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Isolation of a Rhodococcus Soil Bacterium that Produces a Strong Antibacterial Compound.

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Isolation of a *Rhodococcus* Soil Bacterium that Produces a Strong Antibacterial Compound

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by

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ABSTRACT

Isolation of a *Rhodococcus* Soil Bacterium that Produces a Strong Antibacterial Compound

by

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Rhodococci are notable for their ability to degrade a variety of natural and xenobiotic compounds. Recently, interest in *Rhodococcus* has increased due to the discovery of a large number of genes for secondary metabolism. Only a few secondary metabolites have been characterized from the rhodococci (including 3 recently described antibiotics). Twenty-four new *Rhodococcus* strains were isolated from soils in East Tennessee using acetonitrile enrichment culturing and identified using 16S rRNA analysis. Forty-seven *Rhodococcus* strains were screened for antibiotic production using a growth inhibition assay. One strain, MTM3W5.2, had 90% similarity to the *Rhodococcus opacus* 16S rRNA gene sequence and produced a large zone of inhibition against *R. erythropolis* and a large number of closely related species. The antimicrobial compound produced by MTM3W5.2 had a large MW of 911.5452 Da and acts much like a bacteriocin but no amino acids were detected in this molecule based on TLC analysis.

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INTRODUCTION

The Genus *Rhodococcus*

Taxonomic History

The genus *Rhodococcus,* member of the phylum Actinobacteria, was first described by Zopf in 1891 and was then redefined in 1977 to include more strains that did not exactly fit in the genera *Mycobacterium, Nocardia,* and *Corynebacterium* (Bell *et al.* 1998). Subsequently, scientists have continued to reclassify species of this genus. For example, *R. obuensis* was initially a synonym of the species *R. sputi,* which was later on transferred to the genus *Gordonia* (Bell *et al.* 1998). New species have also been discovered and added to the genus *Rhodococcus,* such as *R. roseus, R. zopfii, R. opacus, and R. percolatus* (Bell *et al.* 1998). Currently, there are over 40 species classified under the genus *Rhodococcus* (Euzéby 2011).

Characteristics

Members of the genus *Rhodococcus* are defined as Gram positive, aerobic, and non-motile. Interestingly, their cell morphology varies during different stages of their growth cycle. Some strains can be found as cocci that later on turn into short rods, while others continue transforming into long filamentous rods or start branching out in an elementary or extensive fashion. Rhodococci are also characterized as chemoorganotrophic, catalase positive and can grow well at 30°C. The appearance of rhodococci on standard laboratory media is quite variable. The colonies can be found to be smooth, rough, or mucoid and have different

pigmentations such as white, tan, pink, yellow, orange, red, and translucent depending on their age and the strain type (Goodfellow 1989).

The cell wall of *Rhodococcus* is made up of a mycolyl-arabinogalactan-peptidoglycan complex (Figure 1). Located immediately next to the plasma membrane is a layer of peptidoglycan with A1*γ* type structure. This means that straight cross-linkages are formed between muro peptides by *meso*-diaminopimelic acid, which serves as the diamino acid (Sutcliffe *et al.* 2010). An unusual modification in the glycan strands has led to the N-glycolylation of muramic acid residues, meaning instead of acetate, glycolyl residues are present at the amino group of N-acetylmuramic acid (Schaechter 2009). Linked to the peptidoglycan layer with phosphodiester bonds is the arabinogalactan, which is a heteropolymer made up of homopolymer galactan and arabinan units. This link is made via a linker unit composed of L-rhamnose-D-N-acetylglucosamine phosphate that anchors the galactan to the peptidoglycan. Attached to each galactan are three arabinan domains that branch up and attach to mycolic acids. In *Rhodococcus,* mycolic acids are 28–54 carbons long and branch up into a 10–16 carbon long saturated 2-alkyl chain and a 20–42 carbon long 3-hydroxyl meromycolate chain with up to 4 carbon-carbon double bonds. The mycolic acids act as a second hydrophobic permeability barrier similarly to the outer membrane of Gram negative organisms although distinct in structure and chemistry. Because mycolic acids arrange in a monolayer and are structurally uneven, there are gaps that are plugged by mycolic acid containing-glycolipids. Also, anchored in the membrane of the rhodococcal cell wall are lipoproteins and polysaccharides called lipoglycans, which belong to the lipoarabinomannan (LAM) family. Finally, the cell wall of rhodococci harbors a variety of lipids, usually glycolipids,

lipopeptides, and glycolipopeptides, some of which have surfactant properties and allow them to grow in hydrophobic environments (Sutcliffe *et al.* 2010).

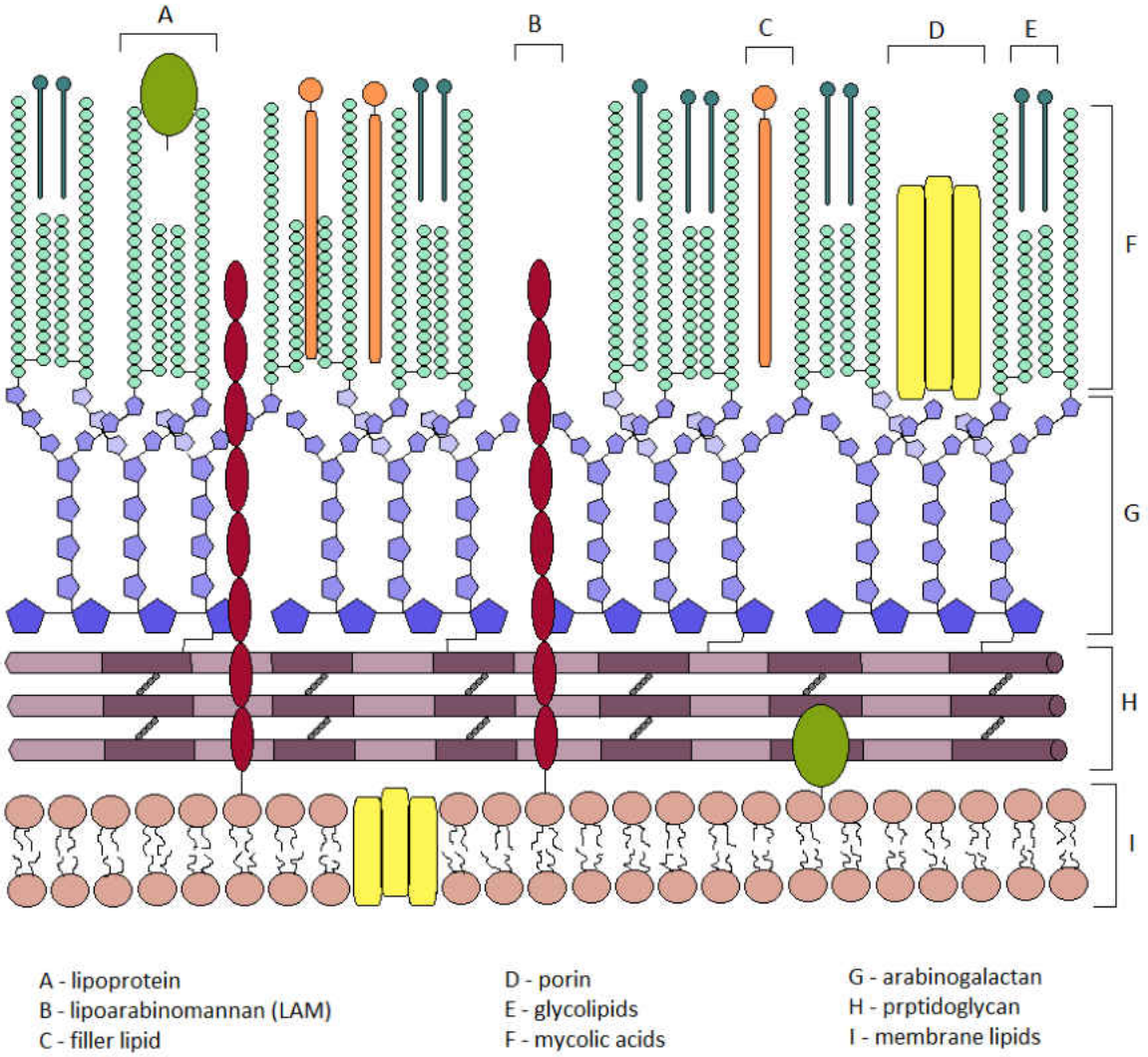


Figure 1: Structural model of the cell envelope of *Rhodococcus* (Sutcliffe *et al.* 2010)

Bacteria from the *Rhodococcus* genus are commonly isolated from soils but they have also been discovered in marine sediments (*R. marinonascens*), fresh water, insects, rocks, and animal excretions (*R. coprophilus*). In addition, some strains have been found to be human and plant pathogens: *R. fascians* causes the tissue in plants to become elongated, crested, or twisted as well as causing leaf lesions; *R. equi* infects domestic animals (horses, swine) and immunocompromised humans; and *R. bronchialis* has been isolated from patients with destruction and dilation of the bronchial tree and from people with pulmonary tuberculosis (Goodfellow 1989).

Industrial Importance

Rhodococci have many enzymes that allow them to carry out a number of chemical reactions that have been useful in the environmental and industrial biotechnology fields. Strains such as *R. erythropolis* are capable of carrying out reactions such as dehydrogenation, hydrolysis, oxidation, desulfurization, hydroxylation, dehalogenation, and epoxidation (de Carvaho 2005). Due to these enzymes, some rhodococci are capable of using gaseous hydrocarbons such as butane, propane, and acetylene as the sole carbon source to grow and are often isolated from soils contaminated with such compounds (Bell *et al.* 1998).

Rhodococci have become a valuable tool in industry due to their ability to produce biosurfactants, molecules that contain both hydrophilic and hydrophobic groups that can be used for the bioremediation of oil pollutants. *Rhodococcus* uses cellular and extracellular surfactants that are more biodegradable and less toxic than synthetic surfactants to ingest hydrophobic compounds and remove them from the environment. This is done with the help of

the mycolic acids present in the cell wall of rhodococci that act as cellular surfactants and allow the bacteria to adhere to oil/water interphases and lower the interfacial tension between the two phases, thereby allowing the hydrophobic compounds to enter the cell. At the same time the hydrophobic compounds are getting dispersed by extracellular surfactants, such as trehalose-containing glycolipids and trehalose tetraesters that increase the surface area available for attachment of the mycolic acids (Bell *et al.* 1998). Another feature of rhodococci is their ability to desulfurize coal and petroleum, which prevents combustion of sulfurous emissions thereby decreasing the occurrence of acid rain (Bell *et al.* 1998).

Nitriles are carboxylic acids with the general structure R–C≡N. They are found both naturally in insects, plants, microorganisms, and oils in the bones and synthetically in herbicides containing benzonitriles and as precursors for the synthesis of polyacrylonitrile plastics (DiGeronimo and Antoine 1976). However, nitriles are quite toxic in nature and there is a great need for their bioremediation. Nitrile transformation reactions using chemicals require extreme conditions, such as high temperatures and acid/alkaline environments. However, microbial enzymes are able to transform nitriles under mild conditions (de Carvalho 2005). Rhodococci contain a nitrile hydratase/amidase system (Figure 2) that works in one or both of two pathways depending on the strain (Heald *et al.* 2001): (1) the direct hydrolysis of nitrile to carboxylic acid and ammonia by nitrilase, or (2) the hydrolysis of nitrile to amide via the enzyme nitrile hydratase and then to carboxylic acid and ammonia via the enzyme amidase (de Carvalho 2005). Members of this genus have been found to have the most active and intriguing enzymes used for nitrile hydrolysis. They are not just used for the degradation of toxic compounds but also for a range of biotransformations, such as the production of amides from nitriles. For

instance, the bacterium *R. rhodochrous* strain J-1 is widely used in the chemical industry to convert acrylonitrile to acrylamide (Brandao *et al.* 2002). The overproduction of nitrilase enzyme has also been used for the production of acrylic acid (Bell *et al.* 1998). The use of bacterial enzymes in the production of these chemicals is favored over synthetic processes because the products are more pure and can be made without the production of unwanted by-products (Brandao *et al.* 2002).

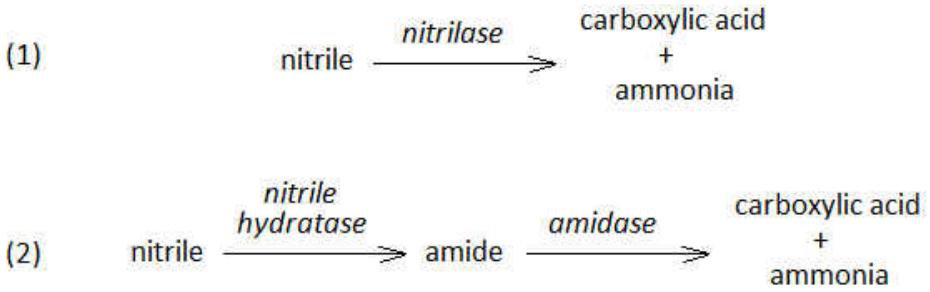


Figure 2: Nitrile hydratase/amidase system in *Rhodococcus*

Genome

Rhodococci were not well characterized in the past and were mainly recognized for their industrial importance. This was until 2006 when McLeod *et al.* sequenced the complete genome of *R. jostii* RHA1 for the first time (McLeod *et al.* 2006). This is a strain of *Rhodococcus* well-known for its ability to transform polychlorinated biphenyls. Based on a 16S rRNA sequence analysis, RHA1 is closely related to *R. opacus*. RHA1 is also capable of using a number of aromatic compounds, nitriles, carbohydrates, and steroids as the only carbon source for growth.

McLeod *et al.* found that the genome of RHA1 was 9,702,737 base pairs long, which makes it one of the largest known bacterial genomes, and it was arranged in 4 linear replicons – 1 chromosome and 3 plasmids (pRHL1, pRHL2, and pRHL3). They determined that RHA1 has a G+C content equal to 67% and 9,145 protein-encoding genes which are rich in ligases and oxygenases. The team also reported that RHA1 has 34 sigma factors.

A very intriguing component of the research was the discovery of a large number of genes for secondary metabolism, including 24 non-ribosomal peptide synthetases and 7 polyketide synthases that may be involved in the synthesis of siderophores, cell signaling molecules, pigments, and antibiotics. This discovery was surprising due to the small number of reported secondary metabolites from rhodococci (McLeod *et al.* 2006). In the past, non-ribosomal peptides from some organisms have been developed into drugs that include vancomycin, daptomycin, cyclosporine A, β-lactams, and teicoplanin (Rokem *et al.* 2007, Gross 2009). Polyketides have been very important in medicine as well and have given rise to drugs like erythromycin and tetracycline (Gross 2009). The discovery of genes for secondary metabolism in rhodococci left some scientists asking the question; are rhodococci capable of producing antibiotics?

Antibiotics

Very few new antibiotics have been introduced into clinical use in the last 30 years. This is partially because recently there has been a decrease in the number of pharmaceutical companies that are willing to pursue research and development of new antibiotics (Baltz 2007, Song 2008). Development of novel antibiotics is an extremely long and hard process and

pharmaceutical companies are finding that there is a larger profit in other disease areas (Payne *et al.* 2007). Nevertheless, there is a growing emergence of antibiotic resistant bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) (Song 2008) that enhances the need for new antibiotics.

The Importance of Actinomycetes

Most antibiotics in the past have been isolated from actinomycetes with the largest contribution from the genus *Streptomyces* but also with input of the less extensively studied genera *Actinomadura, Arthrobacter,* and *Nocardia* (Kitagawa and Tamura 2008a). According to Floss (2007), the screening of natural products yields a higher percentage of suitable drugs than chemical libraries and even if they themselves are not suitable as a drug their structure can be the starting point for the development of semi-synthetic compounds (Clardy *et al.* 2006). This can be seen in the instance of the actinomycete derived drug erythromycin, which was then remodeled into clarithromycin and subsequently into telithromycin (Clardy *et al.* 2006). There is a rich amount of antibiotic producing actinomycetes in soils because the top 10 cm of global soil contains $10^{25} – 10^{26}$ actinomycetes. Only about 10^{7} of them, however, have been screened for antibiotic production, so there are still a lot of actinomycetes waiting to be screened (Baltz 2007).

Secondary Metabolites Derived from *Rhodococcus*

Antibiotics

Rhodococci are actinomycetes that for many years have been used in industry as degraders of toxic hydrophobic pollutants and as producers of acrylamide and acrylic acid. Researchers have been trying more intensively to explore the potential of these bacteria to produce secondary metabolites. Only a few secondary metabolites produced by rhodococci have been characterized thus far, including a few antibiotics.

The first report of an antimicrobial produced by *Rhodococcus* was in 1999, when Chiba *et al.* discovered 5 novel cyclic tetrapeptides exhibiting anti-fungal activity against *Candida albicans* and *Cryptococcus neoformis* but displayed no anti-bacterial activity (Chiba *et al.* 1999). They named these compounds rhodopeptin C1, C2, C3, C4, and B5. The organism producing the rhodopeptins was *Rhodococcus* sp. Mer-N1033, and it was isolated from a soil sample collected at Mt. Hayachine, Iwate Prefecture, Japan. Rhodopeptins were isolated as either colorless solids or white powders and were soluble in acetic acid, dimethylsulfoxide, methanol, and slightly soluble in water but insoluble in chloroform and ethyl acetate. They were composed of 3 *α*-amino acids and 1 lipophilic β-amino acid (Chiba *et al.* 1999). The structure of the 5 rhodopeptins is shown in Figure 3.

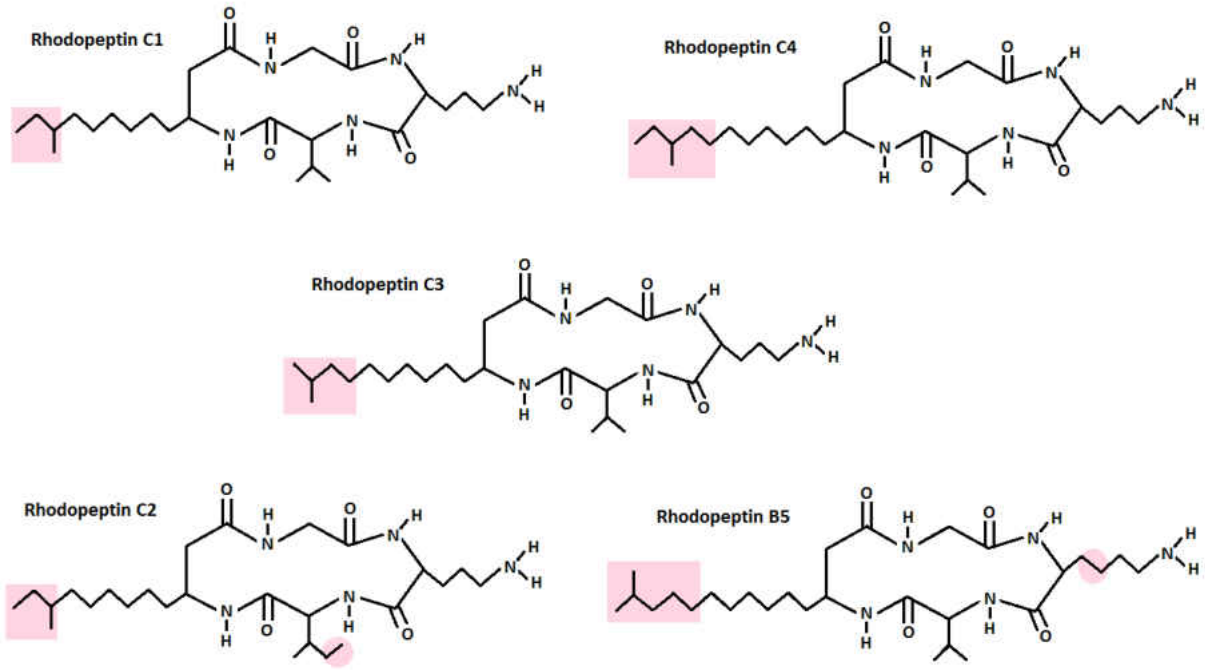


Figure 3: The structure of rhodopeptin C1, C2, C3, C4, and B5 (Chiba *et al.* 1999). The structure differences between the 5 rhodopeptins are highlighted in pink.

In 2006, Iwatsuki *et al.* isolated a strain of *Rhodococcus*, *R. jostii* K01-B0171 from a soil sample in Yunnan, China and discovered that this strain is capable of producing 2 antibacterial compounds that they named lariatin A and B (Iwatsuki *et al.* 2006). Both lariatins had anti-mycobacterial properties against *Mycobacterium smegmatis*; however, lariatin A also inhibited the growth of *Mycobacterium tuberculosis.* The team determined that the lariatins are cyclic peptides with a lasso structure. They contained 18 and 20 amino acids with an internal bond between the *α*-amino group of Gly1 and the *γ*-carboxyl group of Glu8 (Figure 4). Lariatins A and B were isolated as pale yellow powders; they had a MW of 2050 and 2204 respectively and

were soluble in water, methanol, and DMSO while insoluble in chloroform and ethyl acetate (Iwatsuki *et al.* 2007).

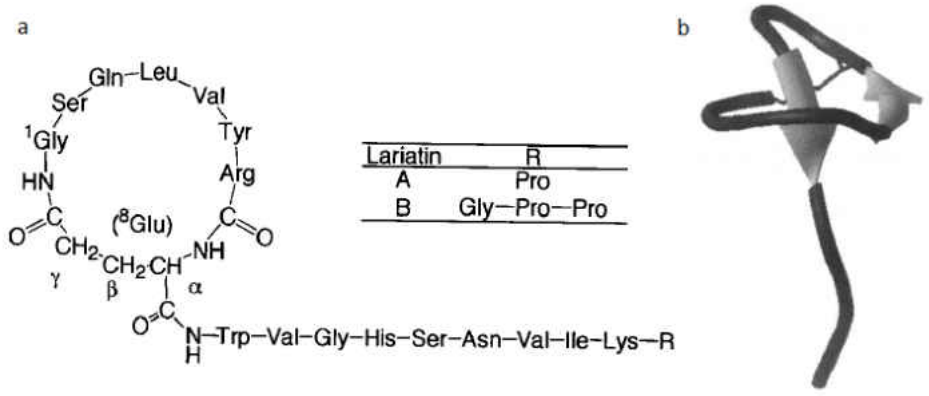


Figure 4: Illustration of Lariatins. (a) The structure of lariatins A and B. (b) The lasso structure of lariatin A (Iwatsuki *et al.* 2007).

In April 2008, another Japanese team, Kitagawa and Tamura (2008a), focused their research on a massive screening for antimicrobial compound producers. They screened about 80 *Rhodococcus* strains acquired from Japanese and German culture collections. For their first round of screening they used *Escherichia coli* and some species of *Pseudomonas, Sinorhizobium, Streptomyces, Corynebacterium, Arthrobacter,* and *Rhodococcus* as the indicator organisms. After their initial screening they discovered 14 *R. erythropolis* strains and 1 *R. globerulus* strain that inhibited at least 1 of the indicator strains. These were then screened against another set of 52 bacterial strains to determine the spectrum of activity of the antibiotics produced. Many of the Gram positive test organisms were inhibited by the antibiotics, while most of the Gram negative test organisms were resilient to them. The antibiotic producers were classified in 3 groups based on the type of antibiotic they produced. Group 1 contained 5 *Rhodococcus* strains that produced antibiotics with activity against a broad

spectrum of Gram positive bacteria; Group 2 included three rhodococci producing antibiotics active against mainly other rhodococci and some other Gram positives; and Group 3, 7 strains exhibiting antibiotic activity against only other *R. erythropolis* strains (Kitagawa and Tamura 2008a).

Kitagawa and Tamura (2008b) continued working on the structure and characteristics of 1 of the Group 1 antibiotics produced by 1 of the 15 antibiotic producing *Rhodococcus* strains, *R. erythropolis* JCM6824, and published their results in October 2008. The antibiotic produced was a new type of quinolone, aurachin RE, that had a very similar structure to aurachin C, an antibiotic derived from *Stigmatella aurantiaca* Sga15 (Figure 5). Both antibiotics were found to inhibit the growth of a wide range of Gram positive organisms, though aurachin RE had a much stronger antimicrobial activity. Aurachin RE was isolated as a gray-brown powder, had a MW equal to 395, and it was found to be soluble in ethanol, methanol, ethyl acetate, acetonitrile, and DMSO (Kitagawa and Tamura 2008b).

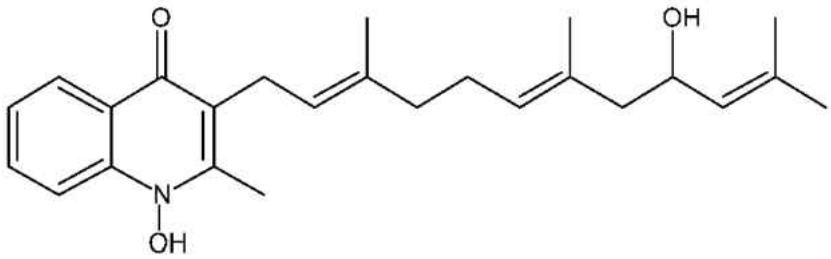


Figure 5: The structure of aurachin RE. Aurachin C has the same general structure except an H instead of 9`-OH (Kitagawa and Tamura 2008b).

In 2008, Kurosawa *et al.* tested the ability of 2 organisms, *Streptomyces padanus* and *R. facians,* to undergo horizontal gene transfer. *S. padanus* is an actinomycete that is known to

produce the antibiotic actinomycin, whereas *R. fascians* does not produce any antibiotics. After co-culturing the 2 actinomycetes, they recovered a new strain of *R. fascians* that they named 307CO and that, they discovered, contained a piece of DNA belonging to *Streptomyces.* Furthermore, the transferred genes allowed the new *Rhodococcus* strain to independently begin producing 2 new antimicrobial compounds. The team named these antibiotics rhodostreptomycin A and B and after characterizing the compounds, they discovered that these antibiotics are 2 isomers of a new class of aminoglycosides. The team reported that the structure of the rhodostreptomycins was significantly different than the one for actinomycins produced by *Streptomyces*. Rhodostreptomycins were able to inhibit the growth of prokaryotes such as *Streptomyces padanus, Escherichia coli, Staphylococcus aureus, Bacillus subtillis,* and *Helicobacter pylori* but not against eukaryotes such as *Saccharomyces cerevisiae* (Kurosawa *et al.* 2008).

Siderophores

The past decade has been productive not only for the discovery of antibiotics produced by rhodococci but also their ability to produce other secondary metabolites such as siderophores. Siderophores are compounds with low molecular weight produced and released by some bacteria to scavenge iron from the environment. When iron is at a low concentration in the environment, the cell releases a siderophore that chelates iron and is then taken back up by the cell using sophisticated transport systems. In the past, 3 classes of siderophores have been described based on their chemical components: catecholates, hydroxamates, and (hydroxyl)-carboxylates (Bosello *et al.* 2011).

In 2001, Carrano *et al.* implemented a search for siderophore producers from the genus *Rhodococcus* and confirmed that rhodococci are capable of producing them. In their study, they discovered that *R. erythropolis* strain IGTS8 was able to produce a new class of siderophores, which they named heterobactins. Using Mass Spectrometry and NMR, the team determined that IGTS8 produces 3 heterobactins, but they only studied the 2 that were more abundant and named them heterobactin A and heterobactin B. Heterobactin A had a mass of 599.2 and heterobactin B was smaller with a mass of 438.2. Further structural analysis showed that heterobactins are "mixed" siderophores, containing both hydroxamate and catecholate donor groups in the same siderophore allowing one part of the molecule to be recognized by a hydroxamate transport system while the other is recognized by a catecholate receptor.

In 2007, Dhungana *et al.* discovered another strain of *Rhodococcus* that produces siderophores. This time the producer was *R. rhodochrous* strain OFS and the siderophore was given the name rhodobactin. With a molecular weight of 830 Daltons, rhodobactin was a mixed ligand hexadentate siderophore containing 1 hydroxamate and 2 nonequivalent catecholate moieties for iron chelation.

In 2008, Miranda-CasoLuengo *et al.* studied the mechanism used by the pathogenic *R. equi* to acquire its iron for growth. The team discovered that during growth at low iron concentrations, *R. equi* produces a chromophore that the team hypothesized to be an iron-siderophore complex. They tested and confirmed their hypothesis, but they did not further analyze the structure and characteristics of the siderophore.

The last *Rhodococcus* derived siderophore discovery was made very recently in 2011 by Bosello *et al*. The team studied the ability of *R. jostii* RHA1 to produce siderophores because

this strain has been shown to possess extensive genetic information for a yet uncharacterized secondary metabolism. As a result of their work, Bosello *et al.* did discover a mixed-type hydroxamate-catecholate siderophore produced by *R. jostii* RHA1 and named it rhodochelin. Structural analysis revealed that rhodochelin is a branched tetrapeptide composed of a 2,3-DHB, threonine, and 2 moieties of *δ*-*N*-formyl-*δ*-*N*-hydroxyornithine and has the molecular mass of 572.2. The researchers also determined the complete set of gene clusters necessary for the biosynthesis of rhodochelin.

Pigments

Biological pigments are substances produced by different types of organisms with the purpose to absorb certain wavelengths of light while reflecting others. In photosynthetic organisms, pigments such as carotenoids are used to absorb light for photosynthesis and protect the chlorophyll from photodamage. Carotenoids are hydrocarbons that are usually 40 carbons long and contain 3 to 15 double bonds, the number of which determines the absorbency spectrum, which ranges between 400 and 500nm. Besides algae, plants, and cyanobacteria, carotenoids are also produced by some non-photosynthetic bacteria and fungi and are also found as natural colorants in insects, birds, fish, and crustaceans (Armstrong and Hearst 1996). One such non-photosynthetic bacterium producing carotenoids is *Rhodococcus.* In 1989, Ichiyama *et al.* conducted a study for the discovery of pigments produced by *Rhodococcus* that had not been studied yet. They tested 16 different species of *Rhodococcus* and discovered 11 different carotenoid-type pigments produced by them, which showed yellow, orange, salmon-pink, and red colorations on Thin Layer Chromatography (TLC) plates.

Four of the species produced β-carotene and 11 species produced a *γ*-carotene-like substance. Some species also produced derivatives of β- and *γ*-carotene, such as myxoxanthophyll-like, zeaxanthin-like, and β-citraurin-like carotenoids as well as some other carotenoids with unknown nature.

Bacteriocins

Bacteriocins are compounds similar to antibiotics produced by some organisms to inhibit the growth of other organisms in their environment in competition for nutrients. What distinguishes them from antibiotics is that they are usually peptides synthesized by ribosomes and have a narrow spectrum of activity. Bacteriocins are produced by all major lineages of bacteria and theoretically it has been determined that 99% of bacteria produce at least one type of bacteriocin. These antimicrobial compounds have been divided into 2 groups – derivatives of Gram-positive and Gram-negative bacteria, and differ in peptide size, mode of action, organisms they target, and release mechanism. Gram-positive bacteriocins are similar to eukaryotic derived antimicrobial peptides. They are usually cationic peptides that are 2 to 6 kDa in size and affect the membrane of the targeted organism by making it more permeable. Gram-positive bacteriocins are in most cases active against Gram-positive bacteria, but their range of activity could vary from very narrow to quite broad. For example, lactococcins A, B, and M are limited to killing only *Lactococcus,* while type A lantibiotics (nisin A, mutacin B-Ny266) are bactericidal against *Actinomyces, Mycobacterium, Corynebacterium, Propionibacterium, Micrococcus, Lactococcus, Enterococcus, Streptococcus, Staphylococcus, Clostridium, Bacillus,*

Listeria, and *Gardnerella* (Gillor *et al.* 2008). Currently, not much is known about bacteriocins produced by *Rhodococcus.*

Current Work

In this study, 83 bacterial strains were isolated from local soils in East Tennessee using a heat shock method and enrichment with acetonitrile. Forty-seven strains were identified as *Rhodococcus* and were further screened for the production of antimicrobial compounds by testing for the growth inhibition of 3 indicator organisms – *R. erythropolis* IGTS8, *Micrococcus luteus*, and *Escherichia coli.* One of the isolates, *Rhodococcus* sp. MTM3W5.2, had a 90% similarity to *R. opacus* and produced a large zone of inhibition against a large number of closely related species. The compound was purified using LH-20 column chromatography and high performance liquid chromatography (HPLC) and then analyzed using Mass Spectrometry and NMR. The characteristics of the compound are described.

CHAPTER 2

MATERIALS AND METHODS

Soil Samples

Soil samples were collected from 8 local areas in East Tennessee including Morristown, Newport, Cosby, Elizabethton, Watauga Forest, and Watauga Lake. Two locations in Morristown were used: soil found in between the driveway of a house and a garden of tomato plants, and soil adjacent to an abandoned car battery, which had been laying upside-down in the grass for years. In Newport, the soil was collected from underneath a lawnmower and in Cosby the collected soil was from the driveway of a house. Two locations in Elizabethton were used: soil next to an abandoned gas station and soil next to a portable toilet in a rental business. Also, near Elizabethton, soils were collected from Watauga Forest and from the shoreline sediment of Watauga Lake. All soil samples were collected and placed in clean Ziploc bags and stored at 4°C until ready to be tested.

Media Used

Rich Medium (RM)

This medium was prepared by first mixing together the following constituents:

1) dH_2O 500ml
2) Glucose 5g
3) Nutrient Broth 4g
4) Yeast Extract 0.25g
5) Agar 7.5g

After mixing all ingredients, the media was autoclaved for 20 minutes at 121°C. The agar was left out when preparing RM broth. When preparing RM agar plates, the media was cooled to 55°C in a water bath and then poured in sterile Petri dishes that were then left to cool to room temperature in order to solidify and were then stored at 4°C for later use.

M3 Medium (M3)

This medium was described by Rowbotham and Cross in 1977. Preparing this growth medium posed some challenges with precipitation. Thus, it was made in 5 separate parts prepared and autoclaved individually and mixed together after cooling to avoid ingredients from precipitating out. These 5 solutions are as follows for a total of 500ml of medium:

Solution A	
dH_2O	100 ml
KH_2PO_4	0.233 g
Na_2HPO_4	0.336 g

Solution B	
dH_2O	100 ml
NaCl	0.145 g
KNO_3	0.05 g

Solution C	
dH_2O	100 ml
$CaCO_3$	0.01 g

Solution D	
dH_2O	100 ml
Na propionate	0.10 g

Solution E	
dH_2O	100 ml
Agar	9 g

The pH of each one of the solutions was adjusted to 7.0 before autoclaving them. After autoclaving the solutions, they were left to cool in a 55°C water bath and were then mixed together. The following trace elements were made into stock solutions due to their low concentrations and were added to the cooled M3 medium:

Stock Solution	Concentration	Amount per 500ml of M3
1) $FeSO_4$	1mg/100ml dH_2O	10 µl
2) ZnCl	2.6g/100ml dH_2O	3.5 µl
3) $MgSO_4$	30g/100ml dH_2O	165 µl
4) $MnSO_4$	10mg/100ml dH_2O	100 µl
5) Thiamine HCl	0.4g/100ml dH_2O	0.6 ml

The final ingredient in the M3 medium is the antibiotic cycloheximide that was added to prevent the growth of fungal organisms on the medium. Cycloheximide was added in solid form directly to the already cooled medium at a concentration of 50mg per 500ml of M3 media. All ingredients were mixed well and then M3 agar plates were prepared as previously described.

Mueller-Hinton Medium (MH)

MH broth was made by adding 11g of BBL™ Mueller-Hinton Broth to 500ml dH_2O and then heating up the medium to a boil, with stirring, before autoclaving.

MH agar plates were made by either adding 11g of BBL™ Mueller-Hinton Broth and 8.5g of Bacto agar into 500ml dH_2O or by mixing 19.5g of Difco™ Mueller-Hinton Agar into 500ml dH_2O. In both cases the mixture was boiled to mix ingredients before autoclaving.

Defined Basal Medium (DE)

This medium was described by Langdahl *et al.* in 1996. This was a highly challenging medium to make due to its many components and precipitation problems. It consisted of 8 main ingredients, a trace element solution, a vitamin mixture, a vitamin B_{12} solution, and thiamine HCl solution. The following DE components are listed with the amounts used to make 500ml of DE medium. Ingredients are also summarized in Table 1.

Main ingredients.

Ingredients	Amt. needed	Working stock sln.	Concentrated sln.	Amt. mixed
1) dH_2O	500 ml			94ml
2) NaCl	10 g	10g/100ml dH_2O		100ml
3) $MgCl_2 \cdot 6H_2O$	1.5 g		30g/100ml dH_2O	5ml
4) KCl	0.25 g	0.25g/100ml dH_2O		100ml
5) $MgSO_4 \cdot 7H_2O$	0.15 g		30g/100ml dH_2O	0.5ml
6) $CaCl_2 \cdot 2H_2O$	0.075 g		15g/100ml dH_2O	0.5ml
7) K_2HPO_4	0.5 g	0.5g/100ml dH_2O		100ml
8) KH_2PO_4	0.375 g	0.375g/100ml dH_2O		100ml
Total				500ml

Due to problems with precipitation, all ingredients were made separately. One-hundred milliliter solutions were made for NaCl, KCl, K_2HPO_4, and KH_2PO_4 and after autoclaving were combined with 100ml sterile dH_2O. The pH of each solution was adjusted to 7.2 before autoclaving. One-hundred times concentrated stock solutions were made for $MgCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ and were added individually to the main ingredients after autoclaving and after letting all ingredients cool to at least 55°C. Even when all ingredients were completely cold, some precipitation usually occurred after adding the $CaCl_2 \cdot 2H_2O$.

Trace element solution (TES-3).

	Ingredients	Amount needed	Concentrated sln.	Amount mixed
1)	dH_2O	100 ml		97ml
2)	$FeCl_2 \cdot 4H_2O$	200 mg	100mg/10ml EtOH	------
3)	$CoCl_2 \cdot 6H_2O$	25 mg		25mg
4)	$MnCl_2 \cdot 4H_2O$	10 mg		10mg
5)	$ZnCl_2$	7 mg		7mg
6)	H_3BO_3	0.6 mg	60mg/100ml dH_2O	1ml
7)	$Na_2MoO_4 \cdot 2H_2O$	4 mg		4mg
8)	$NiCl_2 \cdot 6H_2O$	7 mg		7mg
9)	$CuCl_2 \cdot 2H_2O$	0.2 mg	20mg/100ml dH_2O	1ml
10)	$AlCl_3 \cdot 6H_2O$	6 mg		6mg
11)	$NaWO_4 \cdot 2H_2O$	0.6 mg	60mg/100ml dH_2O	1ml
	Total			100ml

In 100ml dH_2O, the following ingredients of TES-3 were mixed together and after adjusting the pH to 7.2, they were autoclaved: $CoCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnCl_2$, $Na_2MoO_4 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, and $AlCl_3 \cdot 6H_2O$. Concentrated 10x solutions were made for the H_3BO_3, $CuCl_2 \cdot 2H_2O$, and $NaWO_4 \cdot 2H_2O$ due to their low concentrations in the TES-3 solution and 1ml of each was added to the rest of the TES-3 solution. The $FeCl_2 \cdot 4H_2O$ was unable to dissolve in water, so a separate concentrated solution was made by dissolving 100mg of it in 10ml 100% ethanol. A 0.5ml aliquot of the TES-3 solution and 100µl of the $FeCl_2 \cdot 4H_2O$ solution (final concentration of 1mg/500ml DE) were added to the DE main ingredients.

Vitamin mixture (VM).

	Ingredients	Amount needed	10x solutions	Amount mixed
1)	dH_2O	100 ml		80ml
2)	Pyridoxamine dihydrochloride	10 mg		10mg
3)	Calcium D(+)-pantothenate	5 mg		5mg
4)	Nicotinic acid	10 mg		10mg
5)	DL-α-lipoic acid	1 mg	10mg/100ml dH_2O	10ml
6)	Folic acid	3 mg		-------
7)	D-(+)-biotin	1 mg	10mg/100ml dH_2O	10ml
8)	4-aminobenzoic acid	4 mg		4mg
	_____			_____
	Total			100ml

In 100ml dH_2O, the following ingredients of VM were mixed together and then autoclaved: pyridoxamine dihydrochloride, calcium D(+)-pentothenate, nicotinic acid, and 4-

aminobenzoic acid. Concentrated 10x solutions were made for DL-α-lipoic acid and D-(+)-biotin due to their low concentrations and 1ml of each was added to the rest of the VM solution. Thirty milligrams of folic acid was added to 1L dH_2O and was only able to dissolve at this concentration and after autoclaving. A 0.5ml aliquot of the VM solution and 0.5ml of the folic acid solution (final concentration 0.015mg/500ml DE) were added to the DE main ingredients.

Vitamin B_{12} solution. This solution was made by adding 5mg cyanocobalamin to 100ml dH_2O. After autoclaving, 0.5ml of this solution was added to the main DE ingredients.

Thiamine HCl solution.

	Ingredients	Amount needed	100x solution	Amount added
1)	dH_2O	100 ml		99ml
2)	NaH_2PO_4	160 mg		160mg
3)	Na_2HPO_4	0.1 mg	10mg/100ml dH_2O	1ml
4)	Thiamine HCl	10 mg		10mg
	Total			100ml

A 10mM sodium phosphate buffer was made by mixing together 100ml dH_2O, 160mg NaH_2PO_4, and 1ml 100x concentrated Na_2HPO_4 solution. Then, 10mg thiamine HCl was dissolved in the buffer and the pH adjusted to 3.4 with HCl. After autoclaving, 0.5ml of this solution was added to the main DE ingredients.

Table 1: Summary of ingredients and solutions for DE medium

Ingredients		Amt. needed	Working stock solution	100x concentrated solution	10x concentrated solution	Amt. mixed	Amt. per 500ml
Main Ingredients (500ml)	NaCl	10g	10g/100ml	---------	---------	100ml	100ml
	$MgCl_2•6H_2O$	1.5g	---------	---------	30g/100ml	5ml	5ml
	KCl	0.25g	0.25g/100ml	---------	---------	100ml	100ml
	$MgSO_4•7H_2O$	0.15g	---------	30g/100ml	---------	0.5ml	0.5ml
	$CaCl_2•2H_2O$	0.075g	---------	15g/100ml	---------	0.5ml	0.5ml
	K_2HPO_4	0.5g	0.5g/100ml	---------	---------	100ml	100ml
	KH_2PO_4	0.375g	0.375g/100ml	---------	---------	100ml	100ml
Trace Element Solution (TES-3) (100ml)[a]	$FeCl_2•4H_2O$	200mg	----------	---------	100mg/10ml EtOH	-------[a]	100µl
	$CoCl_2•6H_2O$	25mg	---------	---------	----------	25mg	0.5ml
	$MnCl_2•4H_2O$	10mg	---------	----------	----------	10mg	
	$ZnCl_2$	7mg	---------	----------	----------	7mg	
	H_3BO_3	0.6mg	---------	60mg/100ml	----------	1ml	
	$Na_2MoO_4•2H_2O$	4mg	---------	----------	----------	4mg	
	$NiCl_2•6H_2O$	7mg	---------	----------	----------	7mg	
	$CuCl_2•2H_2O$	0.2mg	---------	20mg/100ml	----------	1ml	
	$AlCl_3•6H_2O$	6mg	---------	----------	----------	6mg	
	$NaWO_4•2H_2O$	0.6mg	---------	60mg/100ml	----------	1ml	
Vitamin Mixture (VM) (100ml)[a]	Pyridoxamine dihydrochloride	10mg	---------	----------	----------	10mg	0.5ml
	Calcium D (+) – pentothenate	5mg	---------	----------	----------	5mg	
	Nicotinic acid	10mg	---------	----------	----------	10mg	
	DL-α-lipoic acid	1mg	---------	----------	10mg/100ml	10ml	
	D-(+)-biotin	1mg	---------	----------	10mg/100ml	10ml	
	4-aminobenzoic acid	4mg	---------	----------	----------	4mg	
	Folic acid	3mg	3mg/100ml	----------	----------	--------[a]	0.5ml
B_{12} Solution (100ml)[a]	cyanocobalamin	5mg	5mg/100ml	----------	----------	5mg	0.5ml
Thiamine HCl solution (100ml)[a]	NaH_2PO_4	160mg	----------	----------	----------	160mg	0.5ml
	$Na_2HPO_4•7H_2O$	0.1mg	----------	10mg/100ml	----------	1ml	
	Thiamine HCl	10mg	----------	----------	----------	10mg	
C and N source[b]	Acetonitrile	2.5ml	----------	----------	----------	2.5ml	2.5ml
H_2O	----------	---------	----------	----------	----------	--------	90ml

a: Added separately to the main ingredients of 500ml DE medium. b: 2.5ml added to 500ml of DE medium.

When making DE agar plates, 7.5mg of agar was added to the water of the main DE ingredients before autoclaving. After mixing all parts of the DE media together, 50mg of cycloheximide was added and mixed well with the media after it had cooled. Finally, sterile Petri dishes were poured as previously described.

Enrichment Culturing

Overview

A major problem in discovering new antibiotics is the fact that the most plentiful antibiotic producers have already been discovered, thus rare or small populations of bacteria could yield new antibiotics. One method of recovering presumably rare bacteria from soil is through enrichment culturing (Baltz 2007). In other words, cultures of soil samples containing a large diversity of organisms are grown in the presence of a carbon source that only certain organisms can use for growth, thus eliminating all organisms incapable of using it and yielding cultures with few types of organisms.

Acetonitrile Enrichment

Nitriles are cyanide-containing solvents that are widely used as precursors in the synthesis of acrylic fibers and plastics. In the past, different nitriles have been used by scientists to isolate nitrile-hydrolyzing bacteria via enrichment culturing (Layh *et al.* 1997, Heald *et al.* 2001, Brandao *et al.* 2002). Acetonitrile is a simple form of nitrile with the chemical formula CH_3CN that researchers in the past have used to successfully isolate *Rhodococcus* species from soil and marine sediments samples (Langdahl *et al.* 1996, Heald *et al.* 2001). This is because rhodococci are capable of using acetonitrile as the sole source of carbon and nitrogen for

growth. In order to isolate rhodococci from soil using acetonitrile enrichment, a minimal medium needs to be developed that is free of any carbon and nitrogen sources other than acetonitrile. The soil sample is then added to this medium and after incubation the culture will ideally contain only those organisms that were able to use the acetonitrile, eliminating all that cannot use this for their growth.

In this work, defined basal medium (DE) was used as the enrichment medium and acetonitrile was used as the sole carbon and nitrogen source to supplement the DE medium as described by Langdahl *et al.* (1996). In a 50ml Erlenmeyer flask, enrichment culture was made by adding 1 gram of soil in 10ml DE medium containing 0.5% acetonitrile (Figure 6). The sample was then placed in a 30°C shaking water bath to incubate for 1 week. After 1 week, the sample was removed from the shaker, and 1ml of the grown up culture was transferred to a 50ml Erlenmeyer flask containing 9ml of fresh DE medium plus acetonitrile. After incubating the culture for another week, 100µl of the culture was transferred to 10ml fresh DE medium plus acetonitrile. This transfer was performed 2 more times over 2 additional weeks. The reasoning behind the large number of subcultures is to starve the organisms growing from any carbon or nitrogen that they might have stored and force them to use the acetonitrile instead as the energy source for growth. Thus, any organisms unable to use acetonitrile will simply not grow, while organisms that contain the set of enzymes necessary to hydrolyze acetonitrile will continue growing. After the 4^{th} subculture had grown for a week, 100µl of it was spread on the surface of a DE agar plate using a glass spreader and was then incubated at 30°C until colonies were observed to appear. Colonies from this plate that were different in appearance from one

another were then picked and streaked for isolated colonies on their individual DE agar plates and were incubated at 30°C until growth was apparent.

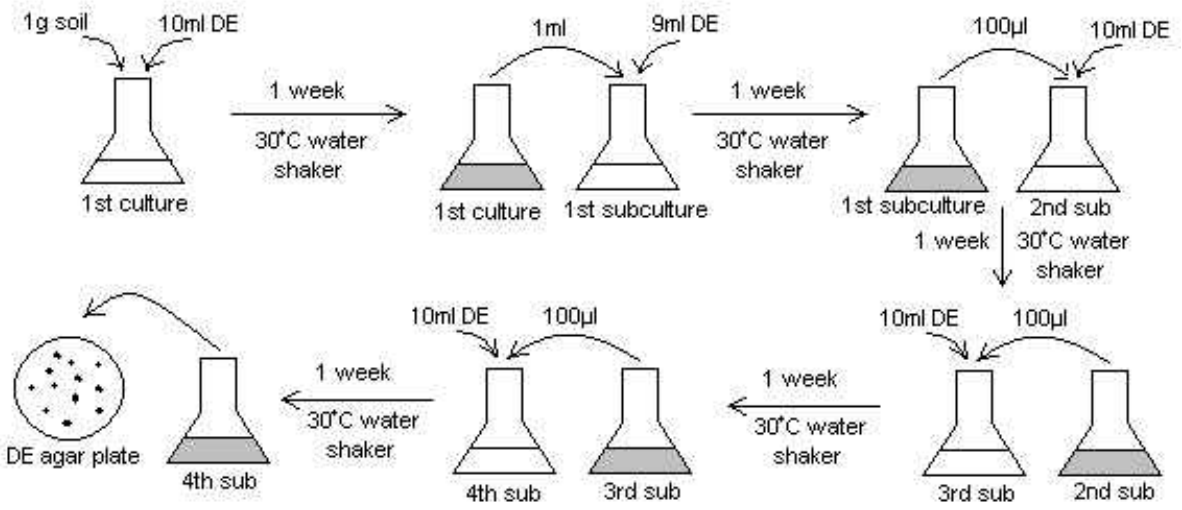


Figure 6: Acetonitrile enrichment using liquid defined basal media (DE)

Acetonitrile enrichment was also performed by using only solid DE media (0.5% acetonitrile was added to the medium right before pouring into petri dishes). In this case, 1 gram of soil was dispersed in 10ml dH_2O and 1ml of this mixture was then added to 9ml dH_2O to make a 1:10 dilution (Figure 7). Then, 100µl of the sample was spread on the surface of a DE agar plate and the plate was incubated at 30°C for about a week. Colonies from this plate that were different in appearance from one another were then picked and streaked for isolated colonies on their individual DE agar plates and were incubated at 30°C until growth was apparent. Isolated colonies from each of these plates were then transferred to fresh DE plates in an attempt to starve them from stored carbon or nitrogen supplies in the same manner as the liquid acetonitrile enrichment. After 2 more transfers to fresh DE plates, the colonies were

transferred to rich medium (RM) plates in order to observe their color. *Rhodococcus* is known for having a colorful appearance on nutrient media that varies from strain to strain. Colonies have been observed in various shades of orange, yellow, pink, tan, and even white and colorless; however, these colors are often not visible on minimal media such as DE.

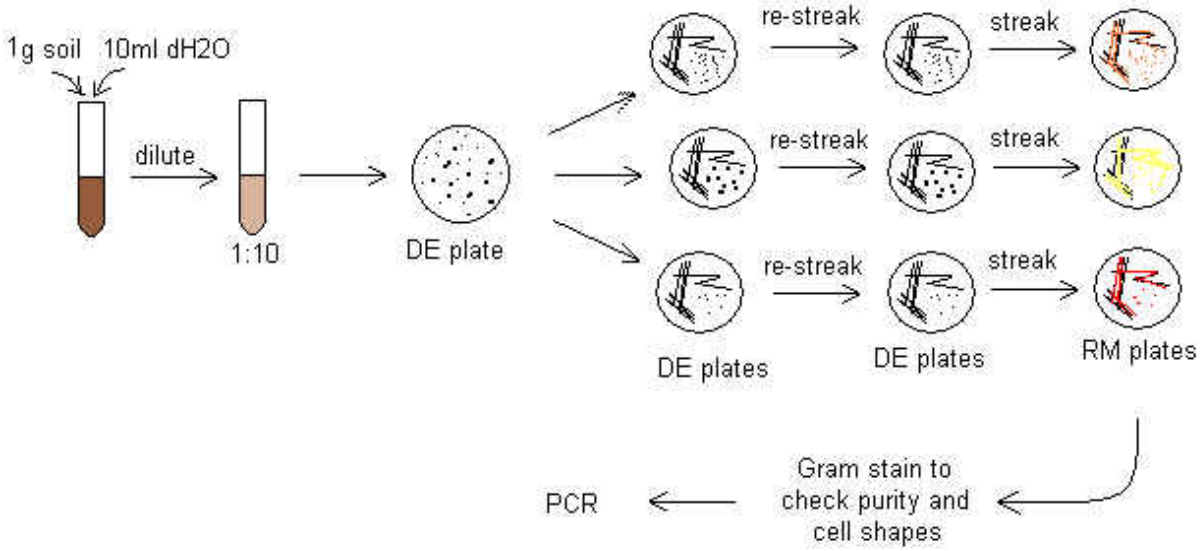


Figure 7: Acetonitrile enrichment using solid defined basal media (DE)

M3 Enrichment

In addition to enrichment with acetonitrile, a more conventional "heat-shock" method of enrichment culturing was performed (Figure 8). In this method, 1 gram of the soil sample was placed in 10ml dH_2O and 1ml of this solution was then placed in 9ml dH_2O to make a 1:10 dilution. This sample was then heat-shocked by being placed in a 55°C water bath for 6 minutes. This eliminates the growth of all organisms unable to withstand the heat-shock and selects for the growth of bacteria like *Rhodococcus* which can withstand it. The sample was

then cooled to room temperature, vortexed, and 100µl of it was spread with a glass spreader on the surface of an M3 agar plate. The plate was then incubated on the bench top at room temperature until colonies were seen to appear (about 1 week). After that, colonies that were different in appearance from one another were picked and streaked for isolated colonies on their individual RM plates in order to enhance the color of the colonies. On M3 agar, some *Rhodococcus* strains are able to produce colored colonies, while others strains do not express pigments.

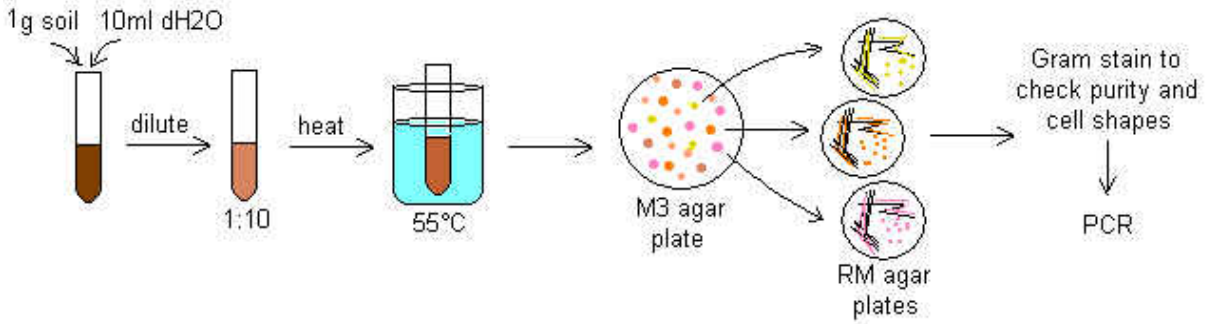


Figure 8: M3 enrichment method

Regardless of the enrichment method used, once colonies were plated on RM media they were all processed in the same manner. Isolated colonies from each plate were Gram stained to check the purity of the colony, the Gram reaction, and the cell morphology. Each Gram stain was checked for purity in 7 – 10 optical fields, e.g. Gram stains which contained consistently the same cell type in all optical fields were considered to be pure. As *Rhodococcus* can be found to have both coccus and bacillus morphology, cells that appeared to be Gram positive cocci or rods were selected for the next step – the identification process.

Identification of Bacterial Strains

Soil isolates identified as pure were grown on an RM agar plate for 48 – 72 hours, after which, for each isolate, a single isolated colony was picked from the plate and suspended in a 1.5ml Eppendorf tube containing 10µl of dH_2O. The cells were dispersed by forcefully spinning the loop against the bottom of the tube. During the next procedure, this cell suspension was used as a DNA template to perform polymerase chain reaction (PCR). This reaction is used to isolate and amplify the 16S rRNA gene from bacterial cells. The sequence of this gene is commonly used to identify unknown bacterial genera. The following PCR reagents were mixed with the bacterial samples to undergo DNA amplification:

1) dH_2O 22 µl
2) 10x PCR Buffer (Go Taq Flexi, Promega) 10 µl
3) 10x Enhancer (Eppendorf) 10 µl
4) 25mM $MgCl_2$ (Promega) 3 µl
5) 10mM Deoxynucleotide Triphosphate Mix (Promega) 1 µl
6) 20µM Forward Primer (63f) 1.25 µl
7) 20µM Reverse Primer (1387r) 1.25 µl
8) Single bacterial colony (DNA template) 1 µl
9) Taq Polymerase (Go Taq Flexi, Promega) 0.5 µl

The sequence of the forward primer,63f, used in this reaction is 5'-CAG GCC TAA CAC ATG CAA GTC-3' and the sequence of the reverse primer, 1387r, is 5'-GGG CGG WGT GTA CAA GGC-3', where W is a code for A or T (http://www.basic.northwestern.edu/biotools/oligocalc.html)

(Marchesi *et al.* 1998). In order to complete the PCR reaction, the 50µl mixture of reagents was then placed in a thermocycler in which they were exposed to the following cyclic temperature changes:

1) 95°C for 3 minutes
2) 95°C for 1 minute
3) 55°C for 1 minute
4) 72°C for 2 minutes
5) Step 2 – 4 are repeated 29 times
6) 72°C for 5 minutes

After this program was complete, the samples were checked on an agarose gel to determine if DNA was successfully amplified by using gel electrophoresis (which should amplify a DNA of about 1.3kb). Then, the amplified DNA was purified using GeneClean Turbo kit. The purified samples were then sent to the DNA Sequencing Service at the University of Tennessee to obtain the whole sequences of the 16S rRNA gene using primers 63f and 1387r. Once the sequences were received, they were processed using the program Chromas that visualized the quality of a sequence and allowed the selection of only the best segment of the sequence to be used for identification. The sequence was then submitted to an online database, Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) that compares it to a number of bacterial DNA sequences. Based on similarity to known 16S rRNA segments, the program determines the genus of the unknown sequence and also estimates the 20 closest species matches and percent

similarity. Once an isolate was identified, it was streaked on RM agar slants for long-term storage at 4°C.

Screening for Antibiotic Production

After soil isolates were identified as *Rhodococcus,* they were ready to be screened for the production of any antimicrobial compounds they might be able to produce. Two methods were used for this process: extraction from liquid cultures via a resin and extraction from RM agar plates. Both methods are described in detail.

Extraction Using Resin

For this method, a 250ml Erlenmeyer flask containing 50ml RM broth and 1g XAD-16 Amberlite absorbent resin was inoculated with 100µl of a seed culture to be tested (Garcia *et al.* 2009, Barber 2010). All cultures were incubated in a 30°C water bath shaker for 1 week, after which the cultures were checked for purity by transferring to an RM plate for isolated colonies. The turbidity of pure cultures was checked on a Klett colorimeter (average reading was about 230) and then they were transferred to Oakridge tubes. Then, they were centrifuged to separate out the resin and cells from the supernatant. The resin and cells were then suspended in 7ml acetone and 7ml methanol and were extracted with spinning for 15 minutes. The acetone and methanol were then collected in a separate Oakridge tube and another 7ml acetone and 7ml methanol were mixed with the resin + cells and extracted for another 15 minutes. After that, the collected extract was pooled with the previous extract in the same Oakridge tube and it was centrifuged to remove the resin and cells. The extract was filtered through a 0.45µm filter into a polystyrene tube and then placed in a Labcono CentriVap to

evaporate to complete dryness. The dry extract was then redissolved in 1ml methanol and was then transferred to a 1.5ml Eppendorf tube to be stored at 4°C for later use. A rough sketch of this process is illustrated in Figure 9.

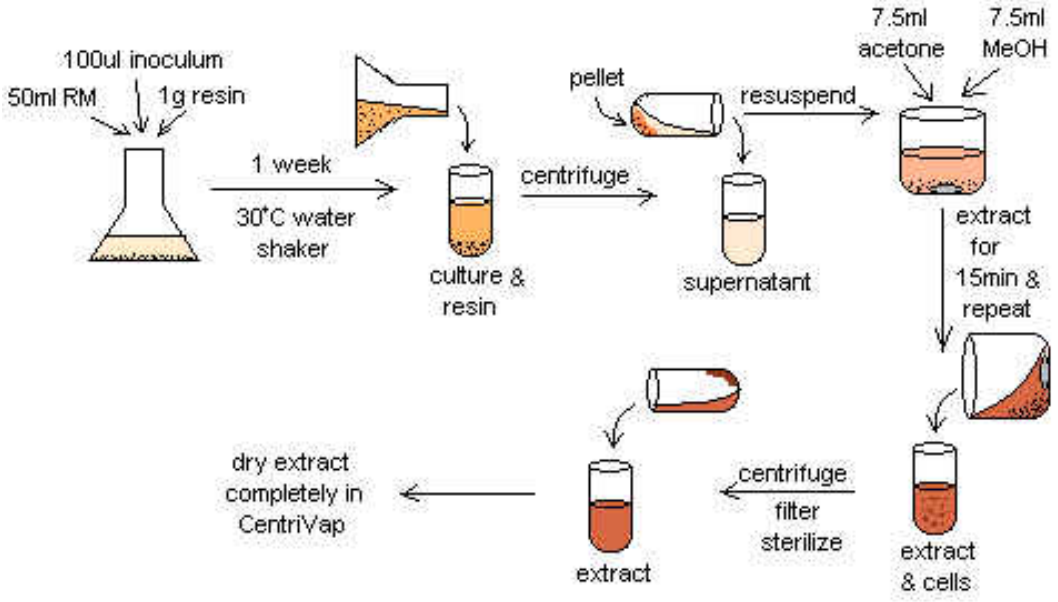


Figure 9: Extraction from liquid cultures using resin

Extraction from RM Plates

For this method (based on Carr *et al.* 2010), an inoculum of the *Rhodococcus* to be tested for antimicrobial production was streaked on an RM agar plate and was incubated at room temperature for 1 week or more (up to 1 month). Many of the RM plates used to check the purity of samples used in the extraction with resin method were afterwards tested using this method. Each plate was cut into small pieces and it was equally split between two 100ml beakers. One beaker was then filled with 50ml ethyl acetate and the other beaker was filled

with 50ml methanol. Both beakers were then covered with a piece of parafilm and left to soak for 24 hours. The beakers were then decanted into clean 100ml beakers and another 50ml ethyl acetate and 50ml methanol were poured into the beakers with agar chunks. After another 24-hour extraction period, the beakers were decanted into the same beakers containing the previous extractions (See Figure 10). The beakers containing the pooled extract were then left to evaporate inside a hood until they reach 20 – 30ml. After that, they were transferred to Oakridge tubes and were centrifuged to separate extracts from particulates (cells + undissolved compounds). After centrifuging, extracts were transferred to clean beakers and left in the hood to air-dry completely, while the particulates were redissolved in 1ml dH_2O and this extract was then tested separately on discs. Once the extracts were dry, 1ml methanol was placed in each beaker and the extracts were redissolved by scratching the bottom and sides of the beakers with a metal spatula. The extracts were then transferred to 1.5ml Eppendorf tubes to be stored at 4°C for later use.

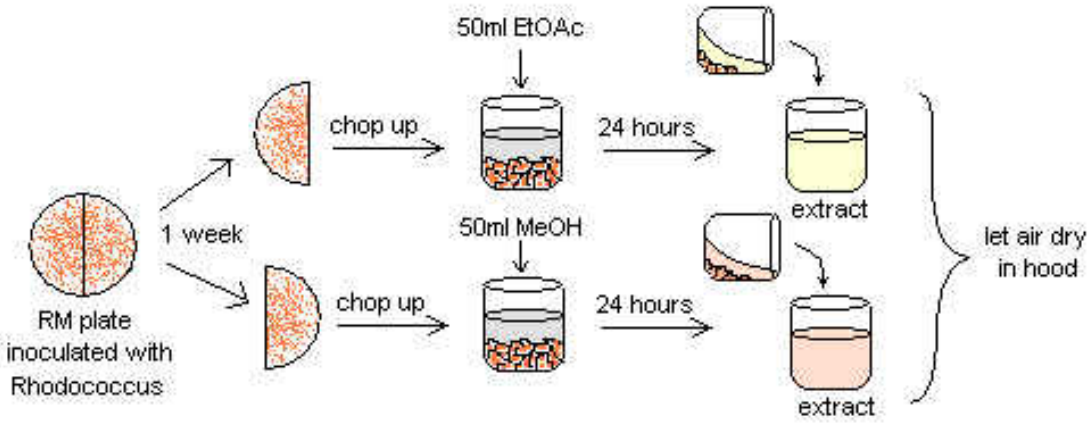


Figure 10: Extraction from RM agar plates

Growth Inhibition Assay

Extracts obtained with either 1 of the 2 methods of extraction were processed the same way in the next step. Paper discs were made using thick Whatman blotting paper GB004 and punched out via a hole-punch. The discs were then autoclaved. After that, 25µl of an extract was placed on a paper disc and after it had dried another 25µl was added to the same disc (50µl/disc). The culture supernatants from the extraction with resin were also placed on paper disc and tested for antimicrobial activity. Extracts were tested against 3 indicator bacteria: *Micrococcus luteus* (another Gram-positive organism), *Escherichia coli* (a Gram-negative organism), and *R. erythropolis* IGTS8 (organism from the same genus). Seed cultures were made by placing 2.5ml of MH broth in 10ml test tubes and then inoculating each test tube with one of the indicator organisms (Barber 2010). *M. luteus* and *E. coli* were then incubated at 37°C with shaking and the *R. erythropolis* IGTS8 was incubated in the 30°C water bath. After shaking for 24 hours, the turbidity of each seed was adjusted to a 0.5 McFarland standard (see Barber, 2010), and each seed was then spread on the surface of an MH plate by dipping a sterile cotton swab in the broth culture and swab-inoculating the entire surface of the plate. The previously prepared and dried discs with extract or supernatant were then placed on the surface of each plate. As before, *M. luteus* and *E. coli* plates were incubated at 37°C, while *R. erythrolpolis* IGTS8 was incubated at 30°C for 24 hours. The plates were then observed for zones of growth inhibition around each disc. Where growth inhibition did occur, the *Rhodococcus* strain responsible for the antimicrobial activity was put through another round of extraction and screening in order to confirm activity.

Scaling-Up Production of the Antimicrobial Compound

Once a *Rhodococcus* strain was found to produce an antimicrobial compound, there was a need to increase the production of this compound so that it can be purified and its identity determined. Scaling up was done by only extracting from agar plates. A seed culture was made by inoculating a 3 ml RM broth with the *Rhodococcus* strain producing the antimicrobial compound. After incubation overnight at 30°C, 200µl of the seed culture was dispersed on the entire surface of a large (150mm) Petri dish containing RM medium using a sterile glass spreader. A single scale-up consisted of 10 such agar plates. Plates were grown at 19°C for 1 week. If a plate was found to have a single colony contamination, the contamination was cut out with a sterile scalpel and the rest of the plate was used for extraction. Next, the *Rhodococcus* cells growing on the surface of the plates were washed off with dH_2O and the help of a glass spreader to scrape off the stubborn cells. The free-of-cells plates were then cut with a sterile scalpel into small cubes about $2mm^3$ in size. These cubes were then placed in a 2L beaker and were soaked with 1L ethyl acetate (100ml ethyl acetate per plate). After stirring the cubes around to remove any air trapped between them, the beaker was covered with a piece of parafilm and was left to soak for 24 hours. The next day the cubes were stirred again and the extract was poured into a clean 2L beaker that was left open in the hood to evaporate. Another 1L of ethyl acetate was added to the cubes for a second round of extraction and was left to soak over another 24 hours. The following day the new extract was added to the beaker containing the previous extract. The combined extract was left to evaporate in the hood until completely dry or it was placed in a 2L round bottom flask and placed in a Büchi Rotavapor R-200, where it was left to reduce to about 30ml. After that, the extract was placed in a 150ml

beaker and left to evaporate in the hood to complete dryness. Once dry, the extract was redissolved in 6ml of methanol by scraping the bottom and sides of the beaker with a metal spatula, after which it was poured into a 15ml polystyrene tube and centrifuged at 8,000RPM for 30 minutes to remove undissolved particulates. The extract was then transferred to a clean 15ml polystyrene tube. A 1ml sample of the extract was then separated out into a 1.5ml Eppendorf tube and was tested for antimicrobial activity. Both the extract and its sample were stored at 4°C for the purification process.

Purification of the Antimicrobial Compound

Sephadex LH-20 Column Chromatography

The 5ml of concentrated extract was initially purified by passing the extract through a column containing Sephadex LH-20 resin submerged in methanol. This allows the compounds present in the extract to be separated based on hydrophobicity and size. Preparation of the column was described in detail by Wright (2009). The 5ml extract was loaded in the column on top of the resin and left to settle for 5 minutes, after which the column was subjected to a constant flow of methanol. Using a Bio-Rad 2110 fraction collector, 50 fractions were collected in aliquots of 175 drops or about 2.5ml in volume. Every other fraction was then tested for inhibitory activity in order to determine the range of fractions containing the antimicrobial compound. Just as before, a total of 50µl were placed on discs from each fraction to be tested and were then placed on MH plates containing the *R. erythropolis* IGTS8 indicator strain. Fractions that showed growth inhibition were pooled together into a 50ml polystyrene tube

and were placed in the Labcono CentriVap to evaporate to dryness. The extract was then redissolved in 7ml of 90% methanol by adding 6.3ml 100% methanol first and then adding 0.7ml dH_2O drop by drop to avoid precipitation. The extract was then stored at 4°C for further purification.

High Pressure Liquid Chromatography (HPLC)

The next step in the purification process of the inhibitory molecule was passing it through a BioRad Biologic Duoflow High Pressure Liquid Chromatography Column (HPLC) for an even more precise separation of the compounds that it contains. The extract was run in 2ml aliquots using a Waters 7.8mm x 300mm Novapak HR C_{18} hydrophobic column as the stationary phase and deaerated dH_2O and methanol as the mobile phases. During initial runs, the sample was run through the HPLC by starting the mobile phase at 90% dH_2O and gradually increasing the methanol content until it reaches 100%. The program was later on adjusted to allow for better separation of the inhibitory compounds by starting the mobile phase at 90% methanol and gradually increasing methanol concentration until it reaches 100%. The presence of each compound in the sample was detected by an ultraviolet light detector that was set at a wavelength of 254nm. The compounds detected were represented by peaks on a chromatogram. During initial runs, 90 fractions were collected and every other one was tested for antimicrobial activity. Once the HPLC program was adjusted, only 35 fractions were collected and only the fractions in close proximity to the inhibitory compounds were tested to confirm the antimicrobial activity. Fractions containing an inhibitory compound were compared to the chromatogram and those that belonged to a single peak were pooled together.

Characterization of the Antimicrobial Compound

Thin Layer Chromatography (TLC)

Detection of amino acids was performed using thin layer chromatography (TLC). After the first scale-up was purified with HPLC, the combined fractions containing the inhibitory compound were dried in the CentriVap and were resuspended in 10ml methanol. Out of this, 1ml was removed and dried back down in the CentriVap. In order to be able to run TLC, the inhibitory compound needs to undergo acid hydrolysis that allows a peptide to be broken down to its individual amino acids. For the acid hydrolysis, the dried down sample was dissolved in 0.5ml 6M HCl + 0.5ml dH_2O (or 1ml 3M HCl) and was then autoclaved at 121°C for 6 hours. Different concentrations (2µl, 4µl, 6µl, and 8µl) of the hydrolyzed sample were then spotted on 3 silica gel TLC plates, 2cm from the bottom of the plate, along with the amino acid isoleucine, which served as a positive control. After the spots were dry, each TLC plate was placed in a closed chamber containing one of 3 solvent systems:

1) Methanol : 0.1M ammonium acetate (60 : 40)
2) Acetonitrile : 0.1M ammonium acetate (60 : 40)
3) N-propanol : ddH_2O (70 : 30)

Three different solvent systems were used because the chemical properties of this compound were unknown, so there was no way to know which solvent will work to separate the amino acids that might be present in the compound. The TLC plates were left inside the chambers until the solvent front reached 1cm from the top of the plate (about 1.25hrs for solvent system 1, about 2.5hrs for system 2, and about 4hrs for system 3). The plates from solvent system 1

and 2 were then air dried completely, after which they were sprayed with 0.5% (w/v) ninhydrin in ethanol and where then incubated at 55°C for 15 minutes. The plate from solvent system 3 was sprayed with 0.25% (w/v) ninhydrin in acetone immediately after taking out of the chamber and was then left to dry completely. When it was dry, it was sprayed with aqueous 0.1% n-cyanoguanidine (pH 10.5) to visualize the amino acids on the plate and was then placed in a 100°C oven to dry completely for about 10 minutes. The sample was run through all 3 solvent systems 3 more times to confirm the results.

Mass Spectrometry

The next step in determining the identity of an unknown antimicrobial compound is finding out its molecular weight. One way to determine the molecular weight of a compound is through mass spectrometry. After the antimicrobial compound was purified by running through HPLC, it was dried down in 1ml screw cap tube and was then sent to the University of Tennessee for structural analysis.

CHAPTER 3

RESULTS

Description of Soil Isolates

Acetonitrile Enrichment Isolates

All soils collected were processed through acetonitrile enrichment (DE media). Isolates were obtained by enrichments in either liquid DE media or on solid DE agar plates. Both methods seemed to be equally effective in obtaining the isolates. However, DE agar plates were harder to make and were a little more costly. Therefore, enrichments were carried out mainly via liquid DE medium. Table 2 is a summary of all isolates obtained using these methods. Acetonitrile enrichments yielded a total of 34 isolates from all soils tested. Out of these, 31 were found to be some kind of actinomycete and out of them 24 belonged to the genus *Rhodococcus.* To put this in perspective, 70% of isolates were *Rhodococcus* and 91% of all isolates belonged to the order Actinomycetales.

M3 Enrichment Isolates

Some of the soils collected were also processed using heat-shock enrichment (M3 medium). This method was less effective in selecting the bacteria of interest. Out of a total of 49 isolates obtained using the heat-shock method, 30 were found to be a kind of actinomycete and out of them 23 belonged to the genus *Rhodococcus.* In other words, 61% of the soil isolates obtained by the heat-shock method were identified as an actinomycete and only 47% of all isolates were found to be a *Rhodococcus* (Tables 3 and 4).

Table 2: Soil Isolates obtained through acetonitrile enrichment

Soil location	Enrichment method	Colony pigmentation	Strain designation[a]	Cell morphology	Genus[b]
Morristown – under battery	Acetonitrile	Tan	CBDET1	G+ coccobacilli	Rhodococcus
	Acetonitrile	Yellow	CBDEY2	G+ rods	Microbacterium[c]
Morristown – between driveway and tomato plants	Acetonitrile	Orange	MTDEO2	G+ rods	Rhodococcus
	Acetonitrile	Yellow	MTDEY3	Large G+ rods	Rhodococcus
	Acetonitrile	Orange	MTDEO9	L/S G+ rods	Rhodococcus
	Acetonitrile	Yellow	MTDEY8	Small G+ rods	Sphingopyxus
Newport –under a lawn-mower	Acetonitrile	Milky	NPDEM14	Large G+ rods	Rhodococcus
	Acetonitrile	Orange	NPDEO11	G+ rods	Rhodococcus
	Acetonitrile	Orange	NPDEO12	G+ rods	Rhodococcus
	Acetonitrile	Milky	NPDEM13	Large G+ rods	Rhodococcus
	Acetonitrile	Orange	NPDEO1	G+ rods	Rhodococcus
	Acetonitrile	White	NPDEW2	Large G+ rods	Cellulomonas[c]
Cosby – grassy driveway	Acetonitrile	Tan	CODET7	G+ rods chain	Rhodococcus
	Acetonitrile	White	CODEW2	Long G+ rods	Rhodococcus
	Acetonitrile	Orange	CODEO8	Long G+ rods	Rhodococcus
	Acetonitrile	Orange	CODEO20	L/S G+ rods	Rhodococcus
	Acetonitrile	Tan	CODET15	Branch G+ rods	Rhodococcus
	Acetonitrile	Yellow	CODEY4	G+ rods	Arthrobacter[c]
	Acetonitrile	White	CODEW12	L/Thin G+ rods	Cellulosimicrobium[c]
	Acetonitrile	White	CODEW14	Thin G+ rods	Microbacterium[c]
	Acetonitrile	White	CODEW5	Long G+ rod ch	Bacillus
	Acetonitrile	White	CODEW11	G- rods	Pseudomonas
Watauga Forest – between trees	Acetonitrile	Milky	WFDEM3	G+ rods	Rhodococcus
	Acetonitrile	Milky	WFDEM6	Large G+ rods	Rhodococcus
	Acetonitrile	Milky	WFDEM2	Long G+ rods	Rhodococcus
	Acetonitrile	Orange	WFDEO8	G+ L/peapods	Rhodococcus
	Acetonitrile	White	WFDEW1	G+ cocci	Rhodococcus
Watauga Lake – shoreline sediment	Acetonitrile	Orange	WLDEO8	G+ coccobacilli	Rhodococcus
	Acetonitrile	Orange	WLDEO9	G+ coccobacilli	Rhodococcus
	Acetonitrile	Tan	WLDET3	G+ L/peapods	Rhodococcus
	Acetonitrile	White	WLDEW1	G+ cocci	Rhodococcus
	Acetonitrile	White	WLDEW2	L/S G+ rods	Cellulosimicrobium[c]
Elizabethton – portable toilets	Acetonitrile	Tan	EZDET1	G+ peapods	Rhodococcus
	Acetonitrile	Clear	EZDEC7	Small G+ rods	Microbacterium[c]

a: The first 2 letters designate the location of the soil, the second two letters are the method of enrichment, the third letter is the color of the colonies (T = tan, Y = yellow, O = orange, M = milky, W = white, C = clear), and the number indicates the order in which the bacterium was isolated. b: Genus was determined by the 16S rRNA sequence analysis as determined by the online Ribosomal Database Project (http://rdp.cme.msu.edu). c: Other actinomycetes closely related to *Rhodococcus.*

Table 3: *Rhodococcus* strains isolated using M3 (heat-shock) enrichment

Soil location	Enrichment method	Colony pigmentation	Strain designation[a]	Cell morphology	Genus[b]
Morristown – under battery	Heat-shock	Milky	CBM3M1a	G+ cocci/rods	Rhodococcus
	Heat-shock	Tan	CBM3T2	G+ rods	Rhodococcus
	Heat-shock	Tan	CBM3T7	G+ rods	Rhodococcus
	Heat-shock	Tan	CBM3T10	Small G+ rods	Rhodococcus
	Heat-shock	White	CBM3W9	G+ rods	Rhodococcus
	Heat-shock	White	CBM3W11.2	G+ rods	Rhodococcus
Morristown – between driveway and tomato plants	Heat-shock	Orange	MTM3O9	Small G+ rods	Rhodococcus
	Heat-shock	Orange	MTM3O9a	Small G+ rods	Rhodococcus
	Heat-shock	Light orange	MTM3O2	Large G+ rods	Rhodococcus
	Heat-shock	Orange	MTM3O6.2	G+ rods	Rhodococcus
	Heat-shock	Tan	MTM3T5	G+ rods	Rhodococcus
	Heat-shock	Tan	MTM3T10	G+ peapods	Rhodococcus
	Heat-shock	White	MTM3W5.2	Small G+ rods	Rhodococcus
	Heat-shock	Orange	MTM3O4	G+ rods	Rhodococcus
	Heat-shock	Orange	MTM3O10	G+ rods	Rhodococcus
	Heat-shock	Orange	MTM3O15	G+ rods	Rhodococcus
	Heat-shock	Tan	MTM3T17	G+ rods	Rhodococcus
	Heat-shock	White	MTM3W12.1a	G+ rods	Rhodococcus
	Heat-shock	White	MTM3W13	G+ rods	Rhodococcus
Newport – under a lawn-mower	Heat-shock	Milky	NPM3M2.2	G+ rods	Rhodococcus
	Heat-shock	White	NPM3W4.1	G+ rods	Rhodococcus
	Heat-shock	Tan	NPM3T8.1	G+ rods	Rhodococcus
	Heat-shock	Tan	NPM3T11	G+ rods	Rhodococcus

a: The first 2 letters designate the location of the soil, the second two letters are the method of enrichment, the third letter is the color of the colonies (as defined in Table 2), and the number indicates the order in which the bacterium was isolated. b: Genus was determined by the 16S rRNA sequence analysis as determined by the online Ribosomal Database Project (http://rdp.cme.msu.edu).

Table 4: Bacterial soil isolates other than *Rhodococcus* obtained via heat-shock enrichment

Soil location	Enrichment method	Colony pigmentation	Strain designation[a]	Cell morphology	Genus[b]
Morristown – under battery	Heat-shock	Yellow	CBM3Y3	G+ cocci/rods	Janibacter[c]
	Heat-shock	White	CBM3W2	G+ rods	Janthinobacterium
	Heat-shock	White	CBM3W8	G+ rods	Janthinobacterium
	Heat-shock	White	CBM3W3a	G+ rods	Janthinobacterium
	Heat-shock	White	CBM3W12	G+ rods	Janthinobacterium
	Heat-shock	White	CBM3W6	G+ rods	Bacillus
	Heat-shock	White	CBM3W3	G+ cocci	Staphylococcus
	Heat-shock	Yellow	CBM3Y2	G+ cocci	Staphylococcus
	Heat-shock	White	CBM3W6a	G+ cocci	Staphylococcus
Morristown – between driveway and tomato plants	Heat-shock	Orange	MTM3O11a	Small G+ rods	Gordonia[c]
	Heat-shock	White	MTM3W2	G+ rods	Arthrobacter[c]
	Heat-shock	Light yellow	MTM3Y1.3	Small G+ rods	Microbacterium[c]
	Heat-shock	Yellow	MTM3Y3	Small G+ rods	Microbacterium[c]
	Heat-shock	Yellow	MTM3Y7	Small G+ rods	Microbacterium[c]
	Heat-shock	Yellow	MTM3W7	G+ rods	Janthinobacterium
	Heat-shock	White	MTM3W6	G+ rods	Janthinobacterium
	Heat-shock	White	MTM3W12.2	G+ rods	Janthinobacterium
	Heat-shock	White	MTM3W7.1	G- rods	Cupriavidus
	Heat-shock	White	MTM3W11	Faint G- rods	Ralstonia
	Heat-shock	Yellow	MTM3Y8a	G+ cocci	Staphylococcus
	Heat-shock	Light yellow	MTM3Y8	G+ cocci	Staphylococcus
	Heat-shock	Yellow	MTM3Y1.1	G+ cocci	Staphylococcus
	Heat-shock	Yellow	MTM3Y2	G+ cocci	Staphylococcus
	Heat-shock	Yellow	MTM3Y12.2a	G+ cocci	Staphylococcus
Newport – under a lawn-mower	Heat-shock	White	NPM3W4.2	G- rods	Stenotrophomonas
	Heat-shock	Yellow	NPM3Y2.1	G+ cocci/rods	Isoptericola[c]

a: The first 2 letters designate the location of the soil, the second two letters are the method of enrichment, the third letter is the color of the colonies (as defined in Table 2), and the number indicates the order in which the bacterium was isolated. b: Genus was determined by the 16S rRNA sequence analysis as determined by the online Ribosomal Database Project (http://rdp.cme.msu.edu). c: Other actinomycetes closely related to *Rhodococcus.*

Comparison Between Enrichment Methods

To better visualize the effectiveness of the 2 methods of enrichment used, the soil isolates obtained are summarized in Table 5. A total of 83 bacterial species were isolated from the 7 soils, out of which 47, about 57%, were identified as *Rhodococcus*. From these results, it seems both enrichment methods are very effective in isolating rhodococci. It appears that acetonitrile enrichment was able to eliminate the growth of bacteria, such as *Staphylococcus* and *Janthinobacterium,* which were present in large numbers after M3 enrichment, though more tests need to be done to confirm this observation. No experiments were performed to determine if rhodococci were using the acetonitrile as a C and N source for growth or if they were simply tolerant to its presence in their environment.

Table 5: Comparison between acetonitrile and heat-shock enrichment

Isolates	Acetonitrile[a]	M3[b]	Total
Rhodococcus	24	23	47
Arthrobacter	1	1	2
Gordonia	0	1	1
Microbacterium	3	3	6
Cellulosimicrobium	2	0	2
Cellulomonas	1	0	1
Isoptericola	0	1	1
Janibacter	0	1	1
Janthinobacterium	0	7	7
Bacillus	1	1	2
Staphylococcus	0	8	8
Cupriavidus, Ralstonia, Sphingopyxus, Stenotrophomonas, Pseudomonas	2	3	5
Total	34	49	83

a: Enrichment culture with acetonitrile as the sole C and N source for growth
b: Enrichment via heat shock

Pigmentation of Soil Isolates

Rhodococci have been found to exhibit various pigmentations on nutrient media. A large difference was seen in the pigmentation of isolates streaked on DE (acetonitrile) agar plates versus RM agar plates. Figure 11 illustrates the difference of appearance of an isolate streaked on both media.

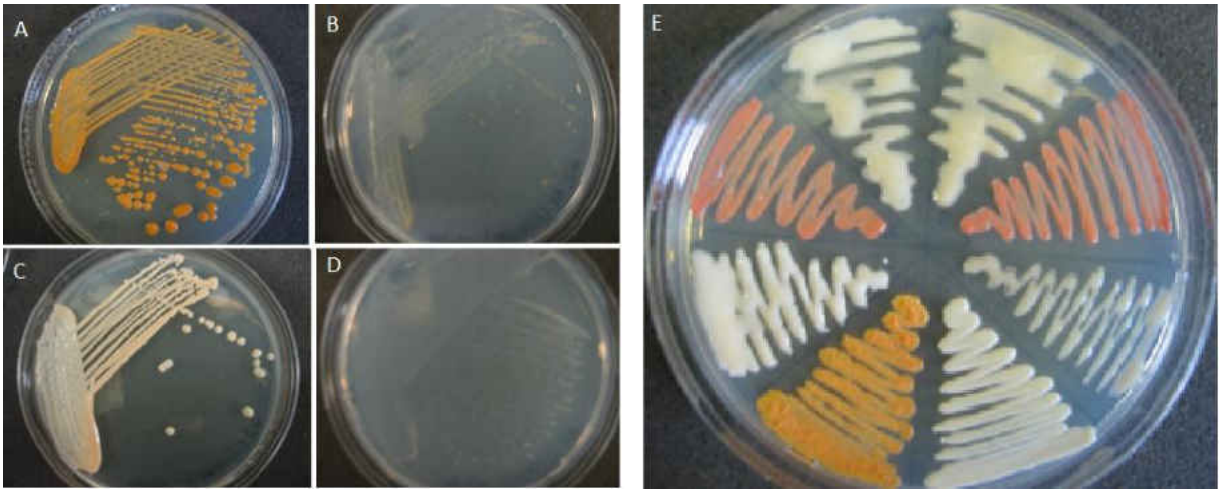


Figure 11: *Rhodococcus* growth appearances. (A) *Rhodococcus* sp. MTDEO2 on RM agar plate. (B) *Rhodococcus* sp. MTDEO2 on DE agar plate. (C) *Rhodococcus* sp. NPDEM14 on RM plate. (D) *Rhodococcus* sp. NPDEM14 on DE plate. (E) 8 *Rhodococcus* strains grown on RM plate.

Screening for Antibiotic Production

Rhodococcus isolates, and some closely related organisms, were screened for the production of antimicrobial compounds. Two methods were used for the production and extraction of potential antibiotics: extraction using resin and extraction from agar plates. Table 6 is a summary of all soil isolates that were screened for antibiotic producers and the method used to screen them. Twenty-nine *Rhodococcus* strains were screened using extractions from resin and 19 were screened using extractions from agar plates.

Table 6: Screening results for Rhodococci and related organisms

Strain	Genus	Extraction method	Indicator organism (Resin/Agar)		
			M. luteus	*E. coli*	*R. erythropolis*
CBM3M1a	Rhodococcus	Resin / Agar	-/-	-/-	-/-
CBM3T2	Rhodococcus	Resin	-	-	-
CBM3T7	Rhodococcus	Resin	-	-	-
CBM3T10	Rhodococcus	Resin	-	-	-
CBM3W9	Rhodococcus	Resin / Agar	-/-	-/-	-/-
CBM3W11.2	Rhodococcus	Resin /Agar	-/-	-/-	-/-
CBM3Y3	Janibacter	Resin	+[a]	-	-
MTDEO2	Rhodococcus	Resin	-	-	-
MTDEY3	Rhodococcus	Resin	-	-	-
MTDEO9	Rhodococcus	Resin / Agar	-/-	-/-	-/-
MTM3O9	Rhodococcus	Resin	-	-	-
MTM3O9a	Rhodococcus	Resin	-	-	-
MTM3O2	Rhodococcus	Resin / Agar	-/-	-/-	-/-
MTM3O6.2	Rhodococcus	Resin / Agar	-/-	-/-	-/-
MTM3T5	Rhodococcus	Resin / Agar	-/-	-/-	-/-
MTM3T10	Rhodococcus	Resin	-	-	-
MTM3W5.2	**Rhodococcus**	**Resin / Agar**	**-/-**	**-/-**	**-/+**
MTM3O4	Rhodococcus	Resin / Agar	-/-	-/-	-/-
MTM3O10	Rhodococcus	Agar	-	-	-
MTM3O15	Rhodococcus	Agar	-	-	-
MTM3W12.1a	Rhodococcus	Resin	-	-	-
MTM3O11a	Gordonia	Resin	-	-	-
MTM3W2	Arthrobacter	Resin	+[a]	-	+[a]
MTM3Y3	Microbacterium	Resin	-	-	-
MTM3Y7	Microbacterium	Resin	-	-	-
NPDEM14	Rhodococcus	Resin	-	-	-
NPDEO11	Rhodococcus	Resin	-	-	-
NPDEO12	Rhodococcus	Resin	-	-	-
NPDEM13	Rhodococcus	Resin / Agar	-/-	-/-	-/-
NPM3T8.1	Rhodococcus	Agar	-	-	-
NPM3T11	Rhodococcus	Resin / Agar	-/-	-/-	+[b]/-
CODET7	Rhodococcus	Resin / Agar	-/-	-/-	-/-
CODEW2	Rhodococcus	Resin	-	-	-
CODEO8	Rhodococcus	Resin / Agar	-/-	-/-	-/-
CODET15	Rhodococcus	Resin / Agar	-/-	-/-	-/-
CODEY4	Arthrobacter	Resin	+[a]	-	+[a]
WFDEM3	Rhodococcus	Resin / Agar	-/-	-/-	-/-
WFDEM6	Rhodococcus	Resin / Agar	-/-	-/-	+[b]/-

a: Results were inconsistent and organisms did not produce antibacterial compounds when they were retested.
b: Halos appeared hazy due to some growth of the indicator strain.

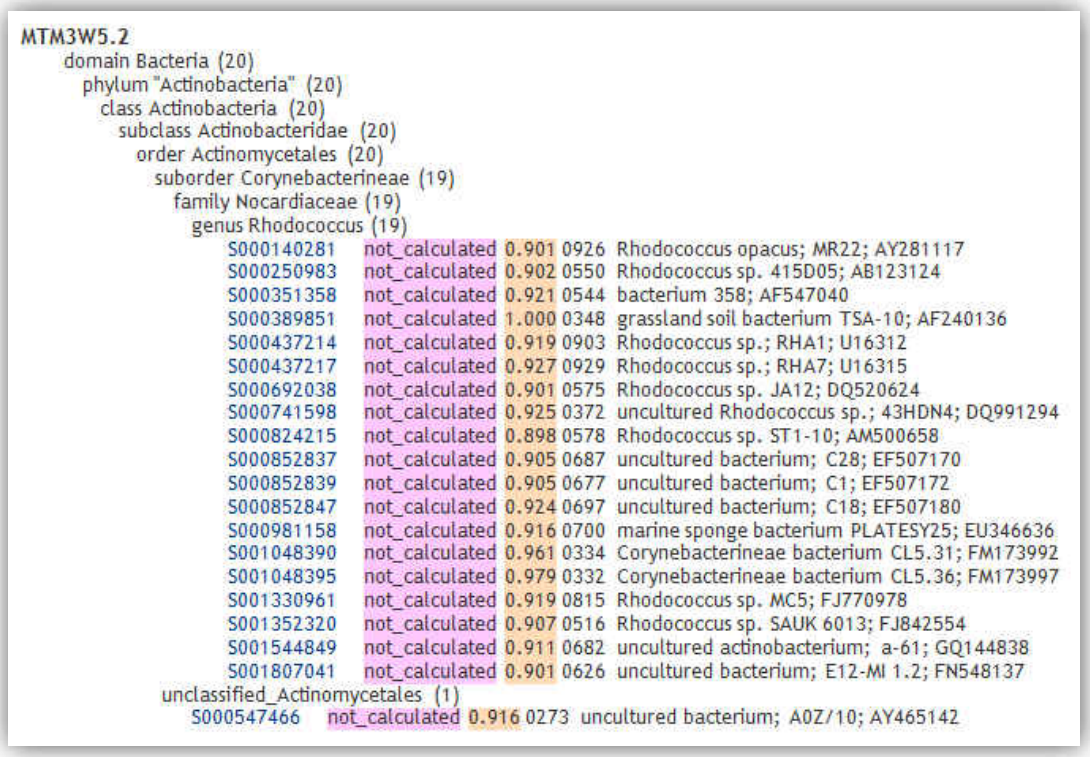
Two *Arthrobacter* species (MTM3W2 and CODEY4) and *Janibacter* sp. CBM3Y3 were found to produce some kind of inhibitory compound when grown in broth cultures in the presence of resin. When the experiment was repeated, however, all 3 of these strains did not inhibit the growth of *M. luteus* or *R. erythropolis* strain IGTS8 as they did the first time around. Due to the inconsistency of the inhibitory compound production, testing of these strains was not pursued further. Another 2 strains, *Rhodococcus* sp. NPM3T11 and *Rhodococcus* sp. WFDEM6, were able to produce weak growth inhibition zones around *R. erythropolis* strain IGTS8 when grown in broth cultures with resin. They were not able to produce an antimicrobial compound when grown on, and extracted from, agar plates. Due to the weak activity of the inhibitory compounds, the testing of these strains was not continued.

One more organism, *Rhodococcus* sp. MTM3W5.2, also produced a zone of growth inhibition against *R. erythropolis* strain IGTS8 when extracts were produced from cells grown on agar plates. The growth inhibition zone was initially about 34mm in diameter but was found to be as large as 50mm in later retesting.

Characterization of *Rhodococcus* sp. MTM3W5.2

MTM3W5.2 was isolated from a soil in Morristown located between the driveway and a tomato garden of a local residence. The soil was processed using M3 (heat-shock) enrichment. Based on the 16S rRNA sequence analysis, strain MTM3W5.2 was most closely related to a strain of *Rhodococcus opacus* with a similarity of about 90% (Figure 12). Gram staining MTM3W5.2 revealed a mixture of shorter and longer rods (Figure 13, A). When streaked on RM agar plates, MTM3W5.2 colonies initially appear white in color, but as they start aging, they

begin producing a tan pigmentation, which becomes apparent after 1–2 weeks of growth (Figure 13, B).



MTM3W5.2
domain Bacteria (20)
phylum "Actinobacteria" (20)
class Actinobacteria (20)
subclass Actinobacteridae (20)
order Actinomycetales (20)
suborder Corynebacterineae (19)
family Nocardiaceae (19)
genus Rhodococcus (19)

S000140281	not_calculated	0.901	0926	Rhodococcus opacus; MR22; AY281117
S000250983	not_calculated	0.902	0550	Rhodococcus sp. 415D05; AB123124
S000351358	not_calculated	0.921	0544	bacterium 358; AF547040
S000389851	not_calculated	1.000	0348	grassland soil bacterium TSA-10; AF240136
S000437214	not_calculated	0.919	0903	Rhodococcus sp.; RHA1; U16312
S000437217	not_calculated	0.927	0929	Rhodococcus sp.; RHA7; U16315
S000692038	not_calculated	0.901	0575	Rhodococcus sp. JA12; DQ520624
S000741598	not_calculated	0.925	0372	uncultured Rhodococcus sp.; 43HDN4; DQ991294
S000824215	not_calculated	0.898	0578	Rhodococcus sp. ST1-10; AM500658
S000852837	not_calculated	0.905	0687	uncultured bacterium; C28; EF507170
S000852839	not_calculated	0.905	0677	uncultured bacterium; C1; EF507172
S000852847	not_calculated	0.924	0697	uncultured bacterium; C18; EF507180
S000981158	not_calculated	0.916	0700	marine sponge bacterium PLATESY25; EU346636
S001048390	not_calculated	0.961	0334	Corynebacterineae bacterium CL5.31; FM173992
S001048395	not_calculated	0.979	0332	Corynebacterineae bacterium CL5.36; FM173997
S001330961	not_calculated	0.919	0815	Rhodococcus sp. MC5; FJ770978
S001352320	not_calculated	0.907	0516	Rhodococcus sp. SAUK 6013; FJ842554
S001544849	not_calculated	0.911	0682	uncultured actinobacterium; a-61; GQ144838
S001807041	not_calculated	0.901	0626	uncultured bacterium; E12-MI 1.2; FN548137

unclassified_Actinomycetales (1)

S000547466	not_calculated	0.916	0273	uncultured bacterium; A0Z/10; AY465142

Figure 12: Ribosomal Database Project sequence analysis of MTM3W5.2

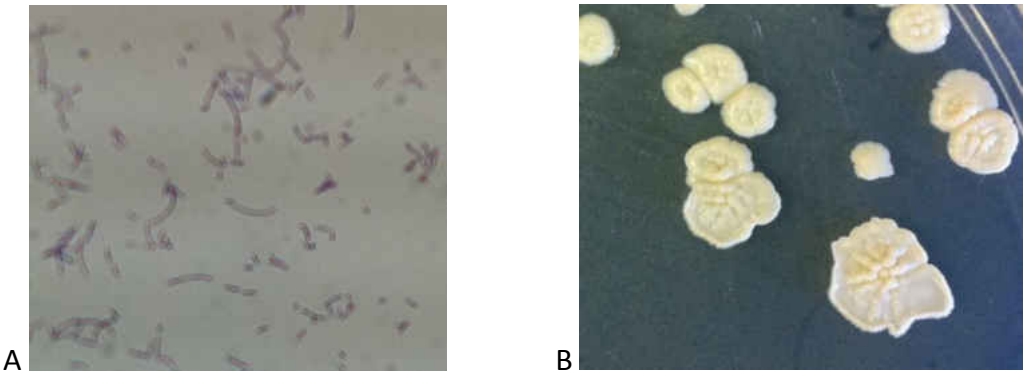


Figure 13: Cell (A) and colony (B) appearance of *Rhodococcus* sp. MTM3W5.2

Strain MTM3W5.2 was initially screened using broth cultures and resin. The cultures, as usual, were grown at 30°C for 1 week with shaking. This culture was not found to produce any kind of inhibitory compound. The RM agar plate used to check for the purity of this culture was left to grow on the bench top at room temperature (varying between 17°C and 20°C) for about 1 month. After that, the plate was processed using extractions with ethyl acetate and methanol and was found to produce growth inhibition against *R. erythropoils* strain IGTS8 (Figure 14). MTM3W5.2 was then tested for the production of this inhibitory compound after growing for 2 weeks, 1 week, and 4 days at 19°C. The size of the zones of growth inhibition of the 1 and 2 week plates were on average the same, whereas the 4 day old plates produced slightly smaller inhibition zones.

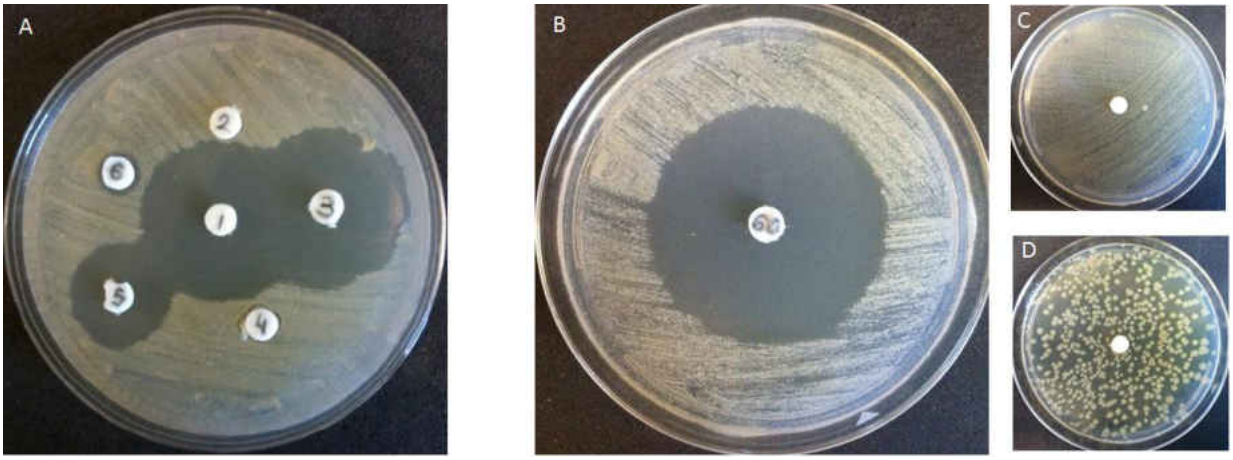


Figure 14: Antibacterial activity against strain IGTS8. (A) Initial discovery of *R. erythropolis* strain IGTS8 growth inhibition after extracting with ethyl acetate (disc 1), methanol (disc 3), and water (disc 5) (discs 2, 4, and 6 contain extract form strain CBM3T7). (B) Activity against *R. erythropolis* strain IGTS8 from a scale-up (60µl of extract on disc). (C) Indicator strain *Micrococcus luteus.* (D) Indicator strain *Escherichia coli.*

Strain MTM3W5.2 was also tested for production of the antimicrobial compound when grown at different temperatures. When MTM3W5.2 was grown at 19°C, 15°C, 10°C, and 4°C, it was able to produce the compound, though at 10°C and 4°C the inhibition zones were a bit

smaller, 26mm and 19mm respectively (Figure 15, A). When MTM3W5.2 was grown at 30°C, however, it was not able to produce a zone of inhibition. Due to this observation, it was speculated that the antimicrobial compound was not produced in the broth cultures with resin because it was grown at 30°C. To test this hypothesis, a broth culture was grown with resin at 19°C and when tested against *R. erythropolis* IGTS8, there was a zone of growth inhibition that was about half the size (20mm) of the regular zone of inhibition (Figure 15, B). There was an inconsistency with the production of this antimicrobial compound (in broth + resin), because a zone of inhibition was not produced when this procedure was repeated to confirm the positive results.

Figure 15: Comparison between zones of growth inhibition. (A) Discs 1 and 4 contained extract from a plate grown at 10°C, discs 2, 3, 5, and 6 contained extracts from plates grown at 19°C. (B) Disc 3 contained supernatant and disc 4 extract from resin grown at 19°C. (C) Discs 7 and 9 contained extract from agar and discs 8 and 10 from cells (ethyl acetate and methanol, respectively).

The next test done was to determine whether using half the amount of the solvents used to extract from the agar plates is sufficient enough to extract as much of the compound as it does using the full 50ml of solvent per half a plate. There was no size difference between the zones of growth inhibition produced when plates were extracted with half the amount of ethyl

acetate versus the full amount. Also, because the ultimate goal was to purify the compound produced and determine what it is, it was important to eliminate as many impurities as possible during the extraction process. Thus, an experiment was done to determine whether the compound produced is being extracted from the agar of the plates or from the cells themselves. After the plates to be tested were grown and ready to extract, the bacterial cells growing on the surface were washed off with 2ml dH_2O into 2 Oakridge tubes and were submerged in 30ml ethyl acetate or methanol. The following day the cells were centrifuged and the extracts were poured into clean beakers and another 30ml ethyl acetate or methanol was added to the cells. After another 24 hours the tubes were centrifuged again and the extracts were added to the first extracts and left to air dry in the hood. The rest of the agar plates were processed as usual. No difference was seen in the size of the inhibition zones produced between the extractions from agar alone versus the extractions from cells (Figure 15, C).

MTM3W5.2 Extract Purification

Sephadex LH-20 Column Chromatography

As previously described, each scale-up was achieved by extracting the antimicrobial compound produced by strain MTM3W5.2 from 10 large plates and then redissolving the dried extract in 6ml methanol (Figure 16, A). About 1ml from each scale-up was saved for other tests, while the remaining 5ml were run through the LH-20 column. The crude extract had a bright yellow appearance, but after passing it through the LH-20 column, the impurities responsible for the yellow coloration were eliminated (Figure 16, A and B). The first scale-up was purified by running the LH-20 column for a total of 50 fractions (175 drops each) or about 2½ hours.

However, by testing the fractions on discs for growth inhibition of indicator strain *Rhodococcus erythropolis* strain IGTS8, it was determined that the antimicrobial compound comes out between fractions 16 and 24 (Figure 17), so the rest of the scale-ups were run for 30 fractions (175 drops each). In a few of the scale-ups, the antimicrobial compound continued coming out in fractions 25, 26, or 27. The fractions containing the antimicrobial compound were then combined and dried down. The dry extract was then redissolved in 7ml 90% methanol in order to be further purified by high pressure liquid chromatography (HPLC).

A  B 

Figure 16: MTM3W5.2 extract before (A) and after (B) LH-20 column purification

Figure 17: Fractions containing the LH-20 column purified extract

High Pressure Liquid Chromatography (HPLC)

Initially, the dried LH-20 column purified extract was suspended in 10ml dH_2O, but most of it was not able to dissolve in the water, so 1ml methanol was added to the extract to make a 10% solution. The extract was syringe filtered to remove the precipitate and was then run through the HPLC using a program that starts the mobile phase at 10% methanol and then gradually increases the methanol content until it reaches 100% over 60ml. Using this program,

0.75ml of LH-20 purified extract was loaded in the HPLC, 90 fractions were collected and were then tested for antimicrobial activity against *R. erythropolis* strain IGTS8. Only one fraction, 81, contained the inhibitory compound (Figure 18). The compound did not come out of the column until the gradient reached 100% methanol.

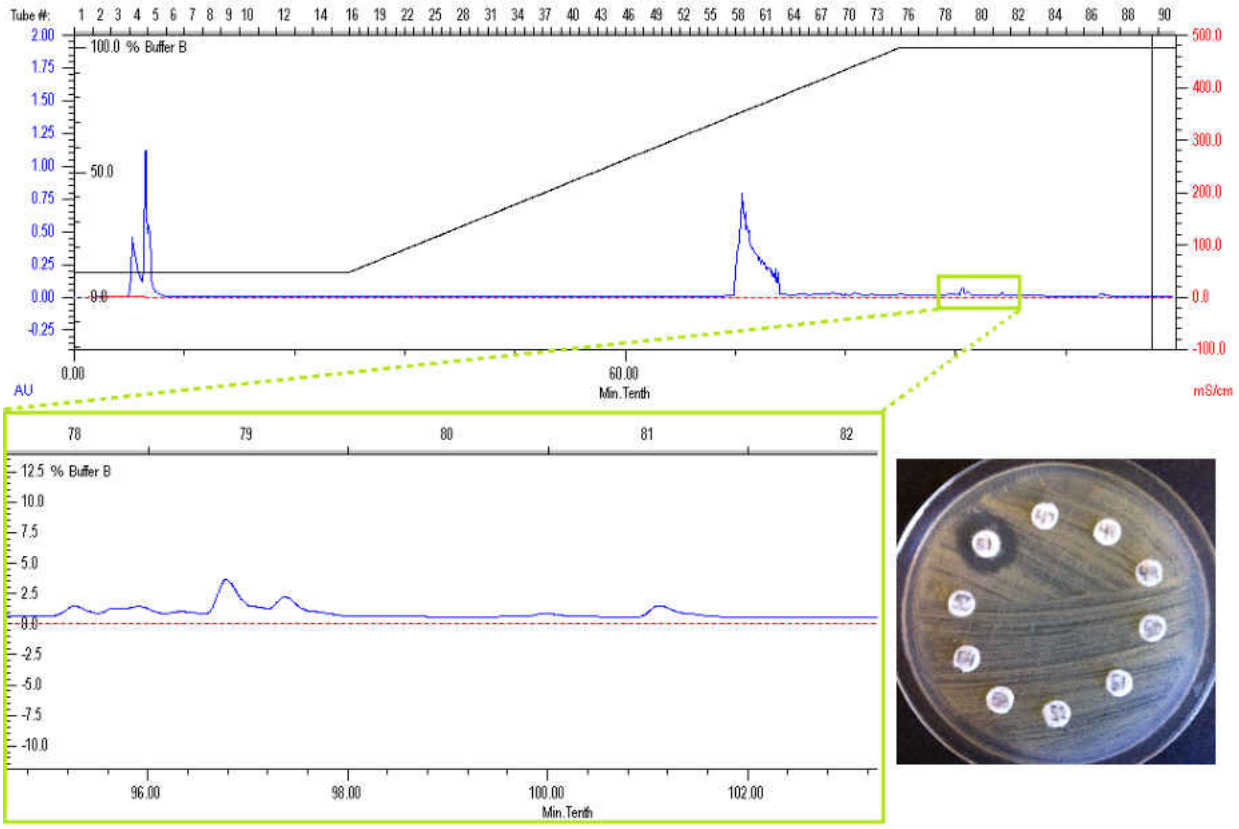


Figure 18: HPLC chromatogram of the first run of MTM3W5.2 extract in 10% methanol. Fraction 81 was the only tube that contained the inhibitory compound.

The small size of the peak in fraction 81 and the small zone of inhibition indicated that the antimicrobial compound produced by strain MTM3W5.2 is highly hydrophobic and that the methanol content of the sample to be run through HPLC needs to be increased. The sample was adjusted to 50% methanol and syringe filtered once again to remove undissolves particles. For this run, the HPLC program was started with 50% methanol as the mobile phase, which was

then gradually increased to 100% over 40ml. A 1.5ml aliquot of the LH-20 purified extract was loaded in the HPLC and 90 fractions were collected and tested for antimicrobial activity. Fractions 53 – 58 contained the antimicrobial compound (Figure 19).

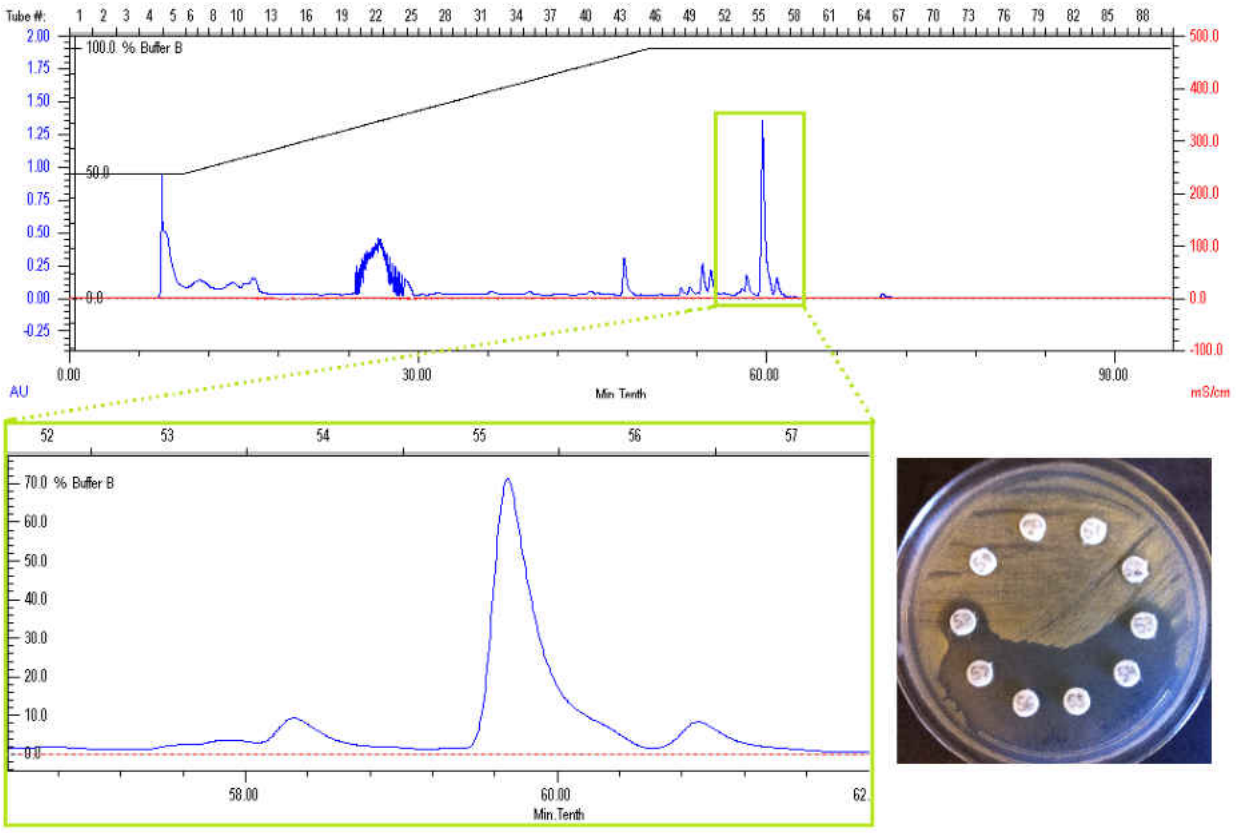


Figure 19: HPLC chromatogram of the second run of MTM3W5.2 extract in 50% methanol. Fractions 53 – 58 contained the inhibitory compound.

The inhibitory compound was not able to come out of the column until the mobile phase reached 100% methanol and the separation between the peaks did not seem adequate. Therefore, the HPLC program was adjusted one more time and the mobile phase was started at initial methanol concentration of 90%, which was then gradually increased to 100% over 30ml. The LH-20 column purified sample was also dissolved in 90% methanol that allowed most of the hydrophobic compound to be dissolved. The sample did not need to be filtered this time

around. A 2.0ml aliquot of the LH-20 purified extract was loaded in the HPLC and 35 fractions were collected and tested for antimicrobial activity. All of the scale-ups were run using this program and usually the inhibitory compound was found in fractions 17 – 28 (Figure 20).

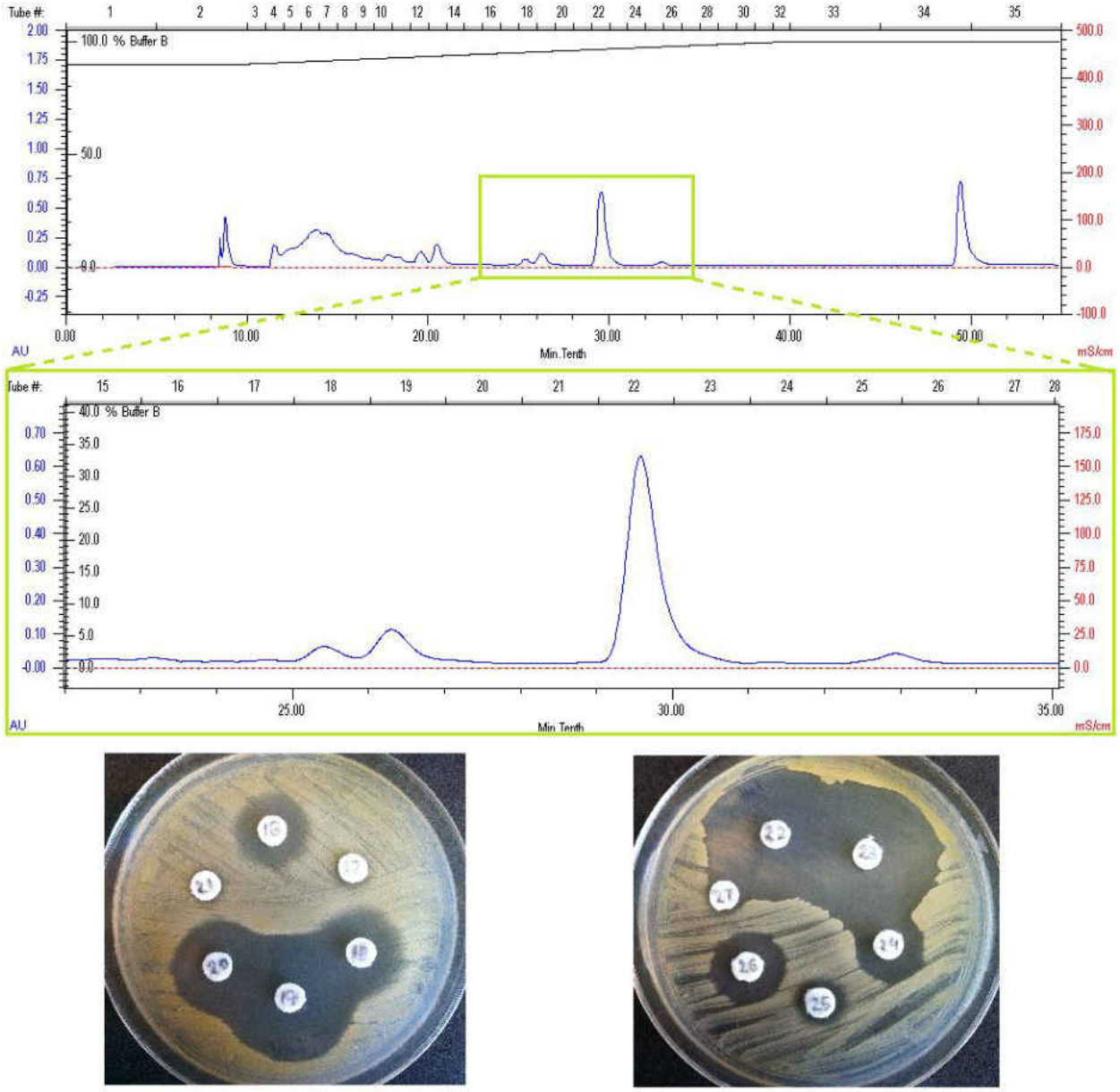


Figure 20: HPLC chromatogram for scale-up 2, run 3. Discs 16, 18, 19, and 20 corresponded to the first set of peaks, discs 22, 23, and 24 correspond to the large peak in the middle, and discs 25 and 26 correspond to the smaller last peak.

Three distinct peaks (or groups of peaks) were distinguished. Peak #1 was a combination of 2 – 3 unseparated peaks and their corresponding fractions 16 – 20 were combined. Peak #2 was the largest peak in the middle, which appeared to stand alone until a later run that indicated that there could be 2 peaks (Figure 21). The corresponding fractions 20 – 23 were combined separately from peak #1. Peak #3 was a single smaller peak that corresponded to fractions 24 – 27 and was combined separately from the other 2 peaks. The peaks came out at a methanol concentration of 95% – 96%. Each one of the combined purified peaks was dried down and stored at 4°C for future testing. From these preliminary results, it was suspected that peak #2 represents the main inhibitory compound and that all other peaks might be degradation products of this compound, though this could not be determined with certainty at this time. Therefore, all further tests were performed only on peak #2.

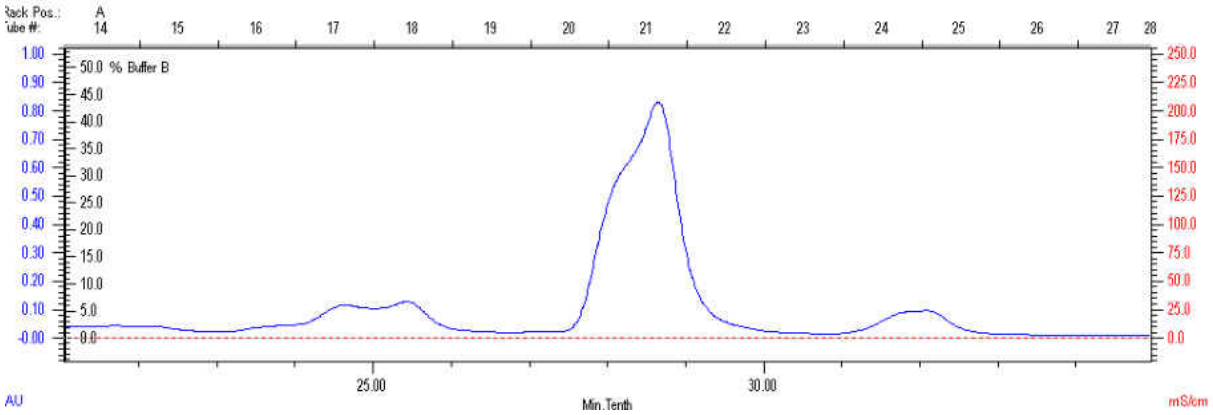


Figure 21: HPLC chromatogram of scale-up 8, run 1 zoomed in on the 3 peaks that produce growth inhibition

Amino Acid Assay of Peak #2

Thin Layer Chromatography (TLC) was performed on acid hydrolyzed Peak #2 using 3 different solvent systems: methanol : 0.1M ammonium acetate (60 : 40), acetonitrile : 0.1M

ammonium acetate (60 : 40), and N-propanol : ddH2O (70 : 30). The sample was first dissolved in 3M HCl and autoclaved at 121°C for 6 hours. It was then run side by side to the amino acid isoleucine in each one of the 3 systems. Figure 22 shows the results of the plates run in the methanol : 0.1M ammonium acetate and the acetonitrile : 0.1M ammonium acetate solvent systems. Both of these plates were sprayed with 0.5% (w/v) ninhydrin in ethanol to visualize amino acids. Isoleucine appeared as a purple spot on the TLC plate; however, no other spots were seen even when these experiments were repeated. From these results it seems that the antimicrobial compound produces by strain MTM3W5.2 is probably not a peptide.

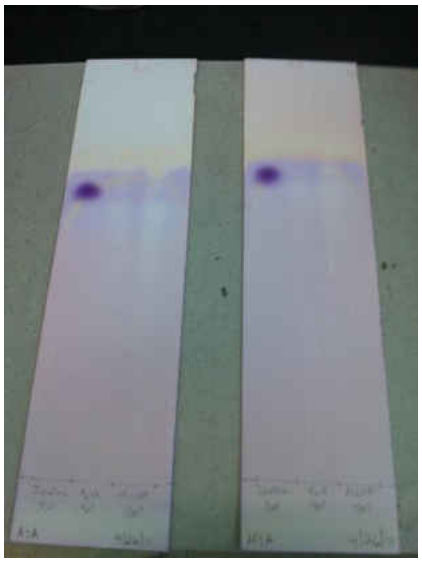


Figure 22: TLC plates of the acid hydrolyzed antimicrobial compound found in Peak #2 (from HPLC) versus the positive control isoleucine (the purple spot). The plate on the left was run in the acetonitrile : 0.1M ammonium acetate solvent system. The plate on the right was run in the methanol : 0.1M ammonium acetate. No amino acid spots appear in the lanes containing sample from Peak #2.

Spectrum of Activity of the Antibacterial Compound

The antimicrobial activity of the compound produced by strain MTM3W5.2, specifically Peak #2 on the HPLC chromatogram, was tested for activity against a number of different

organisms. Discs were soaked with 50µl of the HPLC purified Peak #2 compound and were placed on the surface of MH plates each inoculated with a different indicator organism (Table 7). Growth inhibition of these organisms was limited to bacteria closely related to the producer strain, MTM3W5.2. This included other members of the genus *Rhodococcus* and a few other actinomycetes (Figure 23). The antimicrobial compound did not have any activity against any of the Gram negative organisms tested or against the fungi *Aspergillus niger* and *Candida albicans.*

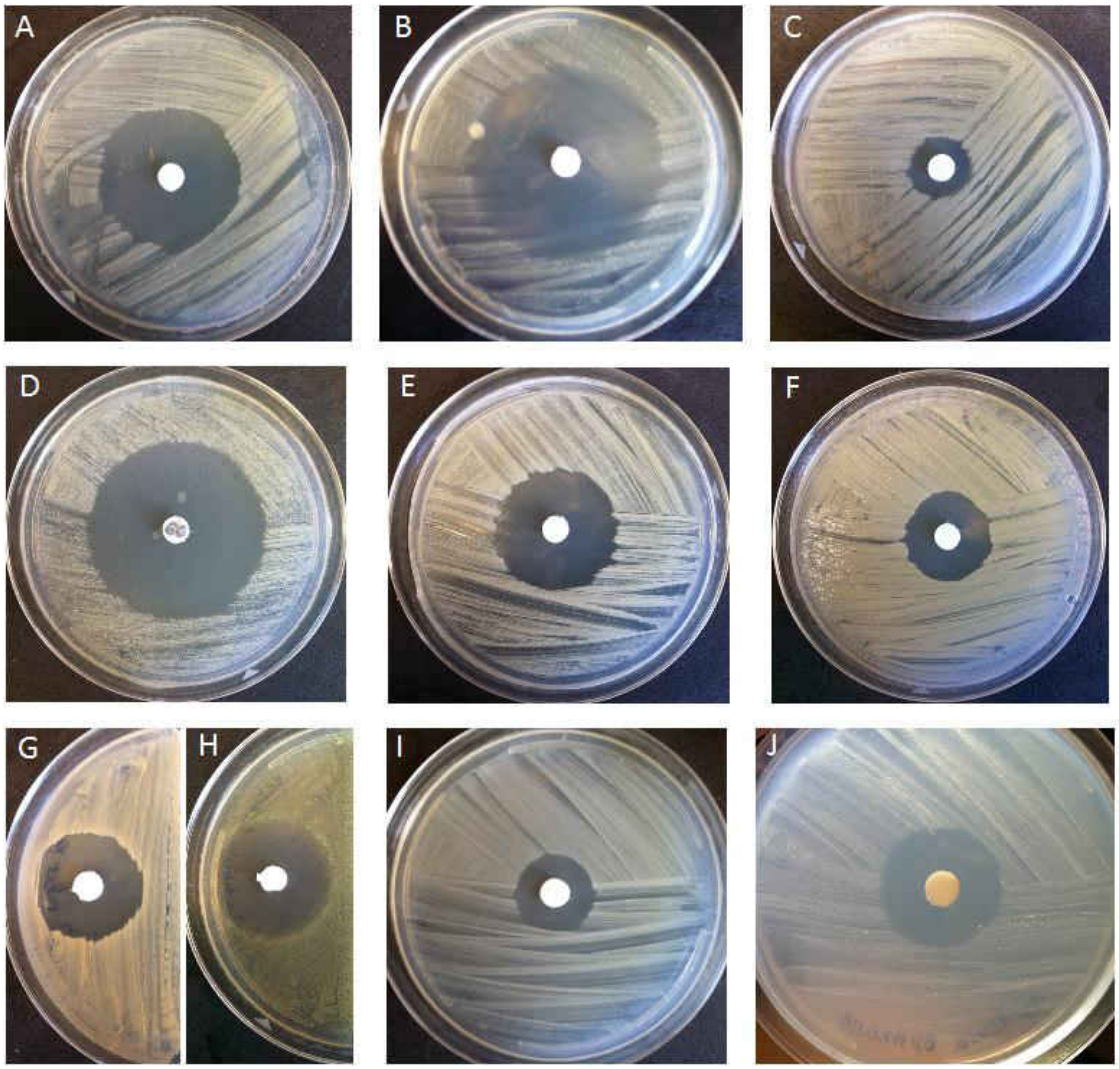


Figure 23: Spectrum of activity of the antimicrobial compound produced by strain MTM3W5.2. Discs were loaded with 50µl of the LH-20 purified extract produced growth inhibition of the following indicator organisms (zone size): (A) *R. australis* (39mm), (B) *R. equi* (50mm), (C) *R. erythropolis* DP-45 (17mm), (D) *R. erythropolis* IGTS8 (50mm), (E) *R. jostii* RHA1 (35mm), (F) *R. rhodochrous* (25mm), (G) *R. ruber* (26mm), (H) *Microbacterium* sp. MTM3Y7 (30mm), (I) *Agromyces* sp. BEM3Y1 (20mm), (J) *Gordonia* sp. BDHXW1B (27mm).

Table 7: Sensitivity of organisms to the antimicrobial compound produced by strain MTM3W5.2

Organism	Gram reaction	Sensitivity to compound	Diameter of zone (mm)
Rhodococcus erythropolis IGTS8	+	+	36 – 50
Rhodococcus erythropolis DP-45	+	+	17
Rhodococcus equi 33701	+	+	50
Rhodococcus australis 087200	+	+	39
Rhodococcus jostii RHA1	+	+	35
Rhodococcus rhodochrous ATCC 33279	+	+	25
Rhodococcus ruber 1979/002000	+	+	26
***Rhodococcus* sp. MTM3W5.2**[a]	**+**	**-**	**-----**
Microbacterium sp. MTM3Y7	+	+	30
Agromyces sp. BEM3Y1	+	+	20
Gordonia sp. BDHXW1B	+	+	27
Gordonia sp. WDM3O5	+	-	-----
Arthrobacter sp. MTM3W2	+	-	-----
Arthrobacter sp. CODEY4	+	-	-----
Arthrobacter sp. AM5-1	+	-	-----
Aeromicrobium sp. SCTEC3	+	-	-----
Cellulomonas sp. NPDEW2	+	-	-----
Methylobacterium sp. A1M3R4	+	-	-----
Alcaligenes faecalis	-	-	-----
Bacillus subtilis	+	-	-----
Citrobacter freundii	-	-	-----
Enterobacter aerogenes	-	-	-----
Escherichia coli T7	-	-	-----
Klebsiella pneumonia	-	-	-----
Micrococcus luteus	+	-	-----
Pseudomonas aeruginosa	-	-	-----
Proteus vulgaris	-	-	-----
Salmonella arizonae	-	-	-----
Salmonella typhi	-	-	-----
Serratia marcescens	-	-	-----
Shigella dysentheriae	-	-	-----
Shigella sonnei	-	-	-----
Staphylococcus aureus	+	-	-----
Staphylococcus saprophyticus	+	-	-----
Aspergillus niger	Fungus	-	-----
Candida albicans	Fungus	-	-----

a: The producer strain

Mass Spectrometry

Mass spectrometry analysis was performed by Amanda May and Shawn Compagna in University of Tennessee. Based on mass spec results, the inhibitory compound has a molecular weight of 911.5452 Da (Figure 24).

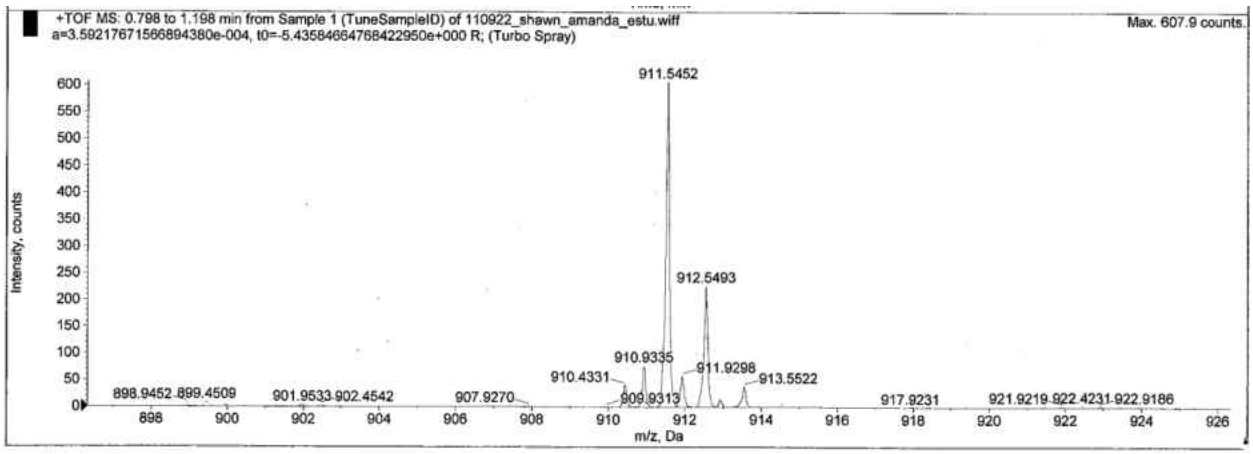


Figure 24: Mass spectrometry of the antimicrobial compound produced by strain MTM3W5.2 (analyzed by Amanda May and Shawn Compagna, UT).

CHAPTER 4

DISCUSSION

For years, the genus *Rhodococcus* has been used in industry for 2 main purposes – degradation of toxic hydrocarbons and synthesis of chemicals like acrylamide (Brandao *et al.* 2002). But after the description of the full genome of this interesting bacterium (McLeod *et al.* 2006), scientists have perceived the potential of *Rhodococcus* to produce antibiotics. In the past decade, production of antibiotics has significantly declined and there is a great need for the discovery of new antibiotics (Baltz 2007, Song 2008). So far, a great number of antibiotics has come from actinomycetes (Kitagawa and Tamura 2008). The rhodococci are closely related to several families (Corynebacteriaceae, Nocardiaciae) in the order Actinomyctales that have, in the past, yielded clinically useful natural products, including antibiotics (Zhu *et al.* 2011). Therefore, *Rhodococcus* is likely to be a candidate to look into for the production of novel antibiotics.

The first challenge of this research was to isolate a large enough number of *Rhodococcus* strains from local soils that can then be screened for antibiotic production. In the past, scientists have used different compounds for enrichment culturing depending on the organism they are trying to isolate. To isolate *Rhodococcus*, scientists in the past have used acetonitrile as the sole carbon and nitrogen source in their enrichment medium (Langdahl *et al.* 1996, Heald *et al.* 2001). To test the effectiveness of this method, 2 different enrichment methods were performed in this research: acetonitrile enrichment and M3 (heat shock) enrichment. A total of 83 isolates of various species were obtained from 7 different soils from East Tennessee. Out of these, 34 were obtained via acetonitrile enrichment and 49 via M3

enrichments. Acetonitrile enrichment yielded 24 rhodococci and M3 enrichment yielded 23. In other words, about 70% of the isolates obtained with acetonitrile enrichment were *Rhodococcus,* whereas only 47% of the isolates obtained with M3 enrichment were rhodococci. The results indicated that acetonitrile enrichment is very effective in the isolation of rhodococci and other actinomycetes that comprised 91% of the isolates, as well as eliminating the growth of other organisms, such as *Staphylococcus* and *Janthinobacterium.*

The second challenge of the research was to find a *Rhodococcus* isolate that is capable of producing an antimicrobial compound. Different methods of extracting compounds produced by microorganisms have been used in the past. Two different methods of extraction were used in this research to increase the chance of detecting an inhibitory compound. First, soil isolates were grown in broth medium containing a resin that binds hydrophobic compounds (because most antibiotics are found to be hydrophobic) and any compounds binding to the resin were extracted from it with methanol. Twenty-nine *Rhodococcus* strains were screened this way and only 2 strains were found to inconsistently produce an antimicrobial compound or produce a very weak inhibitory activity against indicator strains and were not studied further. In the second method of extraction, soil isolates were grown on solid media and were extracted directly from the agar using ethyl acetate. Nineteen *Rhodococcus* strains were screened using this method and one was found to produce a strong antimicrobial compound that consistently inhibited the growth of the indicator strain *Rhodococcus erythropolys* IGTS8. The MTM3W5.2 strain, which produced the antimicrobial compound, was also screened using the resin extraction method but did not produce the inhibitory compound with this method.

Generally, the production of antimicrobial compounds by organisms is very dependent on the environmental conditions in which the organism was grown. There were 2 main differences in the conditions under which an organism was grown when the 2 screening methods were used: (1) with the resin extraction cultures were grown in a broth medium, whereas with extractions from agar plates they are grown on a solid medium, and (2) with the resin extraction cultures were grown at 30°C, whereas with the agar plate extraction they were grown at a temperature of about 19°C. When MTM3W5.2 was grown on agar plates at 30°C, it was not able to produce the antimicrobial compound and when it was grown in a broth cultures with resin at 19°C it inconsistently produced a weak growth inhibition against *R. erythopolis* IGTS8. These results suggested that the production of this antimicrobial compound is not only temperature dependent but it also might be restricted to growth on a solid surface. It is suspected that the reason for strain MTM3W5.2 to be producing the antimicrobial compound on solid agar plates but not consistently in broth is because the natural environment from which this bacterium is isolated is soil, thus the agar mimics its natural environment more closely than the broth. Another researcher in this lab, Megan Carr, observed a similar occurrence with another strain of *Rhodococcus* isolated from soil. Also, secondary metabolites are produced by organisms to possibly give them an advantage over other organisms when competing for nutrients. MTM3W5.2 grows best at 30°C but it does not produce the inhibitory compound at this temperature regardless of the medium used for growth. It is possible that this strain starts producing the antimicrobial compound only when the temperature drops below 20°C to eliminate other organisms that grow well at the lower temperatures.

Strain MTM3W5.2 was isolated from the soil of a residence in Morristown and 16S rRNA analysis revealed it is about 90% similar to *Rhodococcus opacus*. When Gram stained, MTM3W5.2 appears as a combination of shorter and longer Gram positive rods. When grown on RM medium, MTM3W5.2 colonies initially have a white appearance and then gradually become more tan as they age. This strain seems to grow well at 30°C, but it was also slightly psychrotrophic because it was found to slowly grow (and produce the inhibitory compound) in temperatures as low as 4°C.

MTM3W5.2 was grown in different environments in order to determine the conditions in which it produces the largest amount of the antimicrobial compound. The largest amount of the antimicrobial compound was produced when strain MTM3W5.2 was grown between 15°C and 19°C for at least 1 week on a solid medium such as RM agar plates. A total of 10 scale-ups were performed, each consisting of the extraction of about 10 large agar plates. Extracts from these plates were first purified using Sephadex LH-20 column chromatography and were then run through HPLC. The antimicrobial compound was very hydrophobic. It was not able to dissolve in water and when run through HPLC it came out of the column at a methanol concentration of 95% – 96%. Based on HPLC analysis, there were 3 peaks containing the antimicrobial compound. One of the peaks, Peak #2, was the largest and appeared to contain most of the compound. Thus, it was speculated that Peak #2 represented the main antimicrobial compound and that Peaks #1 and #3 were degradation products of this compound or some completely different compounds (such as impurities). An amino acid analysis was also performed on the inhibitory compound found in Peak #2, but no amino acids were detected, indicating that this compound is probably not a peptide. After that, mass

spectrometry was performed on the inhibitory compound and its molecular weight was found to be 911.5452 Da. Based on an online chemical database, Chemspider, this weight closely matches the weight of 7 known compounds, but this does not eliminate the possibility of this compound being novel. The antibacterial compound is currently undergoing fragmentation analysis via Mass Spectrometry. Finally, the spectrum of activity of the inhibitory compound was tested against a broad spectrum of Gram-positive and Gram-negative organisms. The activity of the compound was found to be limited to other members of the *Rhodococcus* genus as well as some other closely related actinomycetes, such as *Microbacterium, Agromyces,* and *Gordonia.* An exciting discovery was that the compound had a strong antimicrobial activity against *Rhodococcus equi,* which is a known horse and human pathogen. Because the compound was inhibitory to all other rhodococci that it was tested against, there is hope that it also has antibacterial properties against the plant pathogen *Rhodococcus fascians.* The spectrum of activity of this interesting compound needs to be tested further in the future.

Currently, the antimicrobial compound is undergoing structural analysis via Nuclear Magnetic Resonance (NMR). Based on preliminary data that the antibacterial compound inhibits the growth of only other rhodococci and closely related bacteria, it was suspected that this compound might be a bacteriocin. However, after amino acid analysis, it was determined that this compound is most likely not a peptide, and thus, probably not a “classic” bacteriocin. The molecular weight and properties of this antimicrobial compound are different from all the previously described antibiotics from *Rhodococcus,* which led to the belief that it might be a novel antibiotic. Interestingly, strain MTM3W5.2 is resistant to this compound. Identifying the resistance genes in this strain could give a clue to the mechanism of action of this compound. In

the past, 3 antibiotics have been discovered form *Rhodococcus,* the biosynthetic genes for which have not been identified yet, so possible work in the future includes identifying the biosynthetic genes for this antimicrobial compound. In most cases, the genes encoding for the production of secondary metabolites are clustered on the chromosome (Banik and Brady 2010). One way to discover these genes is by using PCR to create a library of knockout mutants by inactivating different pieces of the chromosome and then screening the mutants for production of the antimicrobial compound. The resistance gene can be found using a slightly different approach, where pieces of the chromosome can be amplified using PCR and then transferred into a heterologous host, such as *R. erythropolis* IGTS8, which is sensitive to the compound produced by strain MTM3W5.2. After that, the clones can be screened for sensitivity to the antimicrobial compound until a clone is found that is no longer inhibited by the antimicrobial compound.

The traditional screening methods for the discovery of secondary metabolites have proven to be very tedious and based on culture-independent analyses of environmental samples, it is presumed that traditional methods might have missed the majority of metabolites that exist in nature (Banik and Brady 2010). These methods, however, can be extended by newer approaches that include genome mining (Zerikly and Challis 2009) and metagenomics (Banik and Brady 2010). Metagenomics involves extractions of environmental DNA (eDNA) from populations of bacteria from the environment. This eliminates the problems with having to find out the best conditions to culture these organisms as well as the problems associated with determining the right conditions for organisms to produce an antimicrobial compound, which

have limited the discovery of new compounds in the past. This approach also allows for the concurrent analysis of thousands of bacterial genomes.

Genome mining focuses on identifying “cryptic” gene clusters that encode biosynthetic systems that do not produce known metabolites (Zerikly and Challis 2009). Different approaches of genome mining have been used recently to discover new antimicrobial compounds. In some cases, bioinformatics is used to predict the module and domain organization of the non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) and then use this information to predict the substrates and consecutively the structure of the metabolite. Similarly, in a different approach, referred to as “genomisotopic” approach, the predicted substrate is labeled with an isotope of carbon or nitrogen and fed to the microorganism. The metabolites that contain the labeled substrate are then identified by NMR. Another method of genome mining involves inactivation of an important biosynthetic gene within the cryptic gene cluster. The metabolites produced by the knockout mutants are then compared to the ones produced by the wild-type and if any of them are absent in the mutant, they are presumed to be products of cryptic gene cluster and can be then characterized. Also, the entire biosynthetic gene can be cloned and expressed in a heterologous host. The metabolites produced by the clones are compared to the ones produced by the wild-type and the ones that are absent in the wild-type are presumed to be products of the cryptic biosynthetic pathway.

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