Investigating Bacterial Competition in the Leafcutter Ant Microbiome

Abstract

Actinobacteria are well known for their ability to produce antibiotics since most antimicrobials known today originated from bacteria in this phylum. The *Streptomyces* genus in particular, is responsible for most of the antibiotics used today. Therefore, one could assume that an abundance of antimicrobials have also been discovered from the actinomycete *Pseudonocardia,* yet it is the opposite. This leads us to believe that there are a plethora of untapped *Pseudonocardia* antimicrobials waiting to be identified and further studied.

Whole colonies of the leafcutter ant species *Acromyrmex echinatior* have been identified to contain one of two *Pseudonocardia* species on their cuticles. The *P. echinatior* or *P. octospinosus,* along with other bacteria in the ant microbiome, keep the ant colony alive by killing any pathogenic fungi and bacteria through the secretion of antimicrobials, thus protecting the co-evolved fungus *Leucoagaricus gongylophorus,* which in turn produces gongylidia, the sole colony food source.

By isolating the *Pseudonocardia* and *Streptomyces* bacteria from the ant laterocervical plates and performing a series of bioassays, we can obtain these desired antimicrobials. Through amplicon sequencing we can get a better understanding of the ant microbiome that so many bacteria are competing over and yet *Pseudonocardia* have successfully colonised and play such a large role in keeping the ant colony alive alongside other actinomycetes such as *Streptomyces.*

Introduction

Acromyrmex echinatior

Through roughly 50 million years of coevolution between fungus-farming (attine) ants and fungi, a strong symbiosis has formed, which has allowed these insects to thrive and form such large colonies (Aylward *et al*., 2012). Leafcutter ants comprise of the genera *Atta* and *Acromyrmex* which are attine ants. *Acromyrmex echinatior* is a Panamanian leafcutter ant species that gather leaves to feed the co-evolved basidiomycete fungus *Leucoagaricus gongylophorus.* This allows the *L. gongylophorus* to mature and produce hyphal swellings called gongylidia, which acts as the sole source of food to both the *A. echinatior* queen and its larvae (Holmes *et al*., 2016). Without this fungus, these ants would have nothing to consume and starve to death which would result in the loss of the colony. Therefore, *L. gongylophorus* is essential for the colony's survival, and so it is vital that all *A. echinatior* have this fungus. This is possible as the queen vertically transmits the *L. gongylophorus* to all other ants produced when the colony is formed and as it continues to grow (Heine *et al*., 2018, Mueller *et al*., 2001). Gongylidia are rich in lipids and sugars and contain pectinolytic enzymes, which unlike the lipids and sugars, are not digested by the *A. echinatior* but become concentrated in the ant digestive tracts (Aylward *et al.,* 2015). The leafcutter ants then use their faecal matter as manure in their fungal gardens, which allows these plant biomass-

degrading enzymes such as Xyloglucan to breakdown the cell walls of the plant matter located in the fungal gardens. This results in the *L. gongylophorus* having access to the nutrients inside the cells to be able to produce more gongylidia (Grell *et al*., 2013, Kooij *et al*., 2016, Schiøtt *et al.,* 2010). Leafcutter ants are not the only insect to have symbiotically evolved with microorganisms. Just like how the leafcutter ant uses *L. gongylophorus* to get access to nutrients, the beetle *Ambrosiophilus ambrosia* uses the fungus *Flavodon ambrosius* to turn rotted wood into nutrients that the beetles can then consume (You *et al*., 2015). Some insects such as *A. echinatior* ants and beewolf wasps have evolved to form a symbiosis with bacteria in which the bacteria acts as an immune system for the insect colony (Engl *et al*., 2018; Goldstein & Klassen, 2020). These symbiotic bacteria include the genera *Pseudonocardia* and *Streptomyces* which this study focuses on. Symbiosis between ants and microorganisms is not exclusive to *A. echinatior* ants. *Cephalote* ants require nitrogen-recycling bacteria to synthesise nutrients in the gut of the ants which the ants can then digest (Ramalho & Moreau, 2023). *A. echinatior* is not the only fungus-growing ant that relies on symbiosis with the actinomycete *Pseudonocardia*. Both *Apterostigma dentigerum* and *Trachymyrmex cornetzi* (Carr *et al*., 2012, Holmes *et al*., 2016) rely on *Pseudonocardia* bacteria in their microbiome to protect their colonies from pathogenic fungi and bacteria. *Azteca* ants are able to continue their symbiosis with *Cecropia* plants as the *Pseudonocardia* bacteria in these ant's microbiome ensure the *Azteca* colonies are protect from pathogens found on *Cecropia* plants due to the antimicrobials these actinomycetes produce (Fukuda *et al*., 2021).

Pseudonocardia

Pseudonocardia are gram-positive, aerobic, non-motile bacteria that belong to the *Pseudonocardiaceae* family, which includes other genera such as *Amycolatopsis*, *Amycolata*, *Saccharomonospora*, and *Saccharopolyspora* (Warwick *et al.,* 1994). Like *Streptomyces, Pseudonocardia* are actinobacteria which is a phylum known for its ability to produce secondary metabolites (Genilloud, 2017). Species of *Pseudonocardia* can be found in a multitude of vastly different environments such as soil, desert rocks, oceans, or even on living organisms such as plants and insects such as leafcutter ants and termites (Bruner-Montero *et al*., 2021; Jiang *et al*., 2021; Li *et al*., 2012; Parra *et al.,* 2021; Sujarit *et al*., 2017; Trujillo *et al*., 2017; Zhao *et al*., 2012). *Acromyrmex echinatior* contains one of two species of *Pseudonocardia*. The ants either carry *P. echinatior* or *P. octospinosus* on the biofilm in their cuticles on the laterocervical plates (Heine *et al.,* 2018). This may be due to in the past, different *Pseudonocardia* competing with each other to colonise the ant cuticles. Evidence of this is that one strain of *Pseudonocardia* can inhibit the growth of other *Pseudonocardia* that compete to colonise a host by producing a rebeccamycin analog (Van Arnam *et al*., 2015). A study by Zhang, Poulsen & Currie, 2007 showed that A*. echinatior* ants are able to differentiate between their native and foreign *Pseudonocardia* sp. even when the foreign actinomycetes are closely related to their native *Pseudonocardia* sp.. This is believed to be due to the ants being so highly specialised with their native *Pseudonocardia* sp. that they can identify chemicals produced by their *Pseudonocardia* sp.. This may allow the ants to identify invading foreign actinomycetes and remove them to prevent the actinomycetes from competing with each other, which would have caused a decrease in their ability to inhibit pathogens.

A defensive microbiome is made up of these *Pseudonocardia* bacteria, in addition to *Streptomyces* and other bacteria, to protect the ants from pathogenic bacteria and fungi. Antifungal compounds produced by these actinomycete bacteria protect the essential *Leucoagaricus gongylophorus* located on the ants (Holmes *et al*., 2016). These antibacterial and antifungal compounds produced by the defensive ant microbiome protect the *L. gongylophorus* from pathogenic fungi and bacteria such as *Escovopsis*, which if left unregulated is able to quickly wipe out the *L. gongylophorus* in as little time as a few weeks (Barke, *et al*., 2011; Meirelles *et al*., 2013). *Escovopsis* is usually seen as the largest pathogenic threat to ant colonies. The attine ants require the assistance of *Pseudonocardia* sp. to inhibit this pathogen through the production of antifungals. Other pathogens can threaten the longevity of the ant colony, and so it is important that *Pseudonocardia* sp. can inhibit the growth of a variety of pathogens and not just specifically *Escovopsis* (Sen *et al*., 2009). A study (Bruner-Montero *et al*., 2021) has shown *Pseudonocardia* symbiotes are able to inhibit the growth of the common insect fungal pathogen *Metarhizium anisopliae*, as when the ants did not contain *Pseudonocardia* spp. they were more vulnerable to *M. anisopliae.* However, the *A. echinatior Pseudonocardia* species are able to inhibit *Escovopsis* more effectively than their inhibition of other fungi, and are generally much better at inhibiting *Escovopsis* than *Pseudonocardia* found in the environment, such as in the soil (Cafaro *et al*., 2010). It is not just *Pseudonocardia* species that are able to inhibit *Escovopsis. Burkholderia* sp. are able to produce antifungals that inhibit *Escovopsis* without inhibiting *L. gongylophorus* (Francoeur *et al*., 2021). This is due to the biosynthetic gene clusters (BGCs) found in the *Burkholderia* sp. that encode the production of the 2 antifungals burkholderine1213 and pyrrolnitrin (Francoeur *et al*., 2021).

Similarly to *L. gongylophorus, Pseudonocardia* is vertically transmitted from the queen to the larvae. Therefore when new colonies form, all the worker ants will be transmitted *L. gongylophorus* as well as *Pseudonocardia* from the new queen, which would increase the survival odds of the newly formed colony (Heine *et al*., 2018). *Pseudonocardia* bacteria can also be horizontally transmitted between worker ants. Similarly to the queen, worker ants contain tubercles and crypt structures in their cuticles, which are specialised towards harbouring *Pseudonocardia* bacteria (Li *et al.,* 2018). The newly hatched *A. echinatior* ants are quickly colonised by *Pseudonocardia* from older worker ants. This results in a biofilm that covers most of the ant. Approximately a month later, only the laterocervical plates are covered in the biofilm (Andersen *et al.,* 2013). This is due to the specialised structures in the cuticles of the ants that are suited for the colonisation and continued survival of the *Pseudonocardia* on the ants. *Pseudonocardia* are the dominant actinobacteria found on attine ants (Li *et al*., 2018). As *Pseudonocardia* sp. are able to colonise newly hatched worker ants through horizontally transmission from older worker ants, queen ants that hatch are inoculated with *Pseudonocardia* and so can vertically transmit the bacteria through to the offspring when forming new colonies (Marsh *et al.,* 2014). Some male ants have been noted to not possess these specialised structures as male ants are only required for reproduction and not grooming the fungal garden (Li *et al*., 2018; Marsh *et al*., 2014). Culture-independent isolations (Sen *et al*., 2009) were performed on the attine ants *Trachymyrmex septentrionalis, Mycocepurus smithii,* and *Cyphomyrmex wheeleri* which contained a variety of *Pseudonocardia* spp.. *T. Septentrionalis* workers were shown to contain *P. cf. spinosispora* and *P. cf.*

ammonioxydans whilst more *Pseudonocardia* spp. were found on the *M. smithii* and the *C. wheeleri*. Culture-dependent isolations also showed *Pseudonocardia* spp. however less diversity was seen (Sen *et al*., 2009). On *A. echinatior,* the 2 strains of *Pseudonocardia* Ps1 and Ps2 were shown to contain between 11 to 15 secondary metabolite BGCs with 6 of these BGCs being shared between the 2 strains (Holmes *et al*., 2016). Although *Pseudonocardia* Ps1 and Ps2 strains both express some of the same BGCs, all of the *Pseudonocardia* Ps1 strain contained BGCs that result in the production of nystatin P1-like antifungals., whilst all of the *Pseudonocardia* Ps2 strains contained BGCs that resulted in the production of novel variants of nystatin (Holmes *et al*., 2016). Although it is known that the higher attine, ant-associated *Pseudonocardia* bacteria protect the ant's fungal gardens enabling the production of food for the ant larvae, few antibiotics produced by these *Pseudonocardia* species have been identified. It is likely that there remain many attine-associated *Pseudonocardia* antimicrobials to be discovered as the *Pseudonocardia* belong to the *Actinomycetales* order. The *Streptomyces* genus also belongs to this order and so the *Actinomycetales* order is well known for the production of secondary metabolites (Carr *et al*., 2012). However, not many antibiotics have been discovered from *Pseudonocardia* sp., and of those, very few come from fungus-growing-associated ants such as *A. echinatior*. The antibiotics discovered coming from attine-associated ants include dentigerumycins and nystatin-like polyenes (Barke *et al*., 2010; Carr *et al.,* 2012), such as the antifungal polyene Selvamicin (Van Arnam *et al*., 2016).

Streptomyces

Streptomyces are gram-positive, spore-forming actinobacteria that belong to the *Streptomycetaceae* family (Quinn *et al.,* 2020). *Streptomyces* bacteria can be found on plants and insects, and in soil, but as they are also polyextremophilic they can be found in many different environments including extreme environments such as saline or acidic soil, and deserts (Donald *et al.,* 2022). From 1945 to 1978, 55% of all the antibiotics discovered were produced by *Streptomyces* bacteria (Viana Marques *et al*., 2018). 80% of antibiotics used presently come from *Streptomyces* (Hutchings *et al*., 2019; Emerson de Lima ProcópioI *et al*., 2012). This is why the golden age of antibiotics is said to have taken place during that time period and why *Streptomyces* bacteria are so important in the timeline of antibiotics. *Streptomyces* spp. located on *A. echinatior* have been identified to produce candicidin macrolides which have a significant effect on the growth of *Escovopsis* without significantly affecting the growth of the *L. gongylophorus* (Haeder *et al.*, 2009). This is clearly beneficial to the leafcutter ants as their source of food can be protected without being hindered from this protection. This also makes it evident that the *Streptomyces* spp. that have colonised the ant cuticles are also producing antibiotics such as the candicidin. It has been identified that certain *Streptomyces* sp. located on the ants are able to produce actinomycins, valinomycins, and elaiophylins (Boya P. *et al*., 2017; Schoenian *et al*., 2011) which are all known to be antifungal agents. A study by Dhodary & Spiteller, 2021 showed that *Streptomyces* spp. on *Acromyrmex* ants produced high concentrations of ammonia which caused the inhibition of fungi such as *Escovopsis* due to the high pH. The secretion of these antifungals would aid in the protection of the ant colony against pathogenic fungi such as *Escovopsis.* The antibiotic-resistant genes (ARGs) of the *Streptomyces* bacteria allow for these microbes to be able to remain on the ant cuticles due to the resistance that they have acquired to not only

their own antibiotics, but also those of other neighbouring bacteria, such as those produced by the *Pseudonocardia* species (Seipke *et al*., 2012). Therefore the *Streptomyces* bacteria are able to release antibiotics without deteriorating in efficiency. Pathogenic bacteria are able to take these ARGs and become resistant to the antimicrobials produced by the *Streptomyces* via horizontal gene transfer to other soil bacteria (Jiang *et al*., 2017). This increases the level of concern regarding the decline in the effectiveness of antibiotics due to antimicrobial-resistance (AMR) as the antibiotics produced by the *Streptomyces,* and other bacteria, are made much less effective. Identifying these ARGs could create new potential targets in the combat against antibiotic resistance. *A. echinatior* is not the only insect species to have a symbiosis with *Streptomyces.* The cocoon walls of the larva of beewolf wasps contain *Streptomyces* bacteria which increases the probability that the larva survives as the Streptomycetes are able to produce antifungals to protect the larva (Kaltenpoth *et al*., 2005).

Methods

Table. 1 Growth media and broth recipes used for bacteria isolations, spore stocks, bioassays, and Non-*Pseudonocardia* genomic DNA extractions. Each recipe contained a different carbon or nitrogen source to identify a preferred media for the growth and antimicrobial activity of *Pseudonocardia* and *Streptomyces* spp.

Media	**Used for**	**Recipe**	**Source**
Soya flour mannitol (SFM) Solid Liquid	Bacteria isolation Bioassays	20.0 g Mannitol 20.0 g Soya flour 20.0 g Agar Distilled water to 1 L	(Kieser *et al*., 2000)
Maltose-yeast extract-malt extract (MYM) Solid	Bioassays	4.0 g Maltose 4.0 g Yeast Extract 10.0 g Malt Extract 20.0 g Bacto Agar 500 ml Tap Water 500 ml Distilled Water 0.4 ml R2 Trace elements per 200 ml (Post autoclave)	(Stuttard, 1982)
Glucose-yeast-malt (GYM) Solid Liquid	Bacteria isolation Spore Stocks Bioassays	4.0 g Glucose 4.0 g Yeast Extract	(Worsley, *et al.,* 2020)

		10.0 g Malt Extract 2.0 g $CaCl_2$ 15.0 g Agar Distilled water to 1 L	
Peptone Malt Dextrose Agar Solid	Bioassays	10.0 g Peptone 20.0 g Malt Extract 40.0 g Glucose 20.0 g Agar Distilled water to 1 L	
Lysogeny broth (LB) Liquid	Overlay cultures	10.0 g Tryptone 10.0 g NaCl 5.0 g Yeast Extract Distilled water to 1 L	(Bertani, 1951)
SFMNAG Solid	Bioassays	20.0 g Soya flour 20.0 g Mannitol 20.0 g Agar Distilled water to 1 L 400 µl per 200 ml filter sterilised N-acetyl glucosamine (Post autoclave)	
Yeast Peptone (YP) Solid	Bioassays	10.0 g Yeast extract 20.0 g Bacteriological peptone 20.0 g Agarose Distilled water to 1 L	(Jones *et al.*, 2017)
Yeast Peptone Dextrose (YPD) Solid	Bioassays	10.0 g Yeast extract 20.0 g Bacteriological peptone 20.0 g agarose Distilled water to 1 L Autoclave	(Jones *et al.*, 2017)

		10 ml per 100 mL of filter sterilised 20% glucose solution (Post autoclave)	
Yeast Extract-Malt Extract (YEME) Solid Liquid	Bioassays Bacteria isolation	3.0 g Yeast Extract 5.0 g Peptone 3.0 g Malt Extract 10.0 g Glucose 20.0 g Agar Distilled water to 1 L	(Kieser *et al.,* 2000)
SPY Solid	Bioassays	15.0 g Pancreatic digest of casein 15.0 g Peptic digest of animal tissue 2.0 g Yeast Extract 10.0 g $MgSO_4$ 50.0 mg $CaCl_2$ 30.0 mg $ZnSO_4$ 25.0 mg $FeSO_4$ 16.0 mg $CuSO_4$ 12.0 mg $MnSO_4$ 20.0 g Agar 30.0 g Soluble starch Distilled water to 1 L Adjust pH to 7.2 with NaOH	(Imai *et al.,* 2015)
IMA Solid	Bioassays	50.0 g Smash 20.0g Agar Tap water to 1 L	(Joshi *et al.*, 2010)
Minimal Media Solid	Bioassays	0.5 g L-asparagine 0.5 g K_2HPO_4 0.2 g $MgSO_4.7H_2O$	(Kieser *et al.,* 2000)

		0.01 g $FeSO_4.7H_2O$ 10 g agar Autoclave at 121°C for 15 minutes 10 g Glucose (Post autoclave)	
***Streptomyces Media 3* (SM3)** Solid	Bioassays	5.0 g Glucose 50.0 g MD30E Maltodextrin 25.0 g Arkasoy soya flour 3.0 g Molasses (beet) 0.25 g K_2HPO_4 2.5 g $CaCO_3$ 2% Agar Distilled water to 1 L Adjust pH to 7.0 with KOH	This work
***Streptomyces Media 5* (SM5)** Solid	Bioassays	20.0 g Peptone (Oxoid L34) 8.0 g Lab Lemco (Oxoid L29) 15.0 g Glucose 10.0 g Glycerol solution 0.4 g $CaCO_3$ 2% Agar Distilled water to 1 L Adjust pH to 7.0 with KOH	This work
***Streptomyces Media 6* (SM6)** Solid	Bioassays	40.0 g Corn Steep Liquor 20.0 g MD30E Maltodextrin 2.5 g NaCl 0.5 g $MgSO_4$ 2% Agar Tap water to 1 L Adjust pH to 7.0 with KOH	This work
Streptomyces Media 7	Bioassays	20.9 g MOPS	This work

(SM7) Solid		15.0 g L-Proline 20.0 g Glycerol solution 2.5 g Sucrose 1.5 g Sodium L-Glutamate monohydrate 0.5 g NaCl 2.0 g K_2HPO_4 10.0 ml 0.2 M $MgSO_4$ 10.0 ml 0.02 M $CaCl_2$ 5.0 ml R5 Trace Elements 2% Agar Distilled water to 1 L Adjust pH to 6.5 with KOH	
Streptomyces Media 12 **(SM12)** Solid	Bioassays	10.0 g Soya flour (Holland and Barrett) 50.0 g Glucose 4.0 g Peptone (Formedium PEP02) 4.0 g Difco Beef Extract 1.0 g Yeast Extract (Merck) 2.5 g NaCl 5.0 g $CaCO_3$ 2% Agar Tap water to 1 L Adjust pH to 7.6 with KOH	This work
Streptomyces Media 14 **(SM14)** Solid	Bioassays	10.0 g Glucose 20.0 g Soy Peptone (Lab M) 5.0 g Beef extract powder (Oxoid) 5.0 g NaCl 0.01 g $ZnSO_4 \cdot 7H_2O$ 2% Agar	This work

		Distilled water to 1 L Adjust pH to 7.0 with KOH	
***Streptomyces Media 15* (SM15)** Solid	Bioassays	20.9 g MOPS 11.5 g Casamino acids 23.0 g Glycerol solution 0.5 g NaCl 0.52 g K_2HPO_4 0.25 g EDTA 0.49 g $MgSO_4 \cdot 7H_2O$ 0.029 g $CaCl_2 \cdot 2H_2O$ 5.0 ml Trace salts No. 1 2% Agar Distilled water to 1 L Adjust pH to 6.5 with KOH	This work
***Streptomyces Media 18* (SM18)** Solid	Bioassays	15.0 g Glucose 40.0 g Soluble starch (Difco) 20.0 g Molasses (beet) 8.0 g $CaCO_3$ 2% Agar Tap water to 1 L Natural pH 7.2 pre-sterilisation	This work
***Streptomyces Media 19* (SM19)** Solid	Bioassays	40.0 g Tomato paste 15.0 g Oat flour (Avenaflo) 2.0 g Glucose 2% Agar Tap water to 1 L Natural pH 6.0 pre-sterilisation	This work
***Streptomyces Media 20* (SM20)** Solid	Bioassays	20.0 g Maltose 5.0 g Peptone (Oxoid L37)	This work

		5.0 g Beef extract powder (Oxoid) 3.0 g Yeast extract (Oxoid) 3.0 g NaCl 1.0 g $MgSO_4 \cdot 7H_2O$ 2% Agar Tap water to 1 L Natural pH 7.2 pre-sterilisation	
***Streptomyces Media 25* (SM25)** Solid	Bioassays	10.0 g Peptone (Oxoid L34) 21.0 g Malt extract (Oxoid) 40.0 g Glycerol solution 2% Agar Distilled water to 1 L Natural pH 6.3 – 6.5 After autoclaving pH of 6.3	This work
***Streptomyces Media 30* (SM30)** Solid	Bioassays	40.0 g Tomato paste 15.0 g Oat flour (Avenaflo) 2.0 g Glucose 2% Agar Tap water to 1 L Adjust pH to 4.5 with HCl	This work
***Streptomyces Media 32* (SM32)** Solid	Bioassays	10.0 g Peptone (Oxoid L34) 21.0 g Malt extract (Oxoid) 40.0 g Glycerol solution 2% Agar Distilled water to 1 L Natural pH to 4.5 with HCl	This work

Tryptone soy broth (TSB) Liquid	Non-*Pseudonocardia* genomic DNA extraction Bacteria Isolation	30.0 g Tryptone Soy Broth (Oxoid) Distilled water to 1 L	

Isolation of bacteria from the ant cuticle

Bacteria from the laterocervical plates of *Acromyrmex echinatior* leafcutter ants from colonies A.e758, A.e750, and A.e399 were isolated using both live and dead ants. The white bacterial biofilm on the laterocervical plates of the ants was scraped with a sterile needle and streaked over Soya Flour Mannitol (SFM) agar (Table 1). To inhibit the growth of fungi, these SFM plates contained 5 µg/ml nystatin and 100 µg/ml cycloheximide. To inhibit the growth of gram-negative bacteria, 40 µg/ml nalidixic acid was added to the SFM. *A. echinatior* ants from colonies Ae758, Ae750, and Ae399 were also put in 1 ml of 20% glycerol and vortexed. From the glycerol ant solution,100 µl was spread over SFM plates. Another method used was scraping the laterocervical plates of the *A. echinatior* ants with a sterile needle and putting that needle in either 10 ml of liquid GYM, liquid SFM, or a solution of 5 ml liquid YEME and 5 ml TSB (Table 1) to incubate at 30˚C for 10 days. From these liquid cultures, 100 µl was spread over SFM plates containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid. All the SFM plates were then incubated at 30˚C for 21 days. Single colonies were streaked on SFM containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid and incubated at 30˚C for 21 days. The single colony streaking and incubating process was repeated resulting in purified bacteria.

Table 2. The strains used in this project isolated from *A. echinatior* colonies A.e750, A.e758, A.e399, A.e707 and A.e717 and the isolation technique used to isolate each strain. *B. subtilis, E. coli, and C. albicans* were used as indicator strains for bioassays to test for antibacterial and antifungal activity.

Strain Name	Strain Source	*A. echinatior* colony	Isolation Method
A.e750/1/1	This project	A.e750	Dead ant, needle scraped.
A.e750/1/3	This project	A.e750	Dead ant, needle scraped.
A.e750/1/5	This project	A.e750	Dead ant, needle scraped.
A.e750/1/6	This project	A.e750	Dead ant, 20% glycerol
A.e750/1/7	This project	A.e750	Dead ant, liquid YEME + TSB
A.e750/1/8	This project	A.e750	Dead ant, liquid GYM
A.e750/1/9	This project	A.e750	Dead ant, liquid SFM
A.e750/2/1	This project	Ae750	Live ant, needle scraped

A.e750/2/2	This project	A.e750	Live ant, needle scraped
A.e750/2/3	This project	A.e750	Live ant, needle scraped
A.e750/2/4	This project	A.e750	Live ant, needle scraped.
A.e758/1/1	This project	A.e758	Dead ant, needle scraped.
A.e758/1/2	This project	A.e758	Dead ant, needle scraped.
A.e758/1/3	This project	A.e758	Dead ant, 20% glycerol
A.e758/1/4	This project	A.e758	Dead ant, 20% glycerol
A.e758/2/1	This project	A.e758	Live ant, needle scraped
A.e399/1	This project	A.e399	Dead ant, needle scraped.
A.e399/2	This project	A.e399	Live ant, needle scraped.
A.e399/3	This project	A.e399	Dead ant, 20% glycerol
707-CP-A2	Hutchings Lab	A.e707	
A.e717 Ps2	Hutchings Lab	A.e717	
Pseudonocardia Ps1	Hutchings Lab	Unknown	
Bacillus subtilis	Hutchings Lab		
Escherichia coli	Hutchings Lab		
Candida Albicans	Hutchings Lab		

Creation of spore stocks

Following a protocol (Kieser *et al.,* 2000), spore stocks were generated. Glucose Yeast Malt (GYM, Table 1) lawn plates of the purified, isolated bacteria (Table 2) were incubated at 30˚C for 14 days. Following incubation, to each GYM plate, 5 ml of sterile 20% glycerol was applied. The surfaces of the plates were wiped with a cotton swab to free the bacterial spores. This solution of bacterial spores and sterile 20% glycerol was collected and centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded, and the pelleted spores were suspended in 500 µl of sterile 20% glycerol. The bacterial spores were stored at -20˚C.

Dilution streaks were performed using spore stocks of 707-CP-A2, Ae717 Ps2 and *Pseudonocardia* Ps1 (Table 2) on SFM plates containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid before being incubated at 30˚C for 14 days until sporulation was visible. A single colony was vortexed in 100 µl of sterile distilled water, and 50 µl was spread on GYM plates and incubated at 30˚C for 14 days.

Extracting non-*Pseudonocardia* genomic DNA

Following a protocol (Salting Out Genomic DNA Extraction Method - ActinoBase, 2020), the genomic DNA of the bacteria was extracted. Single colonies were incubated in 50 ml of TSB (Table 1) for 3 days at 30˚C whilst being shaken at 200 rpm. Each solution was divided into two 25 ml aliquots and centrifuged at 14000 rpm and 4˚C for 15 minutes. The supernatants were removed, and the pelleted mycelium was resuspended in 5 ml SET buffer (Table 3). To each resuspended sample, 100 µl of 50 mg/ml lysozyme solution was added and then incubated for 3 hours at 37˚C. The samples were then mixed by inversion with 140 µl of 20 mg/ml proteinase K solution and 600 µl of 10% SDS then incubated at 55˚C for 2 hours. The samples were then mixed with 2 ml of 5M NaCl and cooled to room temperature. To each sample, 5 ml of chloroform was added and mixed by inversion at 20˚C for 30 minutes. After centrifugation at 20˚C and 4500 rpm for 15 minutes the samples formed 3 layers. The top layer was removed and transferred to a sterile tube, and the chloroform, inversion mixing, and centrifugation steps were repeated, resulting in the solutions forming three layers again. The top layer was transferred to a sterile tube and incubated on ice for 2 minutes, and the bottom two layers were discarded. To each solution, isopropanol (stored on ice) was added proportional to 60% of the sample volume, and incubated in ice for 3 minutes after being mixed by inversion to allow the DNA to condense. The visible DNA was then collected using sealed Pasteur pipettes, rinsed with 5 ml of 70% ethanol, and air-dried. The DNA was resuspended in 500 µl of sterile distilled H_20.

Table 3. The recipes for each buffers used. Set Buffer was used during the extraction of the non-*Pseudonocardia* genomic DNA, whilst Buffer A, Buffer B, and Phenol-chloroform were used in the Phenol-chloroform DNA extraction.

Buffer	**Used**	**Ingredients**	**Source**
SET Buffer	Extracting non-*Pseudonocardia* genomic DNA	75mM NaCl 25mM EDTA pH8 20mM Tris-HCl pH7.5	Salting Out Genomic DNA Extraction Method - ActinoBase, 2020
Buffer A	Phenol-chloroform DNA extraction	200 mmol/L Tris-HCl pH 8.8 60 mmol/L NaCl, 10 mmol/L EDTA (ethylenediaminetetraacetic acid), 0.15 mmol/L spermine, and 0.15 mmol/L spermidine	Rubin *et al*., 2014
Buffer B	Phenol-chloroform DNA extraction	200 mmol/L Tris-HCl pH 8.8	Rubin *et al*., 2014

		30 mmol/L EDTA 2% SDS	
Phenol-chloroform (pH 8.0)	Phenol-chloroform DNA extraction	phenol/chloroform/isoamyl alcohol at a ratio of 25:24:1	Rubin *et al*., 2014

Extracting *Pseudonocardia* genomic DNA

Pseudonocardia isolates 707-CP-A2, A.e717 Ps2 and *Pseudonocardia* Ps1 were grown on sterile cellophane discs as confluent lawns on GYM agar containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid. Once there was a dense confluent lawn, and the spores were visible, 5 ml of sterile water was applied. the spores were scraped off the cellophane discs and the solution of bacterial spores and water was collected and centrifuged at 4000 rpm for 5 minutes. The supernatants were removed and the pelleted mycelium was resuspended in 5 ml SET buffer (Table 4). The *Pseudonocardia* genomic DNA could then be extracted through the continuation of the protocol for extracting non-*Pseudonocardia* genomic DNA (Salting Out Genomic DNA Extraction Method - ActinoBase, 2020) as described above.

16S rRNA gene amplification by PCR

From the extracted genomic DNA samples, 16S rRNA gene PCR was performed using the universal 16s primers PRK341F and MPRK806R (Table 4). For all 16S rRNA gene PCR, 20 µl reaction volumes were used with 10 µl Taq red polymerase, 7 µl sterile distilled water, 1 µl forward primer PRK341F (Table 4), 1 µl reverse primer MPRK806R (Table 4), and 1 µl genomic DNA sample. For the negative control, sterile water was used instead of the genomic DNA samples, and for the positive control, the genomic DNA of *Streptomyces venezuelae*. The PCR machine lid was preheated to 105 ˚C for 2 minutes. The initial denaturation underwent for 1 minute at 95˚C, and then 30 cycles of 95˚C for 15 seconds, 55˚C for 15 seconds, and 72˚C for 30 seconds. There was a final extension at 72˚C for 2 minutes. Through gel electrophoresis, the DNA fragments of each of the isolates were separated into bands on 1% agarose and the gel was extracted. The bands were excised to purify the PCR product before sequencing.

Table 4. The universal 16S primers used during 16S rRNA gene amplification by PCR and DNA sequencing. The forward primer used was PRK341F and the reverse primer used was MPRK806R.

Primer	Sequence	Use	Paper
PRK341F	5'-CCTACGGGRBGCASCAG-3'	16S rRNA gene amplification by PCR, DNA sequencing	Holmes *et al*., 2016
MPRK806R	5'-GGACTACNNGGGTATCTAAT-3'	16S rRNA gene amplification by PCR, DNA sequencing	Holmes *et al*., 2016

Gel Extraction

The Qiagen gel extraction kit was used to extract all the gels. The bands removed from the 1% agarose gel were incubated at 50˚C with buffer QG proportional to 3 times the volume of the gel band until the gel completely dissolved. Isopropanol was then added proportionally to the gel band volume. Upon being added to a DNA binding column, the samples were centrifuged at 13,000 rpm for 1 minute. The flow-through was removed, and 750 µl of Buffer PE was added. After another centrifugation at 13,000 rpm for 1 minute, the flow-through was removed. The gels were then eluted with 50 µl of heated sterile distilled water, and the DNA yields and $A_{260/280}$ were calculated using a nanodrop.

DNA sequencing

All DNA sequencing of isolates was performed used Eurofins genomics Mix2Seq service. Using the nanodrop, specific volumes of DNA from each isolate were calculated, and water was added to bring the total volume to 15 µl. Finally, 2 µl of the forward primer PRK341F was added.

Bioinformatics

Using the basic local alignment search tool, nucleotide (BLASTn), the DNA sequence was analysed and the database was searched for the closest match in order to identify the ant isolates. The top result was taken, and the identity and coverage were recorded.

Total DNA extraction

The total DNA extraction of the ants was performed using a modified MP Bio spin kit for soil protocol. The laterocervical plates of 40 *Acromyrmex echinatior* leafcutter ants from colonies A.e758, A.e750, and A.e399 were removed using sterile tweezers and placed into lysis matric E tubes. The samples were vortexed for 15 seconds with 978 µl of sodium phosphate buffer. After the addition of 122 µl MT buffer, the samples were vortexed for another 15 seconds. The samples were put in a FastPrep instrument and run for 4 cycles, speed 6 m/s, for 30 seconds, with 30 second breaks in between each cycle. Then centrifuged at 14,000 rpm for 15 minutes. The supernatant was extracted to a sterile Eppendorf, and the pellet was discarded. To each sample, 250 µl protein precipitation solution (PPS) was added and shaken by hand 10 times. After being incubated at room temperature for 10 minutes, the samples were stored in ice. The samples were centrifuged at 14,000 rpm at 4˚C for 5 minutes, and the supernatant was extracted like before, with the pellet being discarded. Binding matrix was then added to the sample proportionally to the volume of the supernatant and inverted by hand for 12 minutes. After being mixed, 800 µl of the sample was added to a SPIN filter tube and centrifuged at 14,000 rpm at 4˚C for 1 minute. The flow-through was discarded, and this was repeated until all of the sample had been passed through the SPIN filter tube. After, 500 µl of SEWS-M was added to the SPIN filter tube and gently shaken to mix and centrifuged at 14,000 rpm at 4˚C for 1 minute. The flow-through was discarded, and the sample was centrifuged again at 14,000 rpm at 4˚C for 5 minutes. The SPIN filter was transferred to a clean catch tube and air-dried at room

temperature for 5 minutes. The pellet was resuspended by adding 150 µl of DES. The resuspended sample was incubated in a heat block at 50˚C for 12 minutes then centrifuged at 14,000 rpm for 2 minutes. The DNA that had been eluted into the catch tube was stored at -20˚C until required.

The total DNA extraction was also performed using the Phenol-chloroform protocol (described in How to Use Phenol / Chloroform for DNA Purification | Thermo Fisher Scientific - UK, n.d., Rubin *et al*., 2014). From each of the 3 *A. echinatior* ant colonies: A.e399, A.e758, and A.e750, the laterocervical plates of 20 ants were extracted, and added to Qiagen tungsten carbide beads together. The larger the number of laterocervical plates used, the greater the DNA concentration and quality. However this had to be balanced against the number of worker ants in the colony as using too many would result in the death of the colony as there would not have been enough worker ants to keep the colony alive. This was then run through a Qiagen TissueLyser at 30 beats per second for 20 seconds to grind up the laterocervical plates. Next**,** 250 µl of buffer A (Table 3) was added and incubated at 65˚C for 15 minutes. After the addition of 250 µl of buffer B (Table 3), the sample was incubated again at 65˚C for 10 minutes. After 100 µg of proteinase K was added, the samples were incubated for 1 hour at 56˚C. The samples were washed with phenol-chloroform (Table 3) by adding an equal volume to the sample and centrifuging at 16,000 rpm for 5 minutes. The upper aqueous phase was transferred to a sterile tube, and the rest was discarded. The samples were then washed using chloroform and once again centrifuged and the upper aqueous phase transferred to a sterile tube. This was repeated until the there was almost no interphase. To the samples,1 µl of glycogen (20 µl/ml**)** was added. Next, 7.5 M NH_4OAc was added proportionally to half the volume of the sample. Finally, 100% ethanol was added proportionally to 2.5 times the volume of the new sample. The sample was then stored overnight at -20˚C to allow for the DNA to precipitate. After centrifugation at 16,000 rpm at 4˚C for 30 minutes the supernatant was discarded, leaving the pelleted DNA. The pellet was resuspended in 150 µl of 70% ethanol and centrifuged at 16,000 rpm at 4˚C for 2 minutes. The supernatant was removed, and the pellet was centrifuged for 16,000 rpm at 4˚C for 30 minutes again. After air-drying the DNA pellet at room temperature for 10 minutes, the pellet was resuspended in 300 µl of TEN buffer. Finally the DNA was stored at -20˚C until required. The total DNA extraction using the Phenol-chloroform protocol was repeated 3 times.

Amplicon Sequencing

For all amplicon sequencing, 16S rRNA gene PCR was performed using the total DNA samples. For all 16S rRNA gene PCR, 50 µl reaction volumes were used with 25 µl Taq red polymerase, 10 µl sterile distilled water, 2.5 µl forward primer PRK341F (Table 4), 2.5 µl reverse primer MPRK806R (Table 4), 10 µl total DNA samples. For the negative control sterile water was used instead of the total DNA samples, and for the positive control the genomic DNA of *Streptomyces venezuelae*. The PCR machine lid was preheated to 105 ˚C for 2 minutes. The initial denaturation underwent for 1 minute at 95 ˚C, and then 35 cycles of 95 ˚C for 15 seconds, 55 ˚C for 15 seconds, and 72 ˚C for 30 seconds. There was a final extension at 72 ˚C for 2 minutes. Through gel electrophoresis, the DNA fragments of each of the isolates were separated into bands on 1% agarose and the gel was extracted. The bands were excised to purify the PCR product before sequencing. To view the relative

abundance of the bacteria Illumina sequencing was performed through the MR DNA Amplicon sequencing service using the forward primer PRK341F (Table 4), and the reverse primer MPRK806R (Table 4).

Bioassays

To test the antimicrobial activity of the bacteria, bioassays were performed using a variety of different media types (Table 1). Each isolate (Table 2) was grown on every media type and tested against the indicator strains (Table 2). This was repeated 3 times for each isolate against each indicator strain. To the centre of each plate 5 µl of bacteria stock was added and incubated at 30˚C for 20 days. Stocks of *Escherichia coli*, *Candida albicans*, *Bacillus subtilis* were each incubated overnight at 30˚C, and 200 rpm in 10 ml of liquid LB. These overlays were then subcultured into 10 ml of liquid LB and incubated at 30˚C, 200 rpm for 4 hours. Overlays were then performed on the 9 plates with 3 plates overlayed with 3 ml of *E. coli*, 3 overlayed with 3 ml of *C. Albicans*, and 3 overlayed with 3 ml of *B. subtilis.* For the negative control the different medias were overlayed with the indicator strains without the presence of any of the isolated strains to see if any of the media was inhibiting the indicator strains. The plates were incubated at 30˚C for 2 days. A zone around the centre colony where no indicator strain grew (zone of inhibition) was used as an indication that antimicrobial activity had occurred. No positive control was used as the zone of inhibition showed the indicator strain growth had been inhibited compared to the negative controls where the indicator strains had not been inhibited.

Gram Staining

A gram stain was performed following the Pro-Lab Diagnostics™ Gram Staining Kit protocol. From the isolate stocks, 2 µl was added to a glass slide and the bacteria was heat-fixed using a flame. Crystal violet was added to the bacteria for 1 minute and then rinsed off with distilled water. The slide was then covered in iodine solution for another minute and then rinsed off. The slide was then covered in safranin for 30 seconds and then rinsed with distilled water. After the slide was gently dried with some absorbent paper, a small bit of oil was added and it was put under a microscope at 100x magnification.

Amplicon Sequencing Data Processing

The amplicon sequencing data was processed through the use of QIIME2. High performing computing (HPC) runs of the amplicon sequencing data were performed. Paired end sequences were made by removing the PRK341F and MPRK806R primers and truncating the forward and reverse sequences. As the number of successful reads depended on how the sequencings were shortened, many different truncations were performed and the truncation with the most reads was then chosen. To assign taxonomy to the sequences a 16S sequence database from silva was used and each truncated sequence was assigned to a 16S sequence from the database. QIMME2 was then used to filter out any unwanted DNA sequences such as archaea DNA sequences that remained. Using RStudio a bar plot was then generated to identify the relative abundance of bacteria located in the laterocervical plate microbiome of the ants from the *Acromyrmex echinatior* colonies.

Aims

This study was focused on the bacterial competition of the *Acromyrmex echinatior* leafcutter ant microbiome. The two bacterial genera *Pseudonocardia* and *Streptomyces* were the focal point of this study since these actinomycetes are found on the ant cuticles and play a large role in protecting the ant colony from pathogens through the production of antimicrobials. One of the main aims of this study was to identify media types that isolates from *A. echinatior* are able to grow and produce antimicrobials on. This would allow for an easier way of studying these actinomyces as it is already known that the *Pseudonocardia* and *Streptomyces* bacteria that have colonised the laterocervical plates of *A. echinatior* are able to express BGCs that result in the production of antimicrobials. The first aim was therefore to isolate *Pseudonocardia* and *Streptomyces* bacteria from the ant cuticles. The following aim was to find a media type on which the *Pseudonocardia* and *Streptomyces* isolates grew and secrete antimicrobials into the media when overlayed with *Bacillus subtilis*, *Candida albicans,* or *Escherichia coli* which act as indicator strains. The *B. subtilis* and the *E. coli* were used as indicator strains to test for antibacterial activity, whilst the *C. albicans* was used to test for antifungal activity.

Results

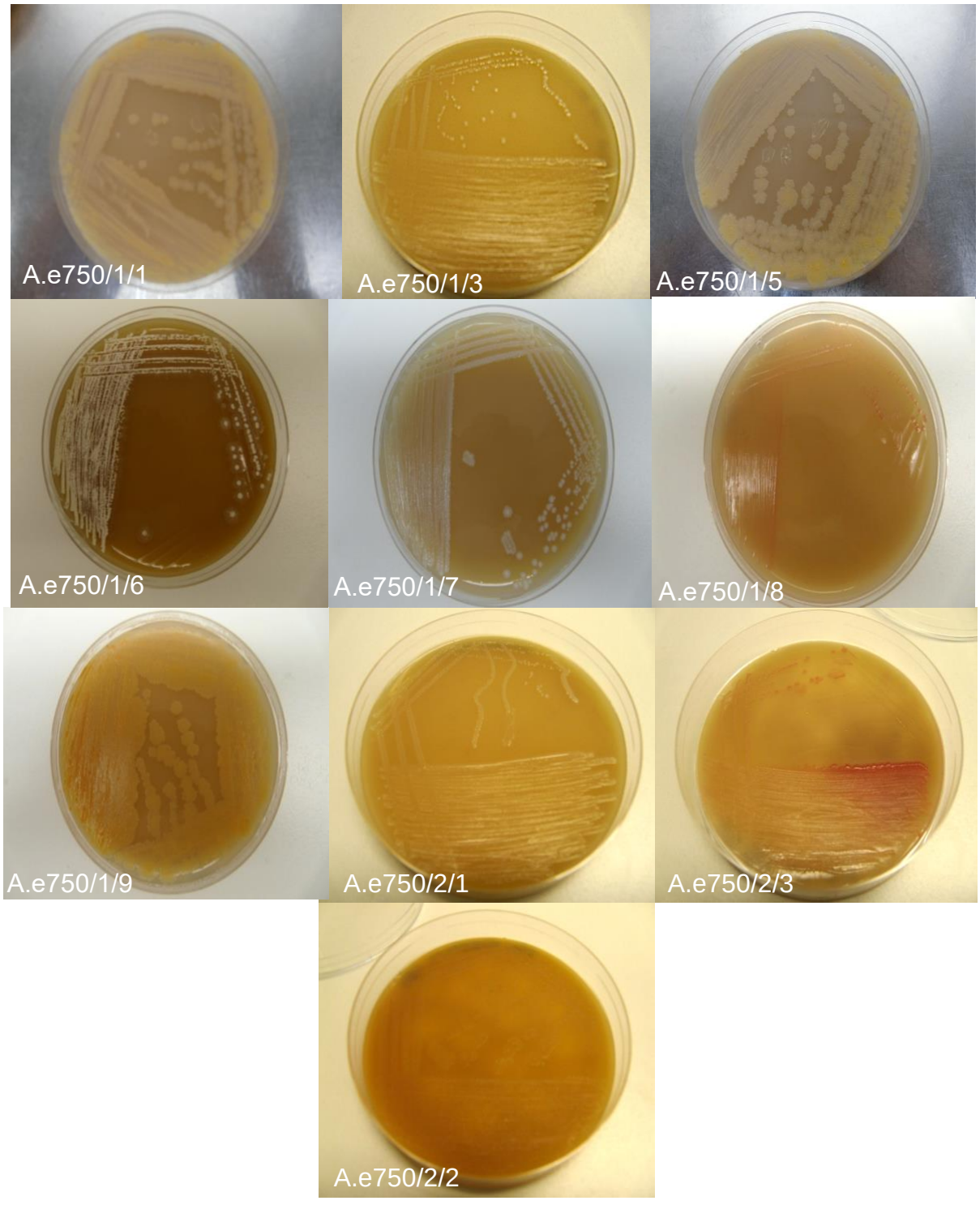

Figure 1. The successful isolation of A.e750/1/1, A.e750/1/3, A.e750/1/5, A.e750/1/6, A.e750/1/7, A.e750/1/8, A.e750/1/9, A.e750/2/1, A.e750/2/2, and A.e750/2/3 from the *Acromyrmex echinatior* colony A.e750. These isolates were grown at 30˚C on SFM containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid for 21 days. Successful isolations from A.e750 are visible as colonies in each image. Each colony of each isolate shared the same phenotypes as other colonies from the same isolate.

Ten *A. echinatior* colony A.e750 isolates were successfully isolated from the ants and grown on SFM plates containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid after incubation at 30˚C for 21 days (Figure 1). None of these isolates had the appearance of *Pseudonocardia* which phenotypically are small, white, hairy colonies. Although A.e750/1/3 had small white colonies, these colonies were not hairy and single colonies had a gelatinous texture unlike *Pseudonocardia* colonies. A.e750/1/1 and A.e750/1/5 colonies were neither white, small, nor hairless and so were not likely to be *Pseudonocardia* species. A.e750/2/1 had small pale mucous-like colonies, whilst A.e750/2/2 were transparent. A.e750/1/8 and A.e750/2/3 colonies were phenotypically small and had a pink colour whilst A.e750/1/9 colonies were yellow. Although A.e750/1/6 had small white colonies, these colonies were not hairy and single colonies contained a ring around them unlike *Pseudonocardia* spp.. A.e750/1/7 colonies were also small. However, the white shade was more of a cream and the isolates had a gelatinous appearance.

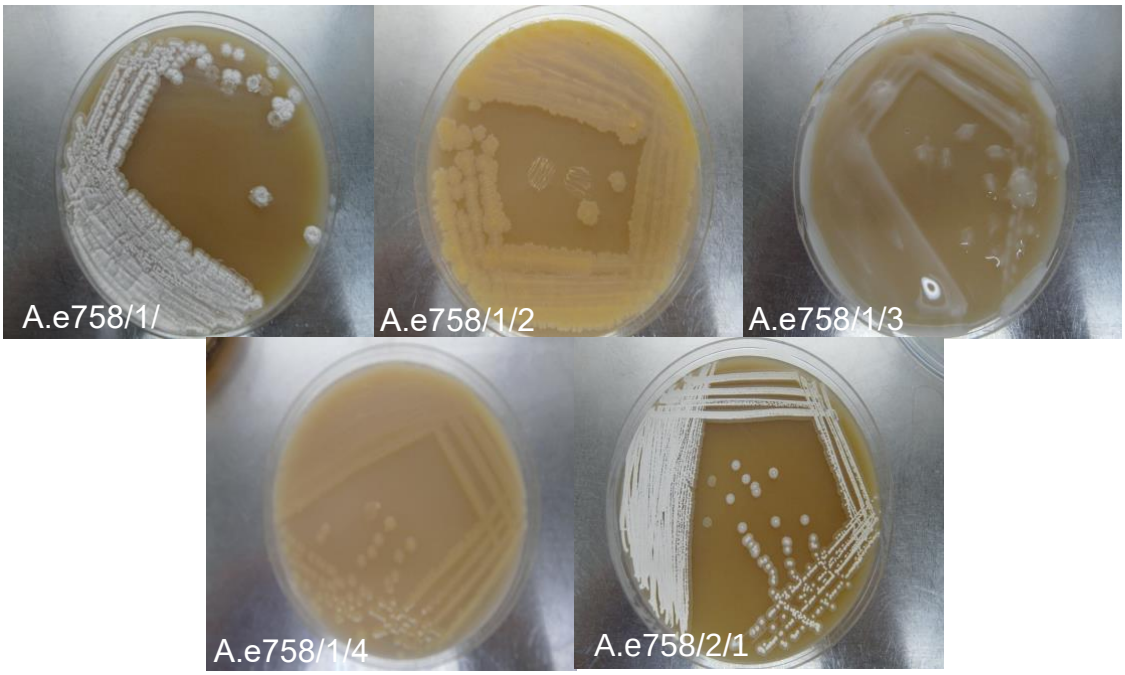

Figure 2. The successful isolation of A.e758/1/1, A.e758/1/2, A.e758/1/3, A.e758/1/4 and A.e758/2/1 from the *Acromyrmex echinatior* colony A.e758. These isolates were grown at 30˚C on SFM containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid for 21 days. Successful isolations from A.e750 are visible as colonies in each image. Each colony of each isolate shared the same phenotypes as other colonies from the same isolate. A.e758/2/1 had the desired phenotypic qualities of small white colonies which are seen in *Pseudonocardia* spp.

Five *A. echinatior* colony A.e758 isolates were successfully isolated and incubated at 30˚C on SFM containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid for 21 days (Figure 2). Only A.e758/2/1 had the phenotypic appearance of *Pseudonocardia*. Although A.e758/1/1 had white, hairy colonies, these colonies were larger and single colonies contained a ring around them which is not a phenotype of *Pseudonocardia* sp.. A.e758/1/2 and A.e758/1/4 colonies were yellow, larger, and hairless and so were not likely to be *Pseudonocardia* sp.. Although

colonies of A.e758/1/3 were white, they had a gelatinous texture unlike *Pseudonocardia* colonies which resulted in the formation of a biofilm over the plate. A.e758/2/1 had small white hairy colonies like *Pseudonocardia* spp. although some colonies had white rings around them.

Figure 3. The successful isolation of A.e399/1, A.e399/2, and A.e399/3 from the *A. echinatior* colony A.e399. These isolates were grown at 30˚C on SFM containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid for 21 days. Each colony of each isolate shares the same phenotypes as other colonies from the same isolate. A.e399/2 and A.e399/3 had the desired phenotypic qualities of *Streptomyces* spp.

Three A.e399 colony isolates were successfully isolated and grown on SFM plates containing 5 µg/ml nystatin, 100 µg/ml cycloheximide and 40 µg/ml nalidixic acid after incubation at 30˚C for 21 days (Figure 3) None of these isolates had all the same phenotypes as *Pseudonocardia* as seen in Figure 4 which are small, white, hairy colonies. A.e399/1 colonies were small, however the white shade was more of a cream and they had a gelatinous appearance. Although Ae399/2 and Ae399/3 were white and hairy during incubation, during sporulation they became green. It is not likely that these two isolates were *Pseudonocardia* bacteria with contamination as these colonies were much too large. It is however possible that these 2 isolates were *Streptomyces* as they had a similar appearance to *Streptomyces* bacteria as well as the change in pigmentation during sporulation can occur with *Streptomyces* spp. (Kelemen *et al*., 1998).

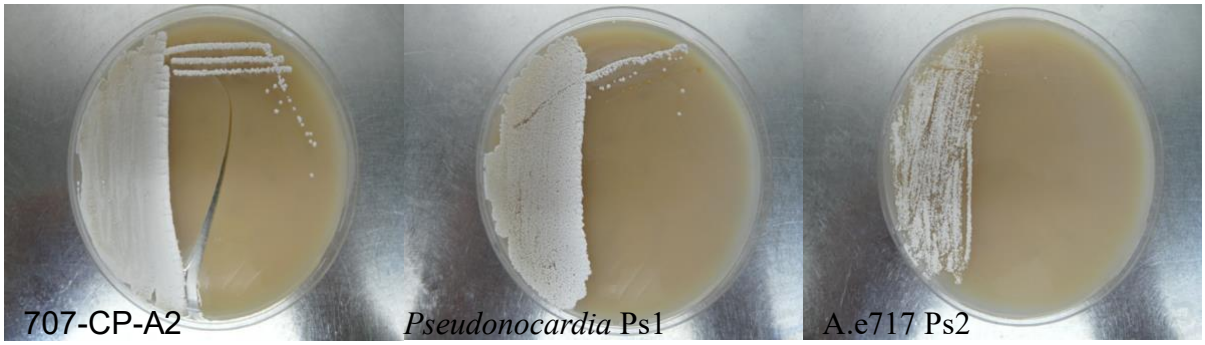

Figure 4. A dilution streak of 707-CP-A2, *Pseudonocardia* Ps1, and A.e717 Ps2 on SFM after 14 days of incubation at 30˚C. These bacteria were known *Pseudonocardia* strains. Successful growth of the colonies demonstrated that these strains had survived being frozen for a large period of time. The phenotypic qualities of *Pseudonocardia* spp. are visible and could be used to compare phenotypes against unidentified strains.

The three *Pseudonocardia* stocks had survived after being kept frozen for many years and could be used to make stocks and for bioassays to identify preferred media for the growth and antimicrobial activity of *Pseudonocardia* spp. (Figure 4). Each *Pseudonocardia* strain shared the same phenotype of small, white, and hairy colonies, which was used as a visual aid to identify potential isolated *Pseudonocardia* sp. from colonies A.e750, A.e758, and A.e399.

Genomic DNA 16S rRNA Gene Amplification by PCR

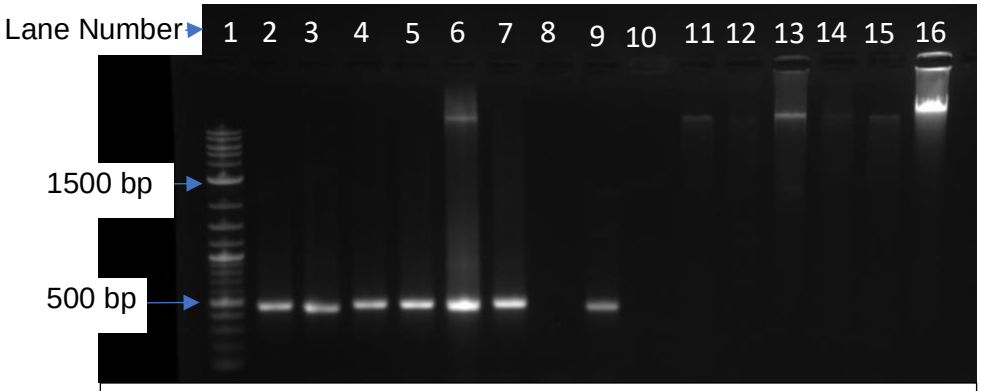

Figure 5. Agarose gel electrophoresis of PCR performed on extracted DNA (lanes 2 – 9) to amplify the 16S rRNA gene of each of the extracted isolates. Lanes 11 – 16 contained 5 µl of genomic DNA without PCR. The 16S rRNA gene was successfully amplified as there are clear visible bands at 500 base pairs and as there was no smearing in lanes 11-16 heavy degradation of the genomic DNA had not occurred so the DNA was of fine quality for sequencing. The lanes contain: 2 µl of GeneRuler 1 kb Plus DNA ladder (1), 20 µl of the genomic DNA solutions A.e750/1/1 (2), A.e750/1/5 (3), A.e758/1/1 (4), A.e758/1/2 (5), A.e758/1/3 (6), A.e758/1/4 (7), the negative control (8), the positive control (9), A.e750/1/1 (11), A.e750/1/5 (12), A.e758/1/1 (13), A.e758/1/2 (14), A.e758/1/3 (15), A.e758/1/4 (16).

The successful 16S rRNA gene amplification by PCR of the 20 µl of the extracted genomic DNA of the 6 strains A.e750/1/1, A.e750/1/5, A.e758/1/1, A.e758/1/2, A.e758/1/3, and A.e758/1/4 indicated by the bright clear bands at 500 bp (Figure 5). These visible bands showed that the genomic DNA could be used for sequencing. As there was not heavy smearing in lanes 11 – 16 which contained genomic DNA that had not undergone PCR it showed that heavy degradation of the genomic DNA had not occurred. Therefore, the genomic DNA was of fine quality for sequencing.

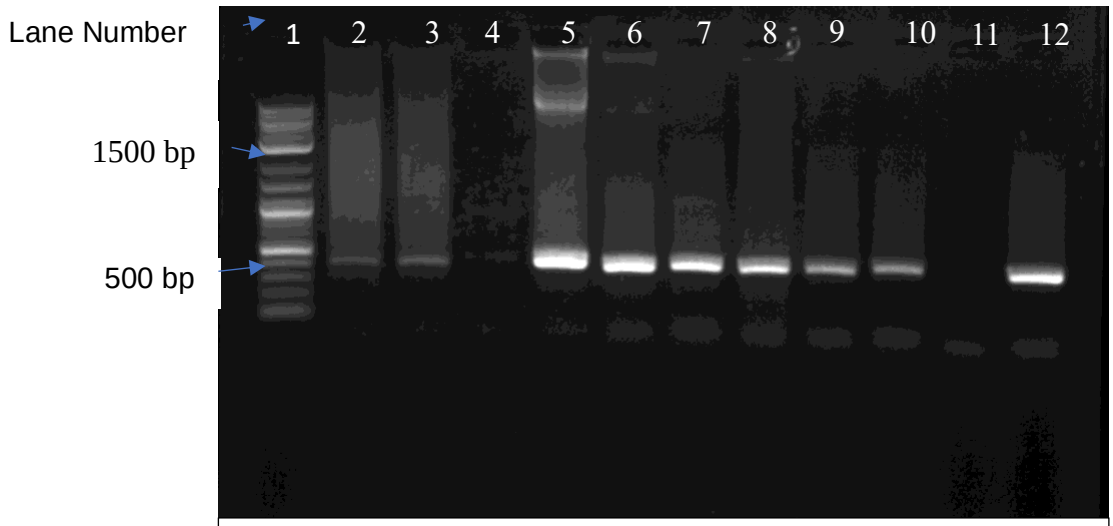

Figure 6. Agarose gel electrophoresis of PCR performed on extracted DNA (lanes 2 – 10). The lanes contain: 2 µl of GeneRuler 1 kb Plus DNA ladder (1), 20 µl of the genomic DNA solutions A.e399/2 (2), 707-CP-A2 (3), A.e399/1 (4), A.e717 Ps2 (5), A.e750/2/2 (6), A.e750/2/3 (7), A.e750/2/4 (8), A.e758/2/1 (9), *Pseudonocardia* Ps1, (10), the negative control (11), the positive control (12).

The 16S rRNA gene amplification of the 20 µl of the extracted genomic DNA of each of the isolates was successful as there were bright, clear bands visible at 500 bp (Figure 6). Since the 16S rRNA gene was successfully amplified for all A.e399/2, 707-CP-A2, A.e717 Ps2, A.e750/2/2, A.e750/2/3, A.e750/2/4, A.e758/2/1, and *Pseudonocardia* Ps1 strains this DNA could be used for sequencing.

DNA sequencing of isolates and Pseudonocardia acquired in the Hutchings Lab

Table 6. The forward and full sequence of *A. echinatior* isolates and *Pseudonocardia* sp. using the BLASTn database. A.e750/1/1, A.e750/1/3, A.e758/1/1, A.e758/1/2, and A.e758/1/4 were only forward sequenced as the reverse sequences were not of high enough quality.

Isolate	Sequence	Top Hit	Coverage (%)	Identity (%)	Length (Base Pairs)
A.e750/1/1	Forward	*Bacillus* sp.	100	100	420
A.e750/1/3	Forward	*Agrobacterium* sp., *Rhizobium* sp.	100	100	375
A.e758/1/1	Forward	*Staphylococcus* sp.	100	99.76	419
A.e758/1/2	Forward	*Bacillus megaterium*	100	100	398
A.e758/1/4	Forward	*Staphylococcus sp.*	100	100	413
A.e399/2	Full	*Streptomyces* sp.	100	99.51	976
707-CP-A2	Full	*Pseudonocardia* sp.	100	99.27	1175
A.e717 PS2	Full	*Pseudonocardia* sp.	100	99.51	1229
A.e750/2/2	Full	*Agrococcus* sp.	100	99.01	1233
A.e750/2/3	Full	*Methylorubrum* sp.	96	96.01	1096
A.e750/2/4	Full	*Enterobacter* sp.	100	99.53	942
A.e758/2/1	Full	*Pseudonocardia* sp.	100	99.28	1176
Pseudonocardia PS1	Full	*Pseudonocardia* sp.	97	99.50	1017

All 1st isolates had a coverage of 100%. These high coverage percentages were good because the coverage shows the percentage of DNA that aligned to a sequence found in the GenBank. Therefore, the larger the coverage the better the match between the DNA sequences of the isolate and top result. Hence table 6 showed that the isolates A.e750/1/1 and A.e758/1/2 were likely to have been a *Bacillus* sp., with A.e758/1/2 likely to have been *Bacillus megaterium* in particular as these isolates had an identity of 100%. It showed that A.e750/1/3 and A.e758/1/4 also had an identity of 100% which indicated that A.e750/1/3 was likely to have been either a *Agrobacterium* sp. or a *Rhizobium* sp. whilst A.e758/1/4 was likely to have been a *Staphylococcus* sp.. Although A.e758/1/1 had an identity of 99.76% it was also likely to be part of the *Staphylococcus* genus as the coverage was at 100% and the identity was close to 100%. Of the five 1st isolated sequenced bacteria, *Staphylococcus* species have been identified to be one of the most common bacteria

found on other leafcutter ant species such as *Mycocepurus smithii* from Panama like *A. echinatior* (Kellner *et al*., 2015). If an actinobacteria is isolated from the *A. echinatior* it is likely to be *Pseudonocardia* as it is the dominant actinobacteria found on attine ants, in particular higher attine ants such as *A. echinatior* (Li *et al*., 2018). A.e750/1 was identified as a *Bacillus* sp.. This is a gut bacteria of *A. echinatior* ants (Zhukova *et al.,* 2017).
As none of the 1st isolates were *Pseudonocardia* or *Streptomyces* bacteria, the 2nd bacteria isolations from *Acromyrmex echinatior* colonies A.e750, A.e758, and A.e399 were isolated and sequenced. The *Pseudonocardia* isolates were also sequenced. The forward and reverse sequences were aligned through the BLASTn database in order to determine the full sequence. This showed that for the top hit, all the 2nd isolates had a coverage of 100% other than the 2nd isolates A.e750/2/3 and *Pseudonocardia* Ps1 which had a coverage of 96% and 97%, respectively. Although no isolate had an identity of 100%, the identity percentages were high with all 2nd isolates other than A.e750/2/3 which had an identity of over 99%. Isolates 707-CP-A2, A.e717 PS2, A.e758/2/1, and *Pseudonocardia* Ps1 were all identified as *Pseudonocardia* species. A.e299/2 was identified as belonging to the *Streptomyces* genus. A.e750/2/2 was identified as belonging to the *Agrococcus* genus, which just like *Pseudonocardia* and *Streptomyces* is an actinomycete. A.e750/2/3 was identified as belonging to the *Methylorubrum* genus whilst A.e750/2/4 was identified as belonging to the *Enterobacter* genus.

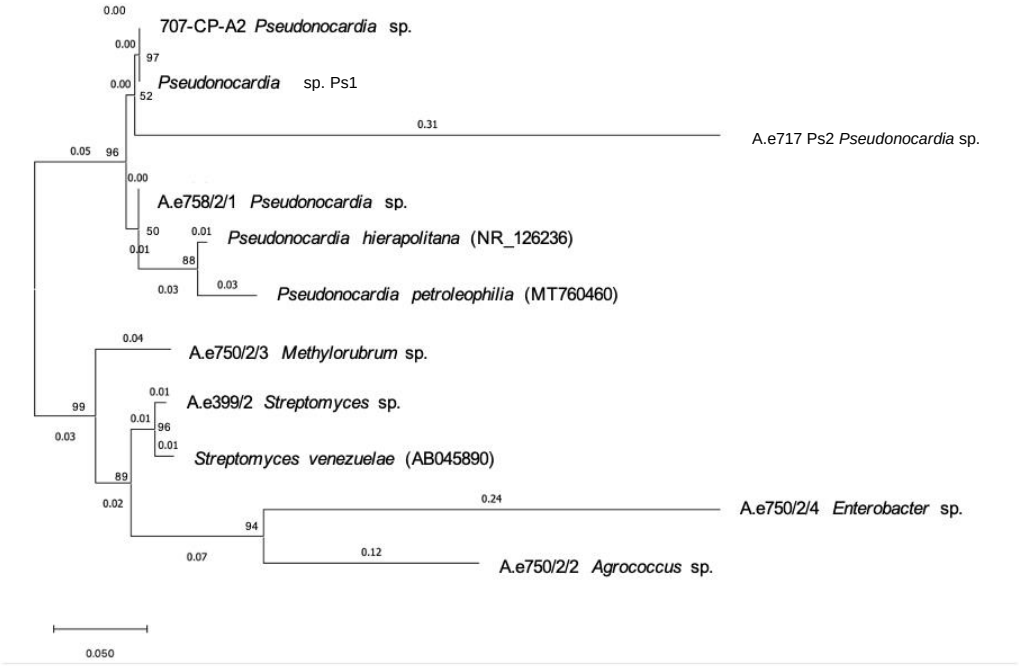

Figure 7. A phylogenetic tree of 16s rRNA gene of ant isolates and reference strains *Pseudonocardia hierapolitana*, *Pseudonocardia petroleophilia*, and *Streptomyces venezuelae* with their GenBank accession numbers. The tree was built with Mega11 using the Nearest-Neighbour-Interchange method with the Kimura-2 model to calculate the distance. The bootstrap confidence value was calculated using 1000 replicates.

All the full sequence strains were then chosen and put in a phylogenetic tree along with *Pseudonocardia hierapolitana*, *Pseudonocardia petroleophilia*, and *Streptomyces venezuelae* which were used as reference strains as seen in Figure 7. This was performed on Mega11 using the Nearest-Neighbour-Interchange method with the Kimura-2 model to calculate the distance. The bootstrap confidence value was calculated using 1000 replicates to demonstrate the confidence levels of the clades on the phylogenetic tree. Upon sequencing, A.e717 Ps2, 707-CP-A2, A.e758/2/1, and *Pseudonocardia* sp. Ps1 were all identified as *Pseudonocardia* species (Table 6). This would explain as to why they are all grouped closely together in the phylogenetic tree in comparison to the other isolates and why the *Pseudonocardia* isolates are closer related to the reference strains *P. hierapolitana* and *P. petroleophilia* which are found in soil (Sahin *et al*., 2014). Since *Pseudonocardia* spp. found in soil are phylogenetically different it would explain as to why the ant *Pseudonocardia* isolates A.e717 Ps2, 707-CP-A2, and *Pseudonocardia* sp. Ps1 are branched away from the *Pseudonocardia* reference strains (Ishak *et al*., 2011). Although A.e717 Ps2 can be seen with the other *Pseudonocardia* isolates, the much larger length of the branch demonstrated that this isolate has more differences in its genetic sequence compared to *Pseudonocardia* sp. Ps1 and 707-CP-A2. However, the bootstrap confidence value is only at 52% and so this this information cannot be trusted to be correct. A.e399/2, which was identified as a *Streptomyces* sp., is seen to be closely related to the reference strain *S. venezuelae* as there is little distance between them and there is a large bootstrap confidence value which further suggests that the identification of A.e399/2 as a *Streptomyces* sp. was correct.

It would be useful to fully sequence the 5 forward strains A.e750/1/1, A.e750/1/3, A.e758/1/1, A.e758/1/2, and A.e758/1/3 (Table 6) and add these isolates to the phylogenetic tree as the more isolates we are able to compare the more accurate the phylogenetic tree would be.

Gram Stain

As A.e758/2/2, 707-CP-A2 and *Pseudonocardia* Ps1 were identified as likely belonging to the *Pseudonocardia* genus, and A.e399/2 was identified as likely belonging to the *Streptomyces* genus, a gram stain was performed to view the cells of these five bacteria. Each of the isolates remained dyed purple as seen in Figure 8 and so were identified to be gram-positive. Each of the isolates were also rod shaped. *Streptomyces* and *Pseudonocardia* species are actinomycetes (Riahi *et al*., 2021), thus they are gram-positive, filamentous, rod shaped bacteria (Rahlwes *et al.,* 2019), which further

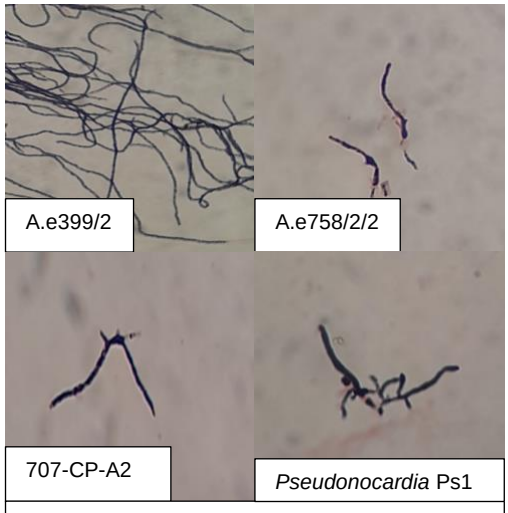

Figure 8. The gram stain of the gram-positive isolates A.e399/2, A.e758/2/2, 707-CP-A2, and *Pseudonocardia* Ps1, which have remained dyed purple. Each of the strains were viewed under 100x magnification with oil.

emphasises that the identification of A.e758/2/2, 707-CP-A2, *Pseudonocardia* Ps1, and A.e399/2 was accurate.

Amplicon Sequencing Data

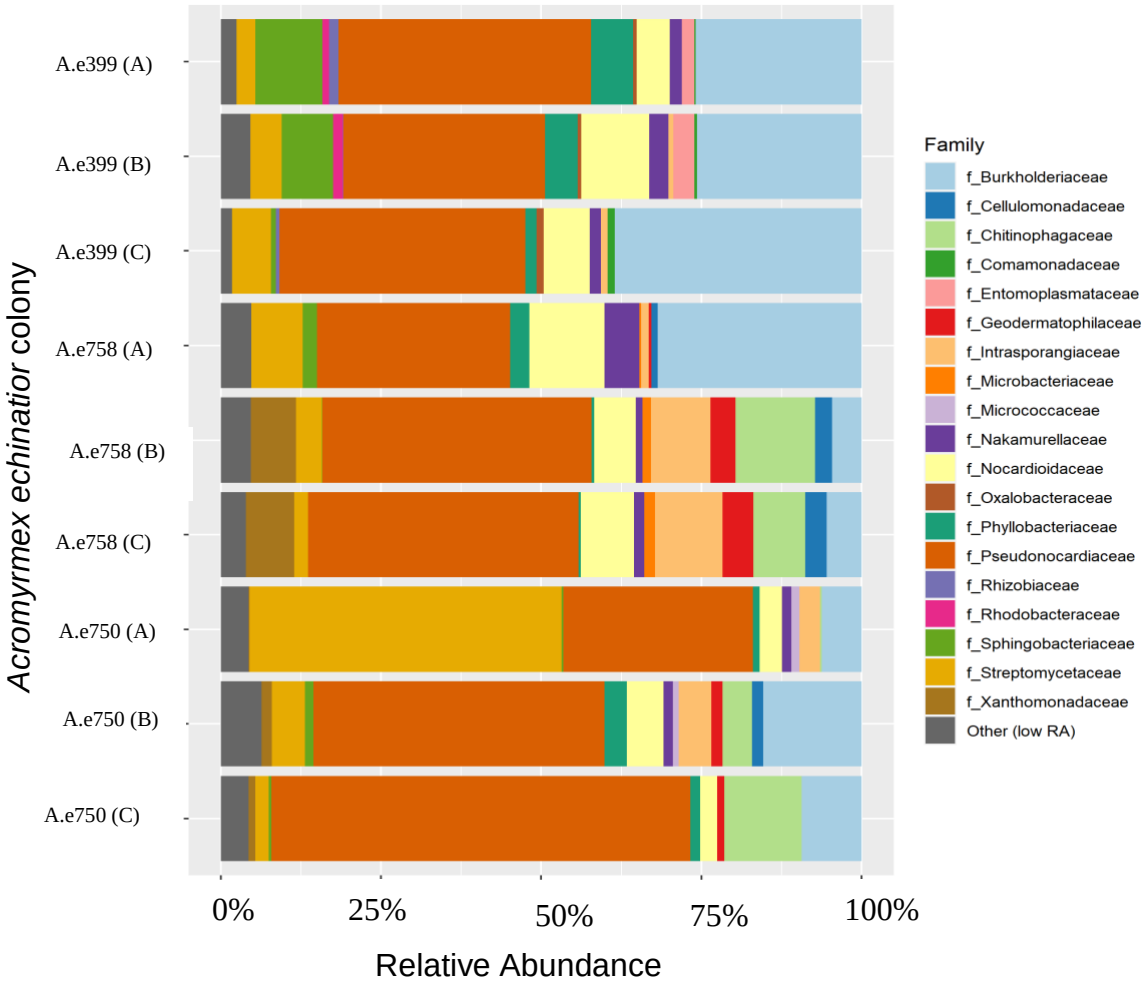

Figure 9. A bar plot which presents the relative abundance of bacteria located in the laterocervical plate microbiome of ants from the *Acromyrmex echinatior* colonies: A.e399, A.e758, and A.e750. For each colony 3 replicates were used. Each of the A. echinatior colonies had a large relative abundance of bacteria from the *Pseudonocardiaceae* family.

Through high performing computing, QIMME2, and RStudio, a bar plot was generated to identify the relative abundance of bacteria located in the laterocervical plate microbiome of the ants from the *Acromyrmex echinatior* colonies A.e399, A.e758, and A.e750 (Figure 9). The largest bacterial family abundance found in one sample was seen in sample A.e750 (C) where *Pseudonocardiaceae* had a relative abundance of 65.4%. The 3 largest *Pseudonocardiaceae* relative abundances across all samples were each identified to be located in the 3 A.e750 replicates where A.e750 (A) and A.e750 (B) had a relative abundance of *Pseudonocardiaceae*

of 48.7% and 45.4%, respectively. In almost every sample the *Pseudonocardiaceae* family had the largest relative abundance and in the few where it does not have the highest relative abundance it still has the second highest. In sample A.e399 (C) the *Pseudonocardiaceae* family has just a 0.1% lower relative abundance than the *Burkholderiaceae* family. In sample A.e758 (A), the *Pseudonocardiaceae* family has just a 1.6% lower relative abundance than the *Burkholderiaceae* family, and in sample A.e750 (A) the *Pseudonocardiaceae* family has a 19.1% lower relative abundance than the *Burkholderiaceae* family.

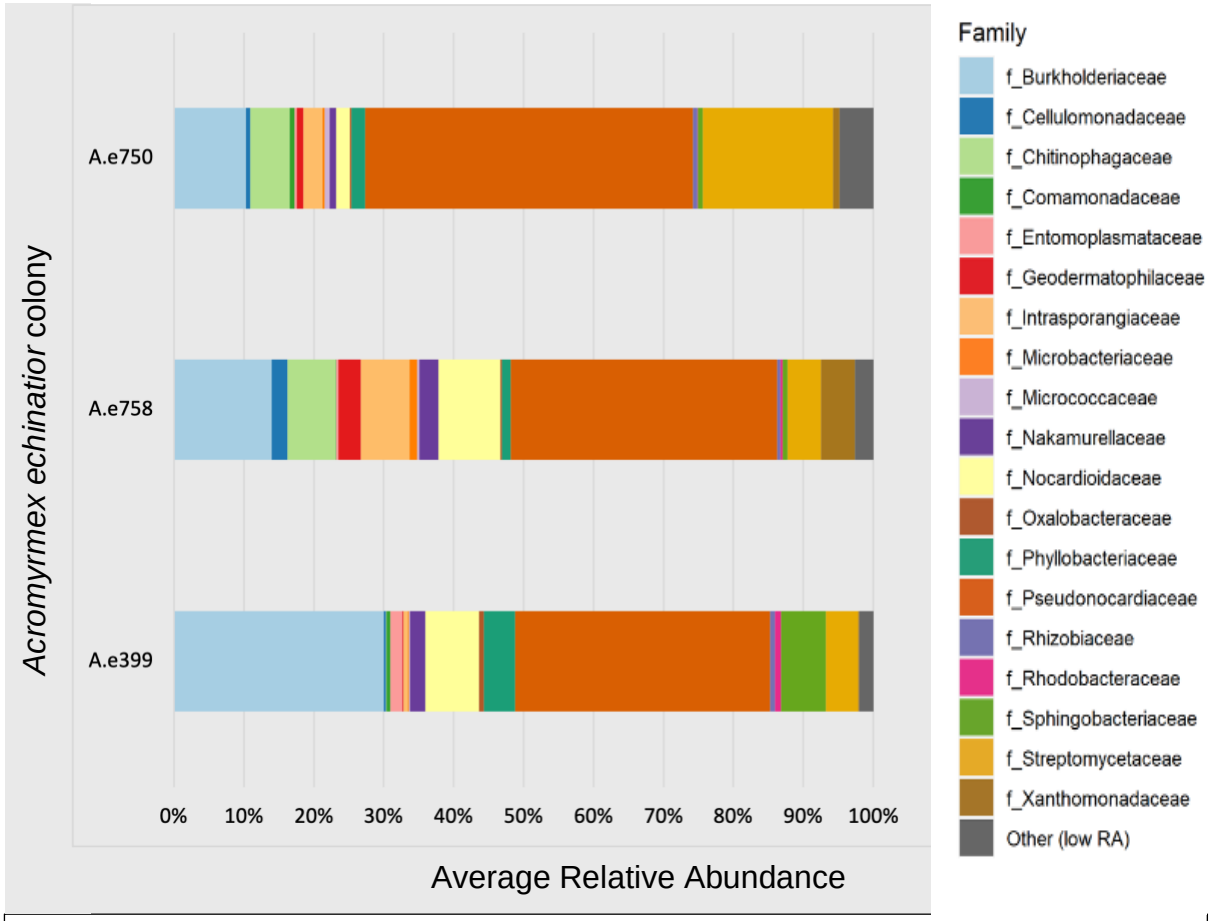

Figure 10**.** A bar plot which presents the average relative abundance of bacteria located in the laterocervical plate microbiome of ants the *Acromyrmex echinatior* colonies: A.e399, A.e758, and A.e750 from the 3 replicates. Each of the A. echinatior colonies had a large relative abundance of bacteria from the *Pseudonocardiaceae* family.

In all 3 of the *Acromyrmex echinatior* colonies the *Pseudonocardiaceae* family had the largest average relative abundance (Figure 10). A.e750 had the largest average relative abundance of *Pseudonocardiaceae* at 46.8% which was 10.4% larger than colony A.e399 and 8.16% larger than colony A.e758. Colony A.e750 also had the largest relative abundance of bacteria from the *Streptomycetaceae* family at 18.7%. This was 14.0% larger than A.e399 and 13.9% larger than A.e758. A.e399 had the largest relative abundance of bacteria belonging to the *Burkholderiaceae* family at 30.0%.

Bioassays

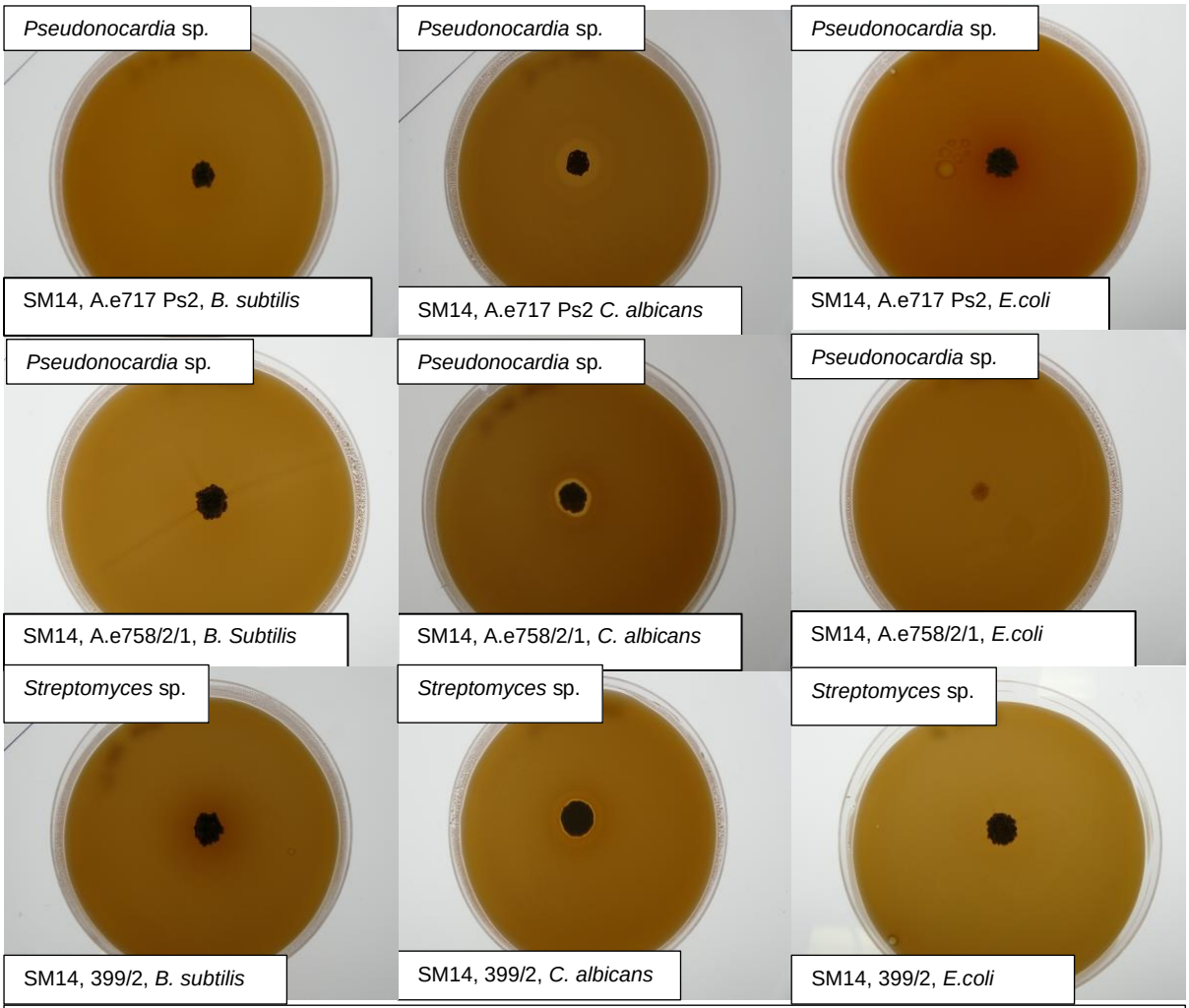

Figure 11. The *A. echinatior* isolates A.e758/2/1 (*Pseudonocardia* sp.), A.e399/2 (*Streptomyces* sp.), and A.e717 Ps2 (*Pseudonocardia* sp.) grown on SM14 agar for 20 days at 30˚C and overlayed with either 3 ml of *C. albicans, B. subtilis,* or *E.coli.* The photos were taken after incubating the isolates with the overlay for 2 days at 30˚C. The production of antifungals from each of the 3 strains is visible due to presence of zones of inhibition for each of the strains when *C. albicans* was used as an overlay.

A.e758/2/1, A.e399/2, and A.e717 Ps2 were able to produce antifungals on SM14 agar as all 3 isolates had a zone of inhibition when overlayed against *C. albicans* as seen in Figure 11. Although all 3 isolates were able to produce antifungals against *C. albicans* on SM14, they were not able to produce antimicrobials against either *B. subtilis* or *E.coli* on SM14. A.e758/2/1 had the clearest zone of inhibition, whilst A.e717 Ps2 had a very feint zone of inhibition. This may indicate that these strains are more specialised against producing antifungals, which would correlate to the

natural threat that these strains protect *A. echinatior* colonies from the fungus *Escovopsis.*

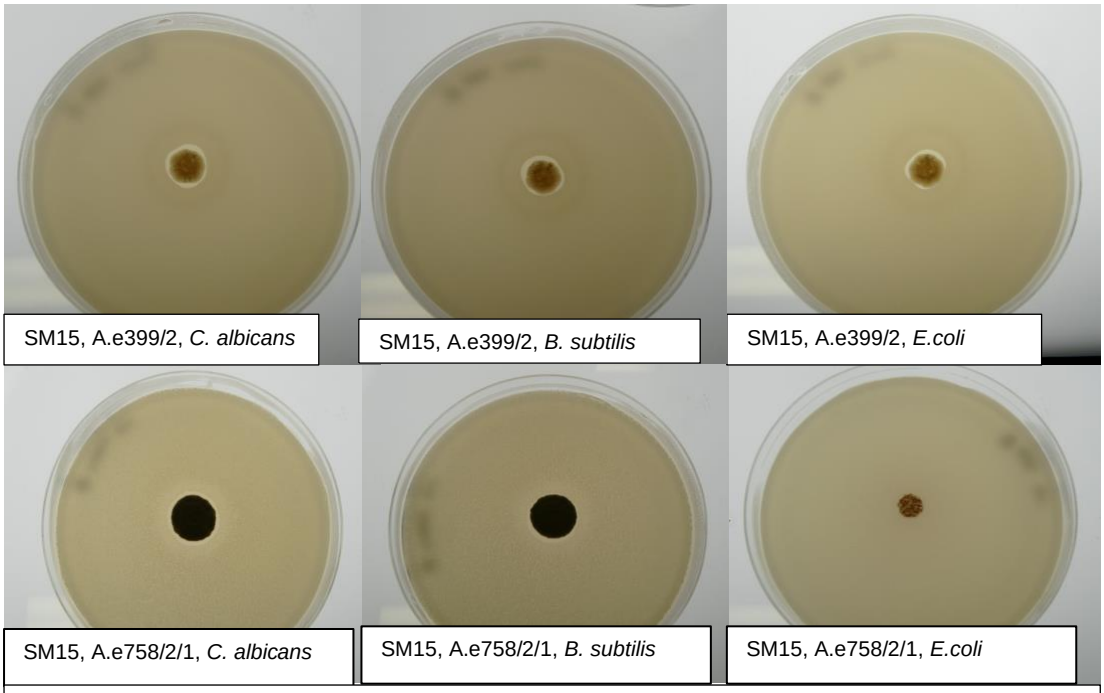

Figure 12. The *A. echinatior* isolates A.e758/2/1 (*Pseudonocardia* sp.) and A.e399/2 (*Streptomyces* sp.) grown on SM15 agar for 20 days at 30˚C and overlayed with either 3 ml of *C. albicans, B. subtilis,* or *E. coli.* The photos were taken after incubating the isolates with the overlay for 2 days at 30˚C.

The *A. echinatior* isolates A.e758/2/1 and A.e399/2 were able to produce antimicrobials on SM15 (Figure 12). A.e399/2 was able to produce a strong zone of inhibition against all 3 indicator strains, whilst A.e758/2/1 was able to produce a small zone of inhibition against *C. albicans* and *B. subtilis,* but not against *E. coli*.

As A.e399/2 was identified as a potential *Streptomyces* sp. these results may indicate that the media SM15 is more suited for the growth and activity of *Streptomyces* spp. than *Pseudonocardia* spp. as A.e399/2 colonies were able to grow and produce not only antifungal, but also antibacterial compounds to inhibit the growth of both bacterial and the fungal indicator strains. The potential *Pseudonocardia* sp. A.e758/2/1 was only able to inhibit the growth of one bacterial indicator strain which may suggest the *Pseudonocardia* sp. is very dependent on its environment to be able to produce different antimicrobials. A.e758/2/1 had a greater zone of inhibition against the fungal indicator strain than against the *B. subtilis* which it was also able to inhibit. This gives further suggestions, as seen in Figure 11, that the *Pseudonocardia* sp. has a greater specialization of the inhibition of fungal pathogens, however is still able to inhibit bacterial pathogens just to a lesser effectiveness.

It would be beneficial to use *Escovopsis* as the indicator strain for antifungal activity in future studies as this pathogenic fungus is a natural threat of *A. echinatior* colonies (Barke, *et al*., 2011), and so the bioassays are a closer representation of the *Pseudonocardia* and *Streptomyces* sp. protecting the ants in the wild. A comparison of the zone of inhibition between a fungal indicator strain such as *C. albicans* and *Escovopsis* would be interesting. A larger zone of inhibition against the *Escovopsis* than other fungal indicator strains could indicate the *Pseudonocardia* sp. is not only more specialised against fungal pathogens than bacterial pathogens but has also co-evolved to be more specialised against specific fungal pathogens such as *Escovopsis.* This would make sense as *Escovopsis* is associated with being a natural pathogenic threat to *A. echinatior* ant colonies. As there were many unsuccessful *Pseudonocardia* isolations during the culture-dependent isolations it would be beneficial to grow the isolated bacteria on SM14 and SM15 since potential *Pseudonocardia* isolates were successfully able to form colonies and produce rings of inhibition against indicator strains on these two media types (Figures 11 and 12). This use of these media during culture-dependent isolations may result in an increase of *Pseudonocardia* isolates as they would be growing on a more suitable medium.

Discussion

The multiple attempts to isolate (Table 2) *Pseudonocardia* sp. from the ant microbiome has shown how incredibly challenging this actinomycete is to work with even though it had the highest relative abundance in the *A. echinatior* colonies (Figure 10). As this actinomycete takes a long time to grow, and even if colonies successfully form, it does not mean that this bacteria will be able to produce antimicrobials in the different media type as shown by figures 11 and 12. This may suggest that the *Pseudonocardia* sp*.* have evolved to be grow and be more efficient against pathogens in the ant microbiome than anywhere else. This may be due to specialised structures on the ants that over millions of years the *Pseudonocardia* sp. has co-evolved to thrive on these structures. This also may explain as to why only one type of *Pseudonocardia* is known to be found in each *A. echinatior* colony. These specialised structures on the ant cuticles contain pores that provide the *Pseudonocardia* sp. with secretions which may provide the perfect environment for the *Pseudonocardia* sp. to grow and produce antimicrobials (Li *et al.* 2018). This may explain as to why the *Pseudonocardia* sp. are so difficult to isolate through culture-dependent methods. These glandular secretions would nourish the *Pseudonocardia* and may even be specialised towards aiding the growth and activity of the *Pseudonocardia*. A future experiment may therefore be worth attempting to isolate these glandular secretions of the *A. echinatior* and identifying them, in an attempt to replicate these secretions. By introducing these secretions to isolates from *A. echinatior* it could create an environment further specialised for the growth and activity of the *Pseudonocardia* sp. which could result in more *Pseudonocardia* isolates and greater zones of inhibition due to the *Pseudonocardia* isolates being more active. A study by Zucchi *et al*., 2011 isolated 20 bacteria from *Acromyrmex subterraneus brunneus*, 17 of which were *Streptomyces* spp. whilst only 1 was a *Pseudonocardia* sp.. Cultured-dependent isolations show less microbial diversity than culture-independent isolations (Sen *et al*., 2009). Culture-independent isolations of *T. septentrionalis* showed two *Pseudonocardia* isolates, whilst culture-dependent

isolations of *T. septentrionalis* only showed one *Pseudonocardia* isolate (Sen *et al*., 2009). Using culture-dependent isolations no *Pseudonocardia* spp. were found on *A. coronatus.* As a Pseudonocardia sp. from only 1 of the 3 A. echinatior colonies was able to be isolated using culture-dependent isolations this correlates with the results of Zucchi *et al*., 2011 & Sen *et al*., 2009. It has been shown by the culture-independent isolations that there is a high abundance of *Pseudonocardia* sp. on all 3 of the ant colonies and a much greater microbial diversity.

It may be interesting to use SFM 14 or SFM 15 to screen the two different *Pseudonocardia* species against one another to see if they produce any antimicrobials to inhibit the colonisation of other *Pseudonocardia* species. This is because *A. echinatior* ants only carry either *P. echinatior or P. octospinosus* and it has been seen that *Pseudonocardia* spp. can inhibit the growth of similar *Pseudonocardia* species that are competing for colonisation (Van Arnam *et al*., 2015). It may be also interesting to screen the *A. echinatior Pseudonocardia* isolates against *Pseudonocardia* species from soil as *Pseudonocardia* can be found in soil *(Wang et al., 2017)* yet only *P. echinatior* or *P. octospinosus* are found on *A. echinatior*. Mueller *et al*., 2010 has shown that environmental *Pseudonocardia* can have close genetic similarities or even matching sequences with *Pseudonocardia* species associated with ants. However, this was not witnessed during this study as shown by Figure 7 where the soil *Pseudonocardia* reference strains branched away from 3 of the *A. echinatior Pseudonocardia* isolates. As there was a high bootstrap value of 96 this branch apart could be accepted. The further away on the phylogenetic tree the more dissimilar the genetic sequence of the bacteria. It is therefore possible that the *Pseudonocardia* reference strains from the soil do not have the same antibiotic resistant genes (ARGs) as the *Pseudonocardia* strains from the *A. echinatior.* If the different *Pseudonocardia* species do not share the same ARGs then different *Pseudonocardia* bacteria would be able to inhibit the growth of each other through the production of antimicrobials. This may suggest why only *P. echinatior* or *P. octospinosus* are the only *Pseudonocardia* sp. found on *A. echinatior* since the attine-associated *Pseudonocardia* sp. would inhibit the colonisation of soil *Pseudonocardia* spp. with antimicrobials.

Isolated *Streptomyces* could be shown to be resistant to the *Pseudonocardia*-produced antibiotics by screening them against each other on SM15 where the *Pseudonocardia* sp. was confirmed to be able to secrete antimicrobials into the agar to inhibit bacteria. As the *Streptomyces* sp. will likely survive unlike the *B. subtilis* and *E. coli* it will show the resistance of the *Streptomyces* to the *Pseudonocardia*-produced antimicrobials. This would demonstrate that the *Streptomyces* sp. on *A. echinatior* likely have antibiotic-resistant genes to the antimicrobials produced by the *Pseudonocardia* sp. on the ants.
Although sporulation of different *Streptomyces* sp. occurs at different rates, some *Streptomyces* sp. are able to sporulate after being incubated for 4-10 days (Shepard, *et al.,* 2010). This occurred with the *Streptomyces* isolate A.e399/2 which is significantly faster than the 21 days it took for the *Pseudonocardia* isolates to sporulate. One could then assume that the *Streptomyces* sp. would be able to inhibit the growth of the *Pseudonocardia* sp. on the *A. echinatior* ants. This would be because the *Streptomyces* sp. would be able to grow at a faster rate and so a greater abundance of *Streptomyces* bacteria would be identified in the microbiome of the *A. echinatior.* The *Streptomyces* would also produce antimicrobials which would

inhibit the growth of other bacteria competing in this microbiome. However, *Streptomycetaceae* bacteria were of a much lower abundance than *Pseudonocardiaceae* in all 3 *A. echinatior* colonies as shown by Figure 10. This may be due to *Pseudonocardia* sp. growing in it's ideal conditions on the ant cuticles. The *Pseudonocardia* sp. is not growing faster as it still takes around one whole month for laterocervical plates to be covered in the *Pseudonocardia* biofilm (Andersen *et al.,* 2013). The more likely assumption is that the *Pseudonocardia* in the *A. echinatior* microbiome are in an ideal environment to secrete antimicrobials which are inhibiting the growth of the other bacteria which would result in more space for the *Pseudonocardia* sp. to grow. This would result in a larger abundance of *Pseudonocardia* bacteria than other bacteria, which can be seen in Figure 10 where it has the largest abundance in 2 colonies A.e750 and A.e758 and the second largest abundance in the colony A.e399.

Conclusion

To summarise, only one isolate from A.758 and no isolates from A.e399 and A.e750 were identified as *Pseudonocardia* sp. which would suggest a lack of abundance of *Pseudonocardia* sp. on the leafcutter ant microbiome. However, the amplicon sequencing data (Figure 10) contradicted this as it demonstrated that the bacterial composition of these three *Acromyrmex echinatior* had a large relative abundance of *Pseudonocardiaceae*. Culture-dependent isolations have been seen to indicate less diversity than culture-independent isolations in other studies (Sen *et al*., 2009). The amplicon sequencing data indicated that there were high relative abundances of bacteria from the *Pseudonocardiaceae*, *Burkholderiaceae*, and *Streptomycetaceae* families on the leafcutter ant laterocervical plates. The bioassays demonstrated that the *Streptomyces* and *Pseudonocardia* sp. could produce antimicrobials on both SM14, and SM15. This proved that *Pseudonocardia* and *Streptomyces* species are able to produce antimicrobials to protect *A. echinatior* colonies from pathogens. This suggested that both media types SM14 and SM15 are preferred medium for *Pseudonocardia* and *Streptomyces* sp.. Therefore, using SM14 or SM15 as the medium rather than SFM during the isolation of the bacteria may result in more isolates being *Pseudonocardia* and *Streptomyces* sp.. To replicate the leafcutter ant microbiome protecting the fungal gardens in the wild it would be useful to use *Escovopsis* as the indicator strain for antifungal activity as this pathogenic fungus is a natural threat of *A. echinatior* colonies (Barke, *et al*., 2011). Through activity-guided fractionation and identify using LCMS and NMR, purification and identification of the antimicrobials produced by the *Pseudonocardia* and *Streptomyces* sp. in the bioassays could then be performed. It may be possible to make a more specialised medium for the *Pseudonocardia* sp. by taking SM14 and SM15 and changing either the carbon or nitrogen source whilst maintaining all other components the same and performing bioassays on these new media. This would allow for the production of a new specialised medium since all of the components of this medium would be specialised for the growth of the *Pseudonocardia* sp.

References:

Actinobase.org. 2020. Salting Out Genomic DNA Extraction Method - ActinoBase. [online] Available at: <http://actinobase.org/index.php/Salting_Out_Genomic_DNA_Extraction_Method> [Accessed 29th March 2021].

Andersen, S., Hansen, L., Sapountzis, P., Sørensen, S. & Boomsma, J. (2013), 'Specificity and stability of the Acromyrmex-*Pseudonocardia* symbiosis', *Molecular Ecology*, 22(16), pp. 4307-4321.

Aylward, F., Currie, C. & Suen, G. (2012), 'The Evolutionary Innovation of Nutritional Symbioses in Leaf-Cutter Ants', *Insects*, 3(1), pp. 41-61.

Aylward, F., Khadempour, L., Tremmel, D., McDonald, B., Nicora, C., Wu, S., Moore, R., Orton, D., Monroe, M., Piehowski, P., Purvine, S., Smith, R., Lipton, M., Burnum-Johnson, K. & Currie, C., 2015. Enrichment and Broad Representation of Plant Biomass-Degrading Enzymes in the Specialized Hyphal Swellings of *Leucoagaricus gongylophorus*, the Fungal Symbiont of Leaf-Cutter Ants. *PLOS ONE*, 10(8), p. 0134752.

Barke, J., Seipke, R., Grüschow, S., Heavens, D., Drou, N., Bibb, M., Goss, R., Yu, D. & Hutchings, M., 2010. A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *BMC Biology*, 8(1), p. 109.

Barke, J., Seipke, R., Yu, D. & Hutchings, M., 2011. A mutualistic microbiome. *Communicative & Integrative Biology*, 4(1), pp. 41-43.

Bertani, G. (1951) 'Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli', Journal of bacteriology. J Bacteriol, 62(3), pp. 293–300.

Boya P., C., Fernández-Marín, H., Mejía, L., Spadafora, C., Dorrestein, P. & Gutiérrez, M., 2017. Imaging mass spectrometry and MS/MS molecular networking reveals chemical interactions among cuticular bacteria and pathogenic fungi associated with fungus-growing ants. *Scientific Reports*, 7(1).

Bruner-Montero, G., Wood, M., Horn, H., Gemperline, E., Li, L. & Currie, C., 2021. Symbiont-mediated protection of *Acromyrmex* leaf-cutter ants from the entomopathogenic fungus *Metarhizium anisopliae, mBio,* 12(6).

Cafaro, M., Poulsen, M., Little, A., Price, S., Gerardo, N., Wong, B., Stuart, A., Larget, B., Abbot, P. & Currie, C., 2010. Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proceedings of the Royal Society B: Biological Sciences*, 278(1713), pp. 1814-1822.

Carr, G., Derbyshire, E., Caldera, E., Currie, C. & Clardy, J., 2012. Antibiotic and Antimalarial Quinones from Fungus-Growing Ant-Associated *Pseudonocardia* sp. *Journal of Natural Products*, 75(10), pp. 1806-1809.

Dhodary, B. & Spiteller, D., 2021. Ammonia production by *Streptomyces* symbionts of *Acromyrmex* leaf-cutting ants strongly inhibits the fungal pathogen *Escovopsis*. *Microorganisms*, 9(8), p. 1622.

Donald, L., Pipite, A., Subramani, R., Owen, J., Keyzers, R.A. & Taufa, T., 2022. *Streptomyces*: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective. *Microbiol. Res,* 13(3), pp. 418-465.

Engl, T., Kroiss, J., Kai, M. & Kaltenpoth, M., 2018. Evolutionary stability of antibiotic protection in a defensive symbiosis. *Proceedings of the National Academy of Sciences*, 115(9).

Fukuda, T.T., Pereira, C.F., Melo, W.G., Menegatti, C., Andrade, P.H., Groppo, M., Lacava, P.T., Currie, C.R. & Pupo, M.T., 2021. Insights into the ecological role of pseudomonas spp. in an ant-plant symbiosis," *Frontiers in Microbiology*, 12.

Francoeur, C.B., May, D.S., Thairu, M.W., Hoang, D.Q., Panthofer, O., Bugni, T.S., Pupo, M.T., Clardy, J., Pinto-Tomás, A.A. & Currie, C.R., 2021. *Burkholderia* from fungus gardens of fungus-growing ants produces antifungals that inhibit the specialized parasite *Escovopsis*. *Applied and Environmental Microbiology*, 87(14).

Genilloud, O., 2017. Actinomycetes: Still a source of novel antibiotics. *Natural Product Reports*, 34(10), pp. 1203–1232.

Giraud, T., Pedersen, J.S. & Keller, L., 2002. Evolution of super colonies: The Argentine ants of southern Europe. *Proceedings of the National Academy of Sciences*, 99(9), pp. 6075–6079.

Goldstein, S.L. & Klassen, J.L., 2020. *Pseudonocardia* symbionts of fungus-growing ants and the evolution of defensive secondary metabolism. *Frontiers in Microbiology*, p. 11.

Grell, M., Linde, T., Nygaard, S., Nielsen, K., Boomsma, J. & Lange, L., 2013. The fungal symbiont of *Acromyrmex* leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and other plant cell wall polysaccharides. *BMC Genomics*, 14(1), p. 928.

Haeder, S., Wirth, R., Herz, H. & Spiteller, D., 2009. Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proceedings of the National Academy of Sciences*, 106(12), pp. 4742-4746.

Heine, D., Holmes, N., Worsley, S., Santos, A., Innocent, T., Scherlach, K., Patrick, E., Yu, D., Murrell, J., Vieria, P., Boomsma, J., Hertweck, C., Hutchings, M. &

Wilkinson, B., 2018. Chemical warfare between leafcutter ant symbionts and a co-evolved pathogen. *Nature Communications*, 9(1).

Holmes, N., Innocent, T., Heine, D., Bassam, M., Worsley, S., Trottmann, F., Patrick, E., Yu, D., Murrell, J., Schiøtt, M., Wilkinson, B., Boomsma, J. & Hutchings, M., 2016. Genome Analysis of Two *Pseudonocardia* Phylotypes Associated with *Acromyrmex* Leafcutter Ants Reveals Their Biosynthetic Potential. *Frontiers in Microbiology*, 7.

Hutchings, M., Truman, A. & Wilkinson, B. 2019. Antibiotics: past, present and future. *Current Opinion in Microbiology*, 51, pp. 72-80.

Imai, Y., Sato, S., Tanaka, Y., Ochi, K. & Hosaka, T. 2015. Lincomycin at Subinhibitory Concentrations Potentiates Secondary Metabolite Production by *Streptomyces* spp.', Applied and Environmental Microbiology. American Society for Microbiology (ASM), 81(11), p. 3869.

Ishak, H.D., Miller, J.L., Sen, R., Dowd, S.E., Meyer, E. & Mueller, U.G., 2011. Microbiomes of ant castes implicate new microbial roles in the fungus-growing Ant *Trachymyrmex septentrionalis. Scientific Reports*, 1(1).

Jaing, H., Chen, Y., Hu, Y., Wang, Z. & Lu, X., 2021. Soil bacterial communities and diversity in alpine grasslands on the Tibetan Plateau based on 16S rRNA gene sequencing. *Frontiers in Ecology and Evolution*, p. 9.

Jiang, X., Ellabaan, M., Charusanti, P., Munck, C., Blin, K., Tong, Y., Weber, T., Sommer, M. & Lee, S., 2017. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. *Nature Communications*, 8(1).

Jones, Stephanie E. & Elliot, Marie A. (2017) 'Streptomyces Exploration: Competition, Volatile Communication and New Bacterial Behaviours', Trends in Microbiology. Elsevier Ltd, pp. 522–531.

Joshi, M.V., Mann, S.G., Antelmann, H., Widdick, D.A., Fyans, J.K., Chandra, G., Hutchings, M.I., Toth, I., Hecker, M., Loria, R. & Palmer, T. (2010) 'The twin arginine protein transport pathway exports multiple virulence proteins in the plant pathogen *Streptomyces* scabies', Molecular Microbiology. John Wiley & Sons, Ltd, 77(1), pp. 252–271.

Kaltenpoth, M., Göttler, W., Herzner, G., & Strohm, E., 2005. Symbiotic bacteria protect wasp larvae from fungal infestation," *Current Biology*, 15(9), pp. 882.

Kelemen, G., Brian, P., Flärdh, K., Chamberlin, L., Chater, K. & Buttner, M., 1998. Developmental Regulation of Transcription of whiE, a Locus Specifying the Polyketide Spore Pigment in *Streptomyces coelicolor* A3(2). *Journal of Bacteriology*, 180(9), pp. 2515-2521.

Kellner, K., Ishak, H., Linksvayer, T. & Mueller, U., 2015. Bacterial community composition and diversity in an ancestral ant fungus symbiosis. *FEMS Microbiology Ecology*, 91(7), fiv073.

Kieser, T., Bibb, M., Buttner, M., Chater, C. & Hopwood, D., 2000. *Practical streptomyces genetics*.

Kooij, P., Pullens, J., Boomsma, J. & Schiøtt, M., 2016. Ant mediated redistribution of a xyloglucanase enzyme in fungus gardens of *Acromyrmex echinatior*. *BMC Microbiology*, 16(1).

Li, H., Sosa-Calvo, J., Horn, H., Pupo, M., Clardy, J., Rabeling, C., Schultz, T. & Currie, C., 2018. Convergent evolution of complex structures for ant–bacterial defensive symbiosis in fungus-farming ants. *Proceedings of the National Academy of Sciences*, 115(42), pp. 10720-10725.

Li, J., Zhao, G., Varma, A., Qin, S., Xiong, Z., Huang, H., Zhu, W., Zhao, L., Xu, L., Zhang, S. & Li, W., 2012. An endophytic *Pseudonocardia* species induces the production of artemisinin in *Artemisia annua*. *PLoS ONE*, 7(12).

Marsh, S., Poulsen, M., Pinto-Tomás, A. & Currie, C., 2014. Interaction between Workers during a Short Time Window Is Required for Bacterial Symbiont Transmission in *Acromyrmex* Leaf-Cutting Ants. *PLoS ONE*, 9(7), p. 103269.

Meirelles, L., Mendes, T., Solomon, S., Bueno, O., Pagnocca, F. & Rodrigues, A., 2013. Broad *Escovopsis*-inhibition activity of *Pseudonocardia* associated with *Trachymyrmex* ants. *Environmental Microbiology Reports*, 6(4), pp. 339-345.

Mueller, U., Ishak, H., Lee, J., Sen, R. & Gutell, R., 2010. Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (*Pseudonocardiaceae, Actinomycetales*): a test of two symbiont-association models. *Antonie van Leeuwenhoek*, 98(2), pp. 195-212.

Mueller, U., Schultz, T. R., Currie, C. R., Adams, R. M., & Malloch, D., 2001. The origin of the attine ant-fungus mutualism. *Q. Rev. Biol.* 76, pp. 169–197.

Parra, J., Soldatou, S., Rooney, L.M., & Duncan, K.R., 2021. *Pseudonocardia abyssalis* sp. nov. and *Pseudonocardia Oceani* sp. nov., two novel actinomycetes isolated from the Deep Southern Ocean. *International Journal of Systematic and Evolutionary Microbiology,* 71(9).

Poulsen, M. & Sapountzis, P., 2012, Behind every great ant, there is a great gut. *Molecular Ecology*, 21(9), pp. 2054–2057.

Procópio, R., Silva, I., Martins, M.K., Azevedo, J. & Araújo, J., 2012. Antibiotics produced by *Streptomyces*. *Braz J Infect Dis*, 16(5), pp. 466-471.

Quinn, G.A., Banat, A.M., Abdelhameed, A.M. & Banat, I.M., 2020. *Streptomyces* from traditional medicine: Sources of new innovations in antibiotic discovery. *Journal of medical microbiology*, 69(8), pp. 1040-1048.

Rahlwes, K.C., Sparks, I.L. & Morita, Y.S., 2019. Cell walls and membranes of actinobacteria. *Subcellular Biochemistry*, pp. 417–469.

Riahi, H.S., Heidarieh, P. & Fatahi-Bafghi, M., 2021. Genus Pseudonocardia: What we know about its biological properties, abilities and current application in biotechnology. *Journal of Applied Microbiology*, 132(2), pp. 890–906.

Ramalho, M.O. &Moreau, C.S., 2023. Untangling the complex interactions between turtle ants and their microbial partners. *Animal Microbiome*, 5(1).

Rubin, B., Sanders, J., Hampton-Marcell, J., Owens, S., Gilbert, J. & Moreau, C., 2014. DNA extraction protocols cause differences in 16S rRNA amplicon sequencing efficiency but not in community profile composition or structure. *Microbiology Open*, 3(6), pp. 910-921.

Sahin, N., Veyisoglu, A., Tatar, D., Spröer, C., Cetin, D., Guven, K. & Klenk, H., 2014. *Pseudonocardia cypriaca* sp. nov., *Pseudonocardia salamisensis* sp. nov., *Pseudonocardia hierapolitana* sp. nov. and *Pseudonocardia kujensis* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology*, 64(Pt 5), pp. 1703–1711.

Schiøtt, M., Rogowska-Wrzesinska, A., Roepstorff, P. & Boomsma, J., 2010. Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. *BMC Biology*, 8(1).

Schoenian, I., Spiteller, M., Ghaste, M., Wirth, R., Herz, H. & Spiteller, D., 2011. Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proceedings of the National Academy of Sciences*, 108(5), pp. 1955-1960.

Seipke, R., Kaltenpoth, M. & Hutchings, M., 2012. *Streptomyces* as symbionts: an emerging and widespread theme?. *FEMS Microbiology Reviews*, 36(4), pp. 862-876.

Sen, R., Ishak, H., Estrada, D., Dowd, S., Hong, E. & Mueller, U., 2009. Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants. *Proceedings of the National Academy of Sciences*, 106(42), pp. 17805-17810.

Shepard., M.D., Kharel M.K., Bosserman, M.A., & Rohr, J., 2010. Maintainance of *Streptomyces* species. *Curr Protoc Microbiol*.

Stuttard, C., 1982. Temperate Phages of *Streptomyces venezuelae*: Lysogeny and Host Specificity Shown by Phages SV1 and SV2, undefined, 128(1), pp. 115–121.

Sujarit, K., Sujada, N., Kudo, T., Ohkuma, M., Pathom-Aree, W. & Lumyong, S., 2017. *Pseudonocardia thailandensis* sp. nov., an actinomycete isolated from a subterranean termite nest. *International Journal of Systematic and Evolutionary Microbiology*, 67(8), pp. 2773–2778.

Thermofisher.com. n.d. *How to Use Phenol / Chloroform for DNA Purification* | *Thermo Fisher Scientific - UK*. [online] Available at: <https://www.thermofisher.com/uk/en/home/references/protocols/nucleic-acid-purification-and-analysis/dna-extraction-protocols/phenol-chloroform-extraction.html#prot1> [Accessed 09 May 2021].

Trujillo, M.E., Idris, H., Riesco, R., Nouioui, I., Igual, J.M., Bull, A.T. & Goodfellow, M., 2017. *Pseudonocardia nigra* sp. nov., isolated from Atacama Desert rock. *International Journal of Systematic and Evolutionary Microbiology*, 67(8), pp. 2980-2985.

Van Arnam, E., Ruzzini, A., Sit, C., Currie, C. & Clardy, J., 2015. A Rebeccamycin Analog Provides Plasmid-Encoded Niche Defense. *Journal of the American Chemical Society*, 137(45), pp. 14272-14274.

Van Arnam, E., Ruzzini, A., Sit, C., Horn, H., Pinto-Tomás, A., Currie, C. & Clardy, J., 2016. Selvamicin, an atypical antifungal polyene from two alternative genomic contexts. *Proceedings of the National Academy of Sciences*, 113(46), pp. 12940-12945.

Viana Marques, D., Machado, S., Ebinuma, V., Duarte, C., Concerti, A. & Porto, A., 2018. Production of β-lactamase inhibitors by streptomyces species. *Antibiotics (Basel)*, 7(3), p. 61.

Wang, R., Zhang, H., Sun, L., Qi, G., Chen, S. & Zhao, X., 2017. Microbial community composition is related to soil biological and chemical properties and bacterial wilt outbreak. *Scientific Reports*, 7(1).

Warwick, S., Bowen, T., McVeigh, H. & Embley, T.M., 1994. A phylogenetic analysis of the family *Pseudonocardiaceae* and the genera *Actinokineospora* and *saccharothrix* with 16S rrna sequences and a proposal to combine the genera *Amycolata* and *Pseudonocardia* in an emended genus *Pseudonocardia. International Journal of Systematic Bacteriology*, 44(2), pp. 293–299.

Woodhams, D.C. & Brucker, R.M., 2013. Disease defence through generations: leaf-cutter ants and their symbiotic bacteria. *Molecular Ecology*, 22(16), pp. 4141–4143.

Worsley, S.F., Innocent, T.M., Holmes, N.A., Al-Bassam, M.M., Schiøtt, M., Wilkinson, B., Murrell, J.C., Boomsma, J.J., Yu, W.D. & Hutchings M.I. (2021), 'Competition-based screening helps to secure the evolutionary stability of a defensive microbiome', *BMC Biology,* 19.

You, L., Simmons, D.R., Bateman, C.C., Short, D.P., Kasson, M.T., Rabaglia, R.J. & Hulcr, J., 2015. New fungus-insect symbiosis: Culturing, molecular, and histological methods determine saprophytic polyporales mutualists of *Ambrosiodmus Ambrosia* beetles. *PLOS ONE*, 10(9).

Zhang, M., Poulsen, M. & Currie, C., 2007. Symbiont recognition of mutualistic bacteria by Acromyrmex leaf-cutting ants. *The ISME Journal*, 1(4), pp. 313-320.

Zhao, G., Li, J., Qin, Y., Miao, C., Wei, D., Zhang, S., Xu, L. & Li, W., 2012. *Pseudonocardia antimicrobica* sp. nov., a novel endophytic actinomycete associated with *Artemisia annua* L. (sweet wormwood)

Zhukova, M., Sapountzis, P., Schiøtt, M. & Boomsma, J., 2017. Diversity and Transmission of Gut Bacteria in Atta and Acromyrmex Leaf-Cutting Ants during Development. *Frontiers in Microbiology*, 8.

Zucchi, T.D., Guidolin, A.S. & Cônsoli, F.L., 2011. Isolation and characterization of actinobacteria ectosymbionts from *Acromyrmex subterraneus brunneus* (hymenoptera, Formicidae). *Microbiological Research*, 166(1), pp. 68–76.