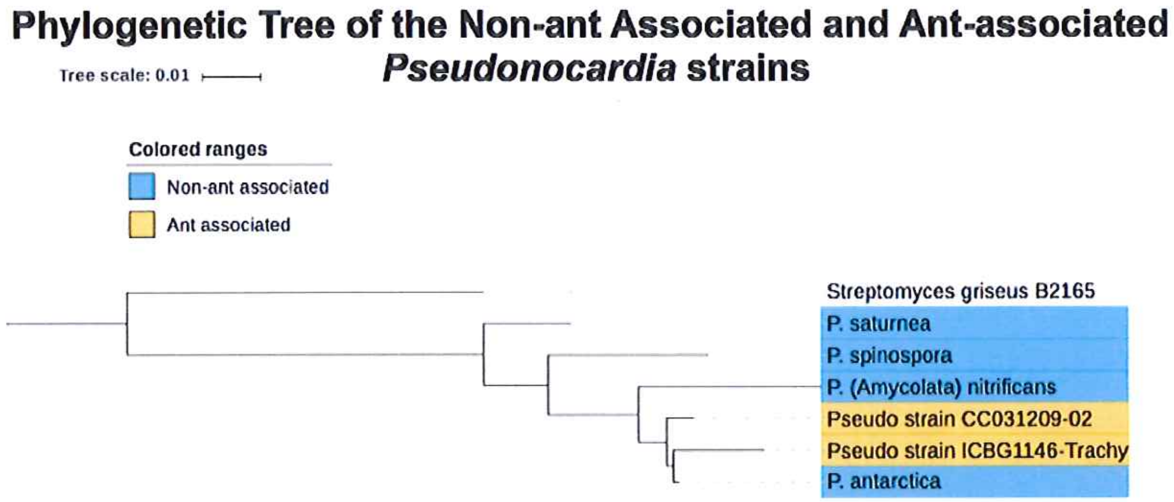

Figure 1. This phylogenetic tree, based on 16s sequences, represents the evolutionary relationship between the non-ant associated *Pseudonocardia* strains (in blue) and ant-associated *Pseudonocardia* strains in yellow. This tree shows that not ant-association does not necessarily predict evolutionary closeness, as *P. antarctica,* a non-ant associated strain, and ICBG1146-Trachy, an ant associated strain, are closely related.

Figure 2a. 1 Day after inoculation of *Pseudonocardia* and *Escovopsis* cocultivation.

Figure 2b. 1 Day after inoculation of *Pseudonocardia* and *Trichoderma* cocultivation. The circumference spread of *Trichoderma* shows evidently more growth than that of the cocultivation plate with *Escovopsis.*

Figure 2c. *Pseudonocardia* without a pathogen.

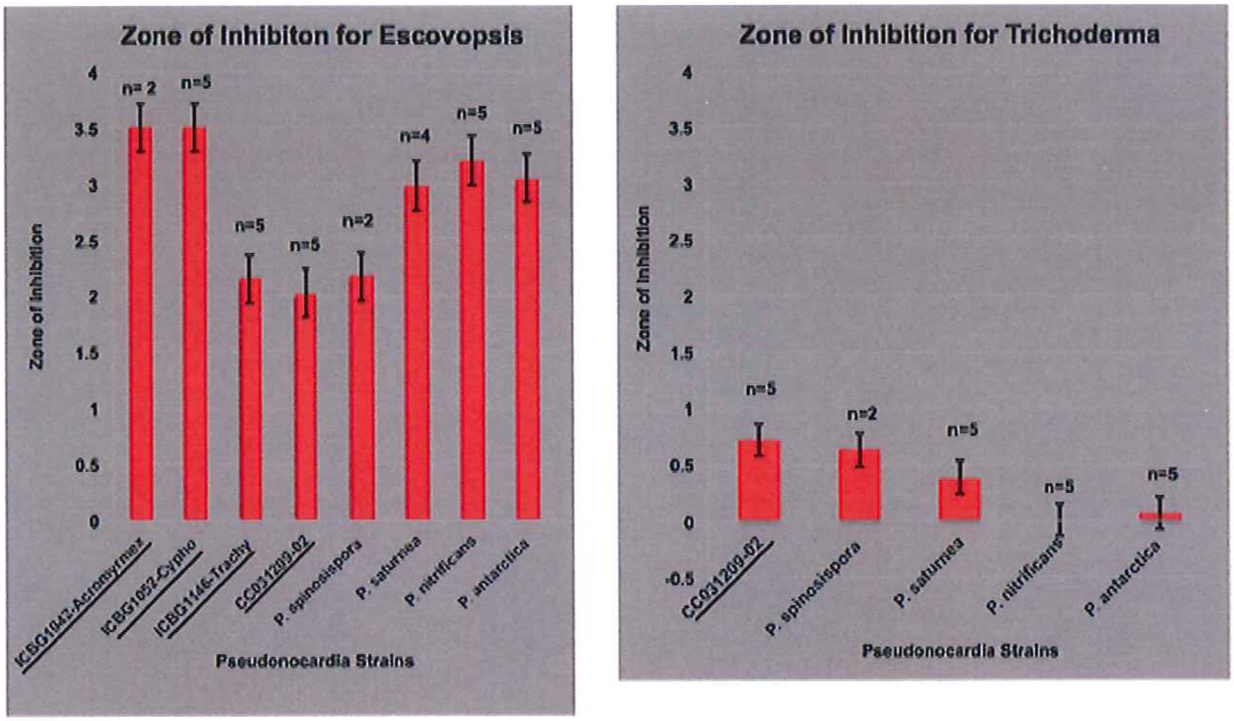

Figure 3. These graphs describe the zones of inhibition measured between co-cultivation treatments and between strains. Across all the strains that had measurements for both pathogens, *Pseudonocardia* had a greater zone of inhibition for *Escovopsis,* than for *Trichoderma.*

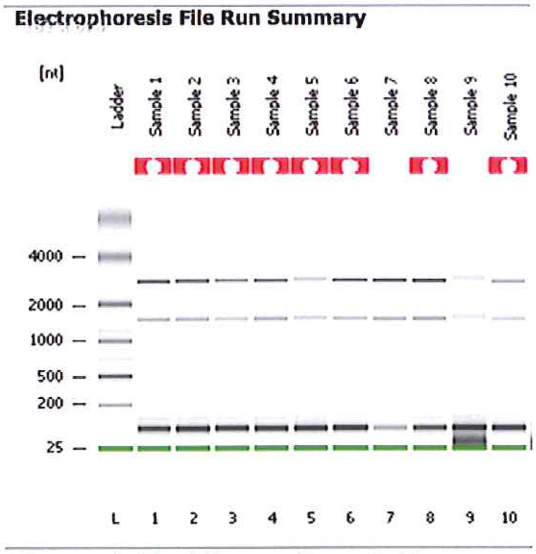

Figure 4. The Electrophoresis Summary obtained from the Bioanalyzer test shows the bold and consistent bands around 3000nt. These bands indicate that the modified RNA extraction method was successful at yielding quality RNA from the *Pseudonocardia* samples.

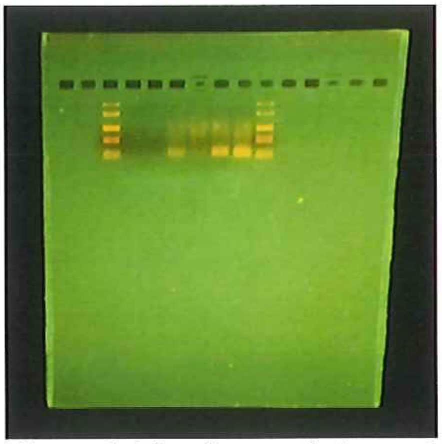

Figure 5. The above gel electrophoresis picture is taken from a primer trail run conducted in the October. Cell 4 was a control sample without a primer or template and cell 5 was a control that included the paaA primer but no template. Because neither of these cells shows amplified PCR product, there is no contamination. In cells 6 and 7, the paaA primer amplified the template because both cells have yellow bands in the pictures. In cell 8 and 9, gyrA and rpoD, respectively, amplified the template. Therefore, this gel picture shows that all three primers amplified the template properly given the thermocycler conditions.

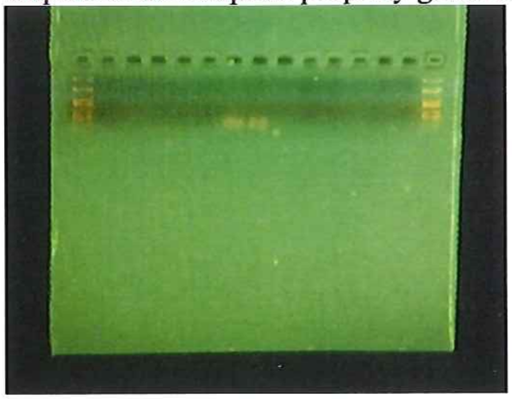

Figure 6. This gel electrophoresis shows that the only samples that amplified properly were 1) cDNA sample 39A with PAA primer that was not diluted and 2) cDNA sample 39A with PAA primer that was a 10x dilution. The other housekeeping primers, gyrA and rpoD, did not amplify the sample properly.

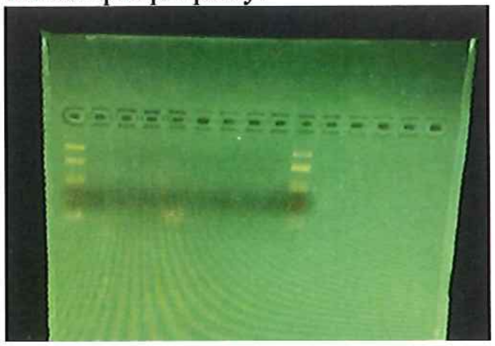

Figure 7. This gel electrophoresis shows that the only sample that amplified properly was with the gyrA primer. The other housekeeping primer, rpoD, did not amplify the sample properly, nor did the experimental primer, paaA, which previously worked.

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Katherine Mary Barnett's RNA Extraction Procedure
CALS Senior Honors Thesis
May 2nd, 2018

Based on Adam Book's 2012 *Streptomyces* RNA Extraction and Paul Airs' Edits

1) Harvest "erasure-size" amount of *Pseudonocardia* cells into an Eppendorf tube. KEEP SAMPLES ON ICE AT ALL TIMES UNLESS OTHERWISE INDICATED.
2) Prepare 2ml screw top conical tube with approx. 100 ul of Zirconia/Silica beads and 200 ul glass beads. Add 700 ul phenol and 700 ul NaOAc to the conical tube with beads.
3) Add 100 ul SDS to sample Eppendorf tube in Step 1 and grind with sterilized pestle.
4) Transfer cell mixture from Step 3 into a bead tube from Step 2.
5) **Lyse cells with bead beater**: shake tubes for 2 minutes in the bead beater and incubate the tubes on ice for 2 minutes. Shake tubes for 2 minutes again.
6) **Centrifuge** tubes at 12,000xg for 5 minutes at 4°C. Transfer aqueous (top) phase to a new 2ml tube.
7) **Phenol/Chloroform Extraction**: Add saturated phenol: chloroform (pH 4.3) to sample tubes in a 1:1: 2 ratio. [FOR EXAMPLE: 300 ul of sample, 300 ul nuclease-free H20, 600 ul phenol: chloroform]. Vortex to mix. Centrifuge tubes at 12,000xg for 5 minutes at 4°C. Transfer top layer to a new 2ml tube.
8) **Chloroform Extraction:** Add saturated chloroform to sample tubes in a 1:1 ratio. Vortex to mix. Centrifuge tubes at 12,000xg for 5 minutes at 4°C. Transfer top layer to a new 2ml tube.
9) **RNA Precipitation:** Add 600 ul of 100% EtOH and 20ml of NaOAc (pH 5.2). Store at -80°C for a minimum of 30 minutes, ideally longer.
10) **Centrifuge** tubes at 12,000xg for 20 mins at 4°C. REMOVE SUPERNATANT. Wash pellet with 70% EtOH. Pellet may be clear or difficult to see. Be careful not to disrupt pellet by making sure that the pipette does not touch the tube's walls.
11) **Centrifuge** tubes at 12,000xg for 5 mins at 4°C.
12) **Pipette off residual liquid**- careful to not have pipette contact sides of the tube because that may cause disruption of the pellet.
13) **Air-dry** 5-10 minutes max.
14) **Re-suspend pellet** with 40 ul of nuclease-free water.
15) **DNAse treatment:** Add 5 ul RQ1 Rnase-Free Dnase 10x Reaction buffer and 5 ul of RQ1 Rnase-free Dnase. Incubate at 37°C for 30-60 minutes.
16) **Phenol/Chloroform Extraction:** Add saturated phenol: chloroform (pH 4.3) to sample tubes in a 1:1:2 ratio. Vortex to mix. Centrifuge tubes at 12,000xg for 5 minutes at 4°C. Transfer top layer to a new 2ml tube. Only do this one time.
17) **Chloroform Extraction:** Add saturated chloroform to sample tubes in a 1:1 ratio. Vortex to mix. Centrifuge tubes at 12,000xg for 5 minutes at 4C. Transfer top layer to a new 2ml tube.
18) **RNA precipitation:** Add 0.1x volume 3M NaOAc and 2x volume 100% EtOH. Incubate at -80°C overnight (min. 1 hour, max long term).
19) **Centrifuge** tubes at 12,000xg for 20 minutes at 4°C. REMOVE SUPERNATANT. Wash with 70% EtOH.

20) **Centrifuge** tubes at 12,000xg for 5 mins at 4°C. Wash with 70% EtOH again. Pipette off residual liquid. Air-dry 5-10 minutes max. Re-suspend with 20 ul nuclease-free water.

21) **Check RNA quality** using Nanodrop, Qubit, or Bioanalyzer.