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Production of an Antibiotic-like Activity by *Streptomyces* sp. COUK1 under Different Growth

Conditions

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A thesis

presented to

the faculty of the Department of Health Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

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by

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August 2014

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Keywords: *Streptomyces*, antibiotics, natural products, bioactive compounds

ABSTRACT

Production of an Antibiotic-like Activity by *Streptomyces* sp. COUK1 under Different Growth

Conditions

by

Olaitan Akintunde

Streptomyces are known to produce a large variety of antibiotics and other bioactive

compounds with remarkable industrial importance. *Streptomyces* sp. COUK1 was found as a

contaminant on a plate in which *Rhodococcus erythropolis* was used as a test strain in a disk

diffusion assay and produced a zone of inhibition against the cultured *R. erythropolis*. The

identity of the contaminant was confirmed as *Streptomyces* through 16S rRNA sequencing. This

Streptomyces produces a strong inhibitory compound in different growth media. A culture

extract from inorganic salts starch agar was found to be very active; producing a large zone of

inhibition against several Gram positive and Gram negative test strains. The active molecules in

this extract have been detected via TLC and bioautography. The difference in the antibacterial

activity and chromatographic properties of extracts recovered from different growth media

suggests that this *Streptomyces* strain could produce more than one type of inhibitory

compound.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Antibiotic Resistance

Many new antibiotics were identified in the 20 years from the late 1940s to the late 1960s, most isolated from the actinomycetes. Some of these compounds became successful drugs and the pharmaceutical industry flourished. The rate of antibiotic discovery then declined sharply, so these productive years came to be called the Golden Age. Most bacterial pathogens were brought under control, but increased use of antibiotics also led to a dramatic rise in antibiotic resistance as these pathogenic bacteria evolved resistance. It became clear that antibiotic discovery was not over but rather an ongoing quest to find new treatments for old infections (Hopwood 2007).

According to the Centers for Disease Control (CDC) threat report of 2013 at least 2 million people are infected with antibiotic resistant bacteria each year in the United States and about 23,000 people die each year from these infections (CDC 2013).

Importance of Natural Products

Natural products are a substance of natural origin. They include (1) an entire organism (e.g., plant, animal or microorganism that has not been subject to processing or preservation, (2) part of an organism (e.g., leaves or flowers of a plant), (3) an extract of an organism or part of an organism and exudates, and (4) pure compounds (e.g., alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, terpenoids) isolated from plants, animals, or microorganisms (Sarker et al. 2006). However, in most cases the term natural product refers to secondary metabolites, i.e., small molecules (molecular weight <2000 Dalton) produced by an organism

(e.g. plant, animal, or microorganism) that are not strictly required for the survival of the organism (Sarker et al. 2006). Natural products can be from any terrestrial or marine source: plants (e.g., paclitaxel (Taxol) from *Taxus brevifolia*), animals (e.g., vitamins A and D from cod liver oil), or microorganisms (e.g., doxorubicin from *Streptomyces peucetius*) (Sarker et al. 2006).

Natural products have been used as therapeutic agents for thousands of years and a large number of modern drugs have been derived from natural sources, many based on their use in traditional medicine. In recent years there has been a renewed interest in natural products as a potential source for new medicines among academia as well as pharmaceutical companies (Sarker et al. 2006).

Approximately 40% of the modern drugs in use have been developed from natural products. More precisely 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives, and 60–80% of antibacterial and anticancer drugs were from natural origins (Sarker et al. 2006). In 2000 approximately 60% of all drugs in clinical trials for a multiplicity of cancers had natural origins. In 2001 eight of the 30 top-selling drugs (i.e. simvastatin, pravastatin, amoxycillin, clavulanic acid, azithromycin, ceftriaxone, cyclosporine, and paclitaxel) were natural products or their derivatives, and these 8 drugs together totaled US $16 billion in sales (Sarker et al. 2006).

Only a small fraction of the world's biodiversity has been explored for bioactivity to date. For example, only about 5 – 10% of at least 250,000 species of higher plants have been examined (Sarker et al. 2006).

Much less is known about marine organisms than other sources of natural products. However, research up to now has shown that they represent a valuable source for novel bioactive compounds. With the development of new molecular targets, there is an increasing demand for novel molecular diversity for screening and natural products will certainly play a crucial role in meeting this demand through the continued investigation of the world's biodiversity, much of which is yet to be explored (Sarker et al. 2006). With less than 1% of the microbial world currently known, advances in technologies for microbial cultivation and the extraction of nucleic acids from environmental samples from soil and marine habitats will offer access to an untapped reservoir of genetic and metabolic diversity (Sarker et al. 2006).

<u>Actinomycetes and Natural Products</u>

Actinomycetes are a taxonomic group of bacteria that are responsible for most antibiotics used today. They account for 45% of the 22,500 biologically active compounds that have been identified from microorganisms (Demain and Sanchez 2009).

Most antibiotics in clinical use are direct natural products or semisynthetic derivatives from actinomycetes or fungi. Many of those products, including erythromycin and derivatives, vancomycin and teicoplanin, cephalosporins, rifamicin, tetracyclines, and daptomycin, were discovered through whole-cell antibacterial screening procedures (Baltz 2007).

It has been estimated that the top 10 cm of global soil contains 10^{25}-10^{26} actinomycetes, but only about 10^7 have been screened for antibiotic production in the past 50 years, leaving plenty of room for further screening (Baltz 2007).

Genus *Streptomyces*

Taxonomic History

The genus *Streptomyces* was proposed by Waksman and Henricii (1943) and was placed

in the family *Streptomycetaceae* on the basis of morphology and cell wall properties.

Incorporation of molecular biology into classification changed early numerical systems using

phenotypic characters (Anderson and Wellington 2001). Stackebrandt et al. (1997) proposed a

classification system in which phylogenetically neighboring taxa at the genus level are clustered

into families, suborders (related organisms ranking between order and family), orders

(comprise of related families), subclasses (related organisms ranking between class and order),

and a class (comprised of related orders) using 16S rDNA phylogenetic analysis and the

presence of taxon-specific 16S rDNA signature nucleotides regardless of the phenotypic

properties used in describing the taxa in the past. For example, prior to this, *Streptomyces* and

Streptoverticillium were 2 separate genera (Anderson and Wellington 2001). Members of

Streptoverticillium were later linked to the *Streptomyces lavendulae* species group through

immunodiffusion assay (Anderson and Wellington 2001). Similarities between the 2 were

confirmed by physiologic tests (Anderson and Wellington 2001). It was later concluded from

16S and 23S rRNA comparison that *Streptoverticillium* could be treated as a synonym of

Streptomyces (Anderson and Wellington 2001).

Characteristics

Streptomyces are Gram positive aerobic bacteria belonging to the phylum

Actinobacteria (Stackebrandt et al. 1997). They have a DNA G+C content of 69-78 % (Anderson

and Wellington 2001). They are in many ways similar to filamentous fungi, growing as

branching hyphae that form a vegetative mycelium and disperse through spores formed on specialized reproductive structures called aerial hyphae, which emerge from the colony surface into the air (Hopwood 2007).

They are able to colonize the soil by growing as a vegetative hyphal mass that can differentiate into spores that assist in dispersal and persistence. The spores are a semidormant stage in the life cycle and can persist in soil for a long time (Kieser et al. 2000). The spores can withstand low nutrient and water availability unlike the mycelial stage that is sensitive to drought (Kieser et al. 2000).

Chemotaxonomic and phenotypic properties are employed in defining the genus *Streptomyces*. The major emphasis is now on 16S rRNA homologies in addition to cell wall analysis and fatty acid and lipid patterns. Detecting the presence of LL-diaminopimelic acid (LL-DAP) (a stereoisomer of diaminopimelic acid) as the diamino acid in the peptidoglycan is one of the quickest methods for identification to the genus level (Kieser et al. 2000). Many studies have attempted to use sequence data from variable regions of 16S rRNA to establish taxonomic structure within the genus, but the variation was regarded as too limited to help resolve problems of species differentiation (Witt and Stackebrandt 1990; Stackebrandt et al. 1991, 1992).

The genus *Streptomyces* has been subjected to several systematic studies over the past 30 years but the identification of unknown isolates is still difficult. The International *Streptomyces* Project in 1964 established a number of standard phenotypic criteria to help in species characterization. However, the criteria turned out to be too minimal, and the

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proliferation of species continued with no real attempt to compare species thoroughly with one another. Williams et al. (1983) came up with a numerical taxonomic system that allowed for comparison of many phenotypic traits concurrently. Species with similar phenotypic properties were clustered together and treated as a single species resulting in the reduction of the large number of described species (Williams et al. 1983).

Developmental Cycle

The life cycle of S*treptomyces* starts with the spores. When environmental conditions become favorable, a germ tube emerges from a spore, grows by tip extension and branch formation giving rise to a substrate/vegetative mycelium. The aerial hypha develops 2 or 3 days later through a process regulated by the *bld* genes (Kieser et al. 2000) (Figure 1).
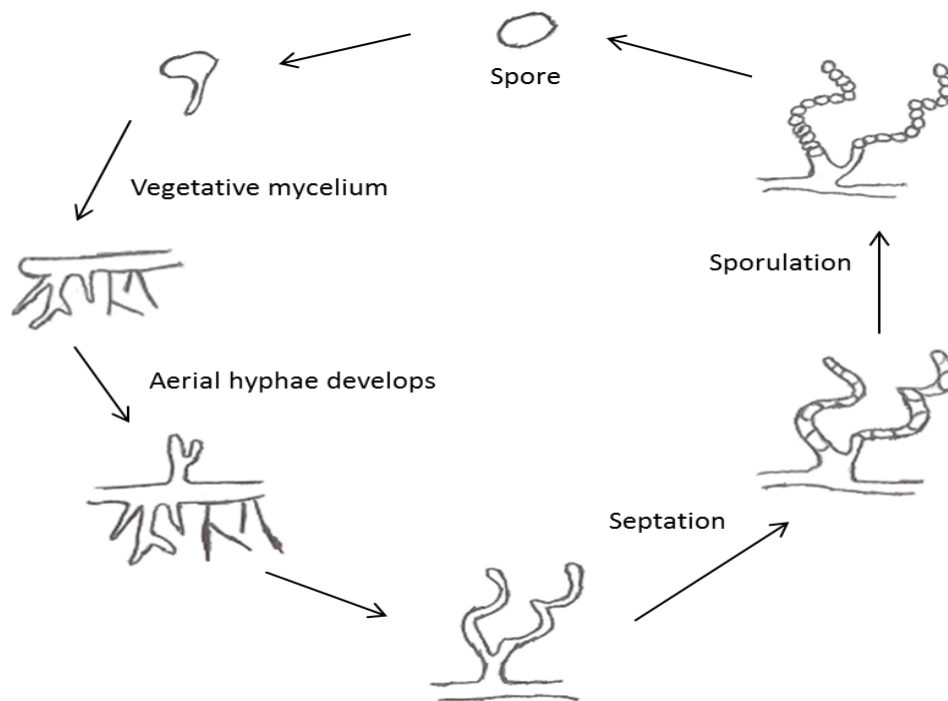
Figure 1. The life cycle of *Streptomyces*

The apical compartment of aerial hyphae forms a spiral syncytium that contains many tens of genomes. As the aerial hyphae become septated and mature into a spore bearing hyphae there is a change in shape as wall thickening occurs and gray spore pigment is deposited to generate desiccation-resistant spores. Sporulation septation is regulated by genes, *whi*A, B, G, H, I and J that are also required for expression of at least 1 (sigF) of 2 late regulatory genes, whiD and sigF. These late genes play an important role in spore wall thickening as well as spore pigmentation that is specified by whiE gene cluster (Kieser et al. 2000) (Figure 1).

Industrial Importance of *Streptomyces*

Streptomycetes synthesize an amazing variety of chemically distinct inhibitors of many different cellular processes. These include antibiotics, fungicides, modulators of the immune response, and effectors of plant growth (Hopwood 2007).

Actinomycetes produce about two-thirds of the known antibiotics and among them 80% are made by members of the genus *Streptomyces,* with other genera trailing numerically. Actinomycetes also account for 60% of secondary metabolites with biological activities other than antimicrobial, and again *Streptomyces* species account for 80% of these (Kieser et al. 2000). Recent evaluations of *Streptomycetes* as potential antagonists of soil borne pathogens have focused on their ability to produce natural antifungal metabolites. *Streptomyces rochei* and *Streptomyces rimosus* from the chickpea rhizosphere were strong antagonists of *Fusarium oxysporum* f. sp. *ciceri* (Kieser et al. 2000).

Despite extensive screening of soil samples, only a small fraction of *Streptomyces* taxa have been discovered and there is strong circumstantial evidence that the discovery of

previously unknown natural products occurs when novel organisms are examined in either established or new pharmacological screening programs (Takahashi and Omura 2003). Nine novel compounds were discovered from actinomycetes strains isolated using unique approaches such as screening soil samples at different depths and from different habitats as well as selective isolation (antibiotic selection) (Takahashi and Omura 2003).

<u>Genome</u>

The National Center for Biotechnology Information (NCBI) genome database lists 56 completed genomes among greater than 50 *Streptomyces* species. For example, *Streptomyces coelicolor* A(3) 2 (Bentley et al. 2002), which is the most widely used laboratory strain (Hopwood 2007), *Streptomyces avermitilis* (Omura et al. 2001; Ikeda et al. 2003), and *Streptomyces griseus* IFO 13350 (Ohnishi et al. 2008). The 8-10 Megabasepair(Mb) linear chromosomes comprise more than 7000 genes about 45% of which are common to the 3 genomes: these are mostly confined to a 6.4-Mb central segment (Ventura et al. 2007). The chromosome and that of some other closely related bacteria represent a novel class of linear replicon capped by terminal proteins covalently bound at the 5' ends (Chaconas and Chen 2005). When the complete genome sequence of *S. coelicolor* was published (Bentley et al. 2002), it was the largest known genome at 8,667, 507 bp carrying 7,825 predicted genes that include more than 20 clusters coding for known or predicted secondary metabolites. It still remains one of the largest bacterial chromosomes and complete genome sequences from other members of the *Streptomyces* genus are also large (Ikeda et al. 2003; Ohnishi et al. 2008).

The *S. coelicolor* genome encodes 819 predicted secreted proteins, including 60 proteases, 13 chitinases/chitosanases, 8 cellulases/endoglucanases, 3 amylases, and 2 pectate

lyases (Bentley et al. 2002). Extracellular proteins from diverse *Streptomycetes* hydrolyze and modify many different high and low molecular weight compounds or have novel function (Schrempf 2007).Comparison of 3 fully sequenced *Streptomyces* genomes revealed a core genome sequence for all 3 *Streptomyces* as well as genes specific to *S. griseus* including gene clusters for secondary metabolite production (Ohnishi et al. 2008).

Similar to other actinomycetes strains, *S. griseus* has many gene clusters that contain a putative polyketide synthetase (PKS), nonribosomal peptide synthetase (NRPS), and PKS-NRPS hybrid genes (Ohinishi et al. 2008). PKSs and NPRSs participate in the synthesis of diverse secondary metabolites by carrying out oligomerization of small building blocks into complex structures. NRPSs use amino or hydroxy acids as building blocks, catalyzing the formation of amide or ester bonds while PKSs generate polyketide chains through the oligomerization of small carboxylic acids (Donadio et al. 2007).

Genes for the synthesis of secondary metabolites probably emerged hundreds of million years ago (Baltz 2006). When natural product chemists analyze the genomes of *Streptomycetes* and closely related organisms, they usually find 20-30 or more gene sets for secondary metabolism, of which perhaps 30% are for antibiotic synthesis (Bentley et al. 2002; Ikeda et al. 2003).

<u>Secondary Metabolites from *Streptomyces*</u>

At least 7000 different secondary metabolites have been discovered in *Streptomyces* isolates (Berdy 2005). These are small molecules, usually between 100 – 3000 Daltons, that are

biologically active outside the producer cell, many being antibiotics that inhibit enzymes and

cellular processes (Chater et al. 2010).

Antibiotics

Some known antimicrobial compounds produced by *Streptomyces spp*. are listed in
Table 1.

Table 1. Bioactivity of some secondary metabolites of industrial importance from *Streptomyces*
(Kieser et al. 2000; Barrios-Gonzalez et al. 2003).

Antimicrobial compounds	Activity	Producer
Cephamycin	Antibacterial	*Streptomyces clavuligerus*
Chloramphenicol	Antibacterial	*Streptomyces venezuelae*
Kanamycin	Antibacterial	*Streptomyces kanamyceticus*
Tetracycline	Antibacterial	*Streptomyces aureofaciens*
Spectinomycin	Antibacterial	*Streptomyces spectablis*
Streptomycin	Antibacterial	*Streptomyces griseus*
Clavulanic acid	Antibacterial	*Streptomyces clavuligerus*
Monensin	Antibacterial/Anticoccidial	*Streptomyces cinnamonensis*
Amphotericin	Antifungal	*Streptomyces nodosus*
Aureofacin	Antifungal	*Streptomyces aureofaciens*
Candicidin	Antifungal	*Streptomyces griseus*
Nystatin	Antifungal	*Streptomyces nourse, Streptomyces aureus*
Oligomycin	Antifungal	*Streptomyces diastachromogenes*
Actinomycin D	Antibacterial/Antitumor	*Streptomyces antibioticus, Streptomyces parvulus*
Mytomycin C	Antibacterial/Antitumor	*Streptomyces lavendulae*

Streptomycin, the first aminoglycoside antibiotic, was discovered in the laboratory of

S.A. Waksman more than 60 years ago (Waksman 1953). This antibiotic cured many people of

tuberculosis and is produced industrially by *S. griseus*. Streptomycin belongs to the glucosides

in which a diguanido-group is linked to a nitrogen-containing disaccharide-like compound. It

was found to be soluble in water and insoluble in organic solvents, such as ether, chloroform, and acetone. Upon hydrolysis it splits into streptidine and streptobiosamine (Waksman 1953).

Siderophores

Most bacteria acquire iron through secreted siderophores (nonribosomally synthesized small peptides with an extremely high affinity for iron (III)). Unusually, it has emerged from genome mining that it is common for *Streptomycetes* to produce more than one type of siderophore. *Streptomyces coelicolor* produces coelichelin and desferrioxamine while *S. tandae* produces desferrioxamine and enterobactin (Challis and Hopwood 2003).

Spore Pigments

Most *Streptomycetes* have pigmented spores whose color has been used as a taxonomic characteristic. Two chemically different types of spore pigments have been described. One common type which is responsible for a range of spore colors is synthesized by a type II polyketide synthetic route, resulting in polycyclic aromatic molecules (Chater et al. 2010). The other is synthesized by a type III polyketide synthetic route to generate a kind of melanin that seems to provide some UV protection (Chater et al. 2010).

Research Problem

Following the discovery and identification of strain COUK1, which was found by accident as a contaminant and identified via 16S rRNA sequencing, efforts have been made to isolate and analyze the antimicrobial compound(s) being produced by this strain. Thus this research tests 2 hypotheses;

1. *Streptomyces* strain COUK1 might be producing more than 1 type of inhibitory compound based on the difference in antibacterial activity of different culture extracts as well as chromatographic analysis of these extracts.

2. Competition with another bacterium during growth could drive production of antibiotics.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains

Streptomyces strain COUK1 was found as a contaminant on a plate in which

Rhodococcus erythropolis was used as a test strain in a disk diffusion assay. It was found to

show inhibitory activity against the cultured *R. erythropolis* strain IGTS8 (Lampson's collection)*.*

Bacterial test strains used in the disk diffusion assay include *Escherichia coli* (Department of

Health Sciences), *Micrococcus luteus* (Department of Health Sciences), *R. erythropolis* (strain

IGTS8), *Citrobacter freundii* (ATCC 8090), *Enterobacter aerogenes* (ATCC 13048), *Shigella*

dysenteriae (ATCC 13313), *Salmonella typhi* (ATCC 14028), *Klebsiella pneumoniae* (ATCC 13883),

Bacillus subtillis (ATCC 6051), *Staphylococcus saprophyticus* (ATCC 15305), *Staphylococcus*

aureus (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 10145), and *Shigella sonnei* (ATCC

29930).

Growth Media

Cultural characteristics were studied using nutrient agar, Mueller Hinton Agar as well as

the following International Streptomyces Project Media (ISP) (Shirling and Gottlieb 1966),

tryptone yeast extract broth (ISP1), yeast malt extract agar (ISP2), inorganic salts starch agar

(ISP4), glycerol asparagine agar (ISP5), peptone yeast extract iron agar (ISP6) and tyrosine agar

(ISP7). The colors of the aerial and vegetative mycelia were noted on these media. Production

of soluble pigments and melanin pigment was checked on ISP2, ISP6, and ISP7 plates.

Carbon/sugar use was studied using the following carbon sources; glucose, arabinose, sucrose, fructose, rhamnose, xylose, inositol, and mannitol.

The growth media used in antibiotic production studies included Mueller Hinton Agar, Rich Medium (RM), Tryptic Soy broth as well as Tryptone yeast extract broth (ISP1) and ISP4. Cultures COUK1 on other ISP media; ISP2, ISP5, ISP6, and ISP7 were also screened for possible antibiotic production.

ISP media, Trace salt solution, Pridham and Gottlieb trace salt solution as well as media used in carbon use studies were prepared as described by Shirling and Gottlieb (1966).

The components/ingredients of all media used are listed below.

Rich Medium (RM) agar

1) Distilled H_2O 500 milliliters(ml)
2) Dextrose   5.0 grams(g)
3) Nutrient Broth   4.0 g
4) Yeast Extract   0.25 g
5) Bacto Agar   7.5 g

Ingredients were mixed together, boiled, and then autoclaved.

Muller Hinton (MH) agar

1) Distilled H_2O 500 mL
2) Mueller-Hinton Broth   11.0 g
3) Bacto agar   7.5 g

The mixture was sterilized by autoclaving

Nutrient Agar

1) Nutrient agar powder   11.5 g
2) Distilled H_2O 500 ml

The mixture was autoclaved

Tryptic Soy Broth

1)	Tryptic soy powder	7.5 ml
2)	Distilled H_2O	250 ml

The mixture was sterilized by autoclaving

Trace Salts Solution

1)	$FeSO_4. 7H_2O$	0.1 g
2)	$MnCl_2.4H_2O$	0.1 g
3)	$ZnSO_4.7H_2O$	0.1 g
4)	Distilled H_2O	100 ml

The pH of the solution was adjusted to 7.0 with 1M KOH. It was then autoclaved and stored at room temperature.

Tryptone Yeast Extract Broth

1)	Tryptone	1.25 g
2)	Yeast Extract	0.75 g
3)	Distilled H_2O	250 ml

Ingredients were mixed together and pH adjusted to 7.0 - 7.2 before autoclaving

Yeast Extract Malt Extract Agar

1)	Yeast Extract	2.0 g
2)	Malt Extract	5.0 g
3)	Dextrose	2.0 g
4)	Distilled H_2O	500 ml

Ingredients were mixed together and pH adjusted to 7.0-7.3 agar was added in the following amount

5)	Bacto agar	10.0 g

It was then sterilized via autoclaving.

Inorganic Salts Starch Agar

Solution 1:

1)	Difco soluble starch	5.0 g
2)	Distilled H_2O	250 ml

Solution 2:

1)	K_2HPO_4	0.5 g

2)	MgSO$_4$.7H$_2$O	0.5 g
3)	NaCl	0.5 g
4)	(NH$_4$)$_2$SO$_4$	1.0 g
5)	CaCO$_3$	1.0 g
6)	Distilled H$_2$O	250 ml
7)	Trace salts solution	0.5 ml

The pH of this solution was adjusted to 7.0-7.4.

Solution 1 and 2 were mixed together and then bacto agar was added as follows

8)	Bacto agar	10 g

The mixture was then autoclaved.

Inorganic Salts Starch Broth

This medium was prepared just like Inorganic salts starch agar (recipe described above) but bacto agar was excluded.

Glycerol Asparagine Agar

1)	L-asparagine	0.5 g
2)	Glycerol	4.0 ml
3)	K$_2$HPO$_4$	0.5 g
4)	Distilled H$_2$O	500 ml
5)	Trace salts solution	0.5 ml

pH of this mixture was adjusted to 7. 0-7. 4, and agar was added in the following amount

6)	Bacto Agar	10.0g

It was then autoclaved

Peptone Yeast Extract Iron Agar

1)	Bacto-Peptone Iron Agar, dehydrated (Difco)	18.0 g
2)	Bacto-Yeast Extract (Difco)	0.5 g
3)	Distilled H$_2$O	500 ml

Ingredients were mixed together and pH was adjusted to 7.0-7.2 before autoclaving.

Tyrosine Agar

1)	Glycerol	6.0 ml
2)	L-tyrosine (Difco)	0.25 g
3)	L- asparagine (Difco)	0.25 g
4)	K$_2$HPO$_4$	0.25 g

5) MgSO$_4$.7H$_2$O	0.25 g
6) NaCl	0.25 g
7) FeSO$_4$. 7H$_2$O	0.005 g
8) Distilled H$_2$O	500 ml
9) Trace salts solution	0.5 ml

Ingredients were mixed together and the pH was adjusted to 7.2-7.4, bacto agar was then added in the following amount

10) Bacto-Agar	10.0 g

Medium was autoclaved.

Pridham and Gottlieb Trace Salts

1) CuSO$_4$.5H$_2$O	0.64 g
2) FeSO$_4$. 7H$_2$O	0.11 g
3) MnCl$_2$. 4H$_2$O	0.79 g
4) ZnSO$_4$.7H$_2$O	0.15 g
5) Distilled H$_2$O	100.0 ml

Solution was stored at 4^{0}C until required

Basal Mineral Salts Agar

1) (NH$_4$)$_2$SO$_4$	1.32 g
2) KH$_2$PO$_4$ anhydrous	1.19 g
3) K$_2$HPO$_4$.3H$_2$O	2.83 g
4) MgSO$_4$.7H$_2$O	0.5 g
5) Pridham and Gottlieb trace salts	0.5 ml
6) Distilled H$_2$O	500 ml

Ingredients were mixed together and the pH was adjusted to 6.8-7.0, agar was added in the following amount

7) Agar (Difco)	7.5 g

Mixture was autoclaved.

Carbon Use Media

A 10% solution of 8 different sugars (glucose, arabinose, sucrose, fructose, rhamnose,

xylose, inositol, and mannitol) was made by dissolving 10 g of each sugar in 100 ml of distilled

water. The solution was then autoclaved. Freshly made sterile Basal mineral salts agar was

allowed to cool to about 50^{0}C, then 10 ml of a 10% sterile sugar solution was added to 100ml of

basal salts mineral agar to give a final concentration of 1%. The mixture was stirred and then poured into sterile petri dishes.

After autoclaving, the media bottle or flask was placed in a 50°C water bath until cool enough to hold. Agar plates were poured into sterile plastic petri dishes. The poured plates were left to solidify and dry at room temperature overnight. They were then stored at 4°C until ready for use. Broths were stored at room temperature or at 4^{0}C.

Identification of Strain COUK1

Following the discovery of a contaminant (designated strain COUK1) with inhibitory activity, one of the contaminant colonies was isolated and streaked out on Mueller Hinton Agar (medium on which contaminant was originally found). This was repeated until a pure culture was obtained. A colony from the pure culture was Gram stained to determine the Gram reaction as well as the cell morphology.

16S rRNA Analysis

To identify the unknown isolate, the 16S rRNA gene was amplified through the Polymerase Chain Reaction (PCR) and sent to University of Tennessee for DNA sequencing. A single colony from a 24 hours old culture was picked up with a sterile loop and dispersed in 10 µl of sterile water. The resulting cell suspension was used as a DNA template. The template and other reagents were mixed in a PCR tube as shown below

1) Double Distilled H2O   22.0 microliters(µl)

2) 10x Buffer (Promega, Go taq flex)   10.0 µl

3) 25 mM $MgCl_2$ (Promega)   3.0 µl

4) 10 mM Deoxynucleotide Triphosphate Mix (dA, dT, dG, dC)   1.0 μl

5) 10x Enhancer (Eppendorf)   10.0 μl

6) 20 μM Forward primer 63F   1.25 μl

7) 20 μM Reverse primer 1387R   1.25 μl

8) DNA template (single bacterial colony)   1.0 μl

9) Taq polymerase (Promega, Go taq flex)   0.5 μl

The sequence of the forward primer, 63f, used in amplification of the 16S rRNA gene 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' as according to Marchesi et al. (1998). The reaction mixture totaling 50 μl was placed in a thermocycler and was subjected 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 were repeated 29 times

6) 72° C for 5 minutes

After the cycling was completed, the amplification of the 16S rRNA gene was checked for size and concentration by subjecting the PCR reaction mix to agarose gel electrophoresis.

The PCR product was then cleaned using GeneClean Turbo PCR cleaning kit and then sent to the

University of Tennessee sequencing center to obtain DNA sequences using 63F and 1387R as

primers. The program Chromas was used to select the best segment (based on quality) of the

sequence. The sequence was then put into the Michigan State online Ribosomal Database

Project (RDP) (http://rdp.cme.msu.edu) for comparison and identification at the genus level.

The database compares the query sequence to previously sequenced bacterial isolates based

on similarity of the 16S rRNA gene sequence and will provide 20 most closely related organisms

to the unknown 16S rRNA gene.

Phylogenetic Analysis

The 16S rRNA sequences of the 20 closely related species that came up following a

sequence similarity search using the Michigan State RNA database were pulled from the

GenBank database and aligned with that of strain COUK1 using Molecular Evolutionary Genetic

Analysis (MEGA) software, version 5.2.2 (Tamura et al. 2011). A neighbor-joining tree was

employed to show phylogenic relationships between strains.

Preparation of Spore Suspension

All growth media used in antibiotic production, cultural characteristics as well as carbon

use studies were inoculated with spore suspensions. ISP4 broth was inoculated with a 2 cm by 2

cm agar piece cut from a 2 days old COUK1 MH plate culture (which was inoculated with spore

suspension).

To make a spore suspension, 10 ml of sterile distilled water was placed on the surface of

a 4 days old plate (Mueller Hinton agar) culture of COUK1. A sterile scalpel was used to scrape

30

cells off the agar surface suspending the cells in water. The plate was tilted slightly and a sterile

syringe was used to suck up the resulting cell suspension from the plate. The suspension was

then dispensed into a sterile test tube and the test tube was capped. Sterile nonadsorbent

cotton wool was inserted into another sterile syringe. The cell suspension in the test tube was

then poured into the syringe and filtered through the nonadsorbent cotton wool. The filtrate

was collected in micro-centrifuge tubes that were centrifuged for about 30 minutes at 10,000

revolutions per minute to pellet the spores. The supernatant was removed using a sterile glass

Pasteur pipette. The pelleted spores was then resuspended in 20% or 40% glycerol and then

stored at -20^0 C.

Preparation of Seed Cultures

Seed cultures of test organisms used in disk diffusion assays or seed culture of *R.

erythropolis* used in cocultivation were prepared by picking up a single colony from a plate with

a sterile loop and then dispersing the cells into 2.5 ml of sterile MH broth or tryptone yeast

extract broth (for cocultivation) in a test tube (Barber 2010). The cultures were then incubated

at 37^0 C for 24 hours. The seed cultures for *Rhodococcus erythropolis* were incubated at 30^0 C

for 48 hours.

Cocultivation

It's been shown that coculturing microorganisms could induce competition or

antagonism which could bring about the accumulation or increased production of new

secondary metabolites that are not present in the pure cultures of the producing strain (Ola et

al. 2013; Marmann et al. 2014).

To determine the effect of cocultivation on strain COUK1; 250 ml of Tryptone yeast extract broth was inoculated with 150 μl of a *R. erythropolis* seed culture and was incubated for 18-24 hours or until the optical density (OD) of the culture was 0.2-0.3. The culture was checked for purity by streaking, and then 2 or 3 pieces of agar (2 cm by 2 cm each) cut from a 2-days old COUK1 culture plate were added to the broth culture. This was incubated for about 1 to 2 weeks or until growth of the *Streptomyces* was confirmed by a color change in the broth and streaking. The broth culture was then extracted; the extract was dried and then resuspended in water or methanol for sensitivity testing. The zone of inhibition of extracts from cocultures and pure cultures were compared.

Extraction and Recovery of Antimicrobial Compound(S)

Broth Extraction

A 250 ml volume of tryptone yeast extract broth, tryptic soy broth, or inorganic salts starch broth was inoculated with 150 μl of spore suspension and then incubated at 30^0C with agitation at 200 revolutions/minute for 4 days. The culture was then put in Oakridge tubes and spun for 15 minutes at 10,000 rev/min. The spent broth was recovered by decanting it into an Erlenmeyer flask. A volume of 30 ml of methanol was added to the cell pellet, and the mixture was vortexed before centrifugation for another 15 minutes. The methanol extract was collected in a beaker and another 30 ml of methanol was added to the cell pellet. This was spun for another 15 minutes and the methanol extract was added to the one already in the beaker. The pooled methanol extracts were dried in a chemical hood or spun to dryness in a vacuum evaporator (Figure 2)
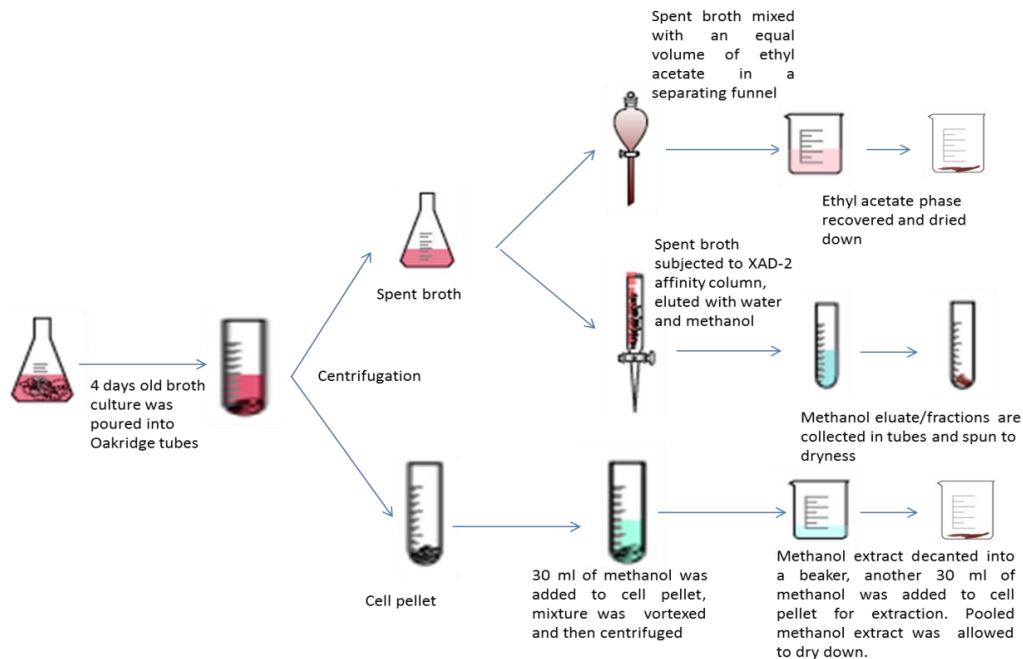
Figure 2. Extraction from broth culture

The spent broth recovered was mixed with an equal volume of ethyl acetate in a separating funnel which was shaken vigorously every 5 minutes for about 30 minutes or the spent broth was mixed with an equal volume of ethyl acetate in an Erlenmeyer flask placed on a rotatory shaker for about an hour. The organic/ethyl acetate phase was then collected in a beaker and allowed to dry under a chemical hood (Figure 2).

Agar Extraction

Small Scale

Sterile plates were inoculated by spreading out 50 µl of spore suspension on the agar surface using a sterile glass spreader. Plates were incubated at 30^0C for 1 week. One week old plate cultures were chopped into pieces/chunks and then soaked in 50 ml of methanol in a beaker and then covered with parafilm for 24 hours and was shaken or stirred at random times. The methanol extract was decanted into a separate beaker and agar chunks were soaked in

another 50 ml of methanol for another 24 hours. The methanol extract was then pooled with the previous methanol extract in the beaker. The pooled extracts were allowed to dry under a chemical hood (Figure 3).

Dried extracts were resuspended in 2 ml of methanol or water and stored at 4^0 C for sensitivity testing.

1. One week old plate culture was chopped into chunks

2. Agar chunks are soaked in 50ml of methanol for 24 hours

3. Methanol extract was decanted into a separate beaker. Step 2 was repeated and methanol extract was decanted into beaker containing previous extract

4. pooled methanol extract is allowed to dry down
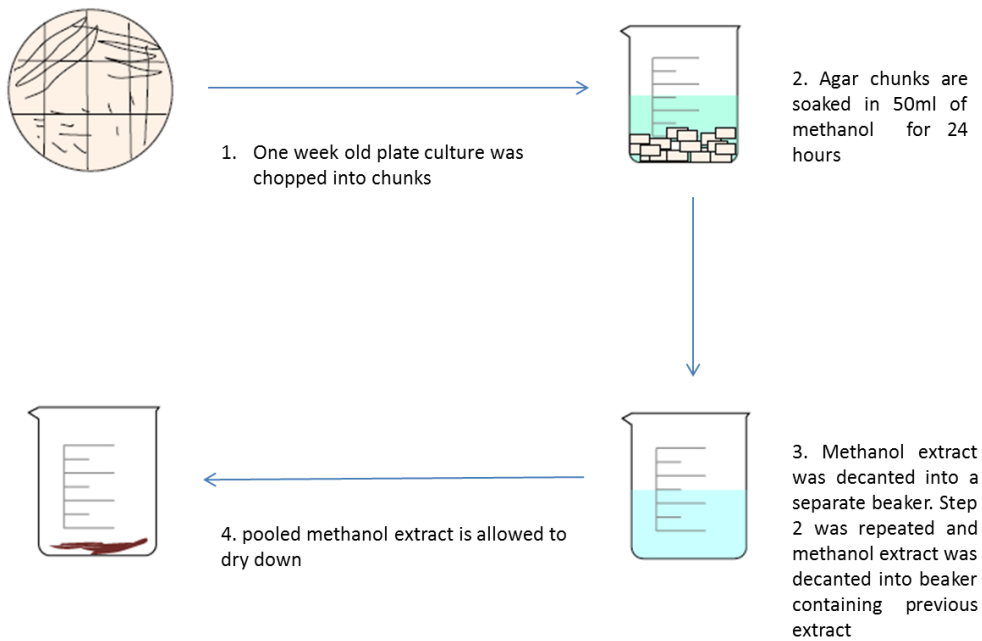
Figure 3. Extraction from agar/solid culture (small scale)

Large Scale

After the detection of an inhibitory compound in Inorganic salts starch agar, a large scale culture was initiated to maximize bacterial production. A volume of 100 µl of spore suspension was used to inoculate a 150 mm Inorganic salts starch agar plates. Twenty of these plates were inoculated and incubated at 30^0 C for a week. Each plate was then chopped into pieces and soaked in 100 ml of methanol for 24 hours. The methanol extracts were recovered

in separate beakers and the agar chunks were soaked in methanol for another 24 hours. The methanol extracts from all 20 plates were pooled together and allowed to dry down under a chemical hood. Extracts were resuspended in water, tested for activity, and stored at 4^0C.

Disk Diffusion Assay/Sensitivity Testing to Detect Antibacterial Activity

Inhibitory activity of all extracts and chromatographic fractions were checked against all test organisms (listed under bacterial strains). A volume of 50 µl of a crude extract/fraction was applied to a paper disc and was allowed to dry. Paper discs were made from Whatman Blotting Paper GB004 with the aid of a standard hole punch and sterilized by autoclaving. A seed culture of each test strain with turbidity adjusted to a 0.5 McFarland standard was swabbed on Mueller Hinton agar and then paper discs soaked with 50 µl of extracts/fractions were placed on the swabbed agar surface. The plates were incubated overnight at 37^0C for 24 hours. Plates with *R. erythropolis* were incubated at 30^0C for 48 hours. Discs showing a halo/clear zone of no growth indicate the possible presence of an inhibitory compound.

Silica Gel Column Chromatography

Size exclusion chromatography allows compounds to be separated based on size. Thirty-two grams of silica gel resins (mesh 70-230) was soaked in water to make a free flowing slurry that was then poured into a 1.8 cm (width) by 50 cm (height) column to a give a bed volume of about 100 ml. The water was slowly drained to obtain a well packed column. Crude extract from ISP4 large scale culture (same as large scale agar extraction described above) was dried and resuspended in 1 ml of water; this was then loaded gently on top of the column using a Pasteur's pipette and was allowed to drain into the column before water was applied for

elution. Fractions of 2.5 ml each were collected and tested for activity until the last active

fraction was collected.

XAD-2 Column Chromatography

In this type of affinity chromatography, aromatic and hydrophobic compounds of

interest are expected to bind to the resin that is then eluted using an organic solvent or a

suitable buffer. In this case some of the spent broth from broth cultures were reserved and

subjected to XAD-2 column chromatography (Figure 2) with the aim of recovering the inhibitory

compound contained in spent broths. Fifty grams of XAD-2 resin was soaked in water overnight

and packed in a column (3.5 cm (width) by 50 cm (height)) to make a bed volume of

approximately 80 ml. Spent broths showing activity were passed through the column and were

allowed to drain out of the column at a flow rate of 1 drop/second to allow for retention of

inhibitory compound(s). The eluate was saved. The column was then washed with about 200 ml

of distilled water; the water eluate was also saved. The column was then eluted with about 100

ml of methanol. Fractions of 25 ml each were collected. All saved eluate/fractions were tested

for inhibitory activity. Methanol fractions were dried in a vacuum evaporator and then

resuspended in 2 ml of methanol or water for sensitivity testing.

Thin Layer Chromatography (TLC)

TLC silica gel 60 F_{254} plates from Merck KGaA, Germany were employed in the

separation of crude extracts and chromatographic fractions. Extracts in varying amounts were

spotted on the baseline which is 1 cm from the bottom of a plate cut to a height of 15 cm and a

varying width (depending on the number of samples to be analyzed). This was then developed

using different solvent systems that allowed best separation of the extract. The plates were

removed from the solvent system when the solvent front reached about 1cm from the top of the plates. The plates were dried and then observed under UV light for bands or spots. Extracts from ISP4 agar or broth were developed on TLC plate using butanol: acetic acid (1:1), butanol: acetic acid: water (6:4:1), propanol: acetic acid: water (5:1:3) as these were the solvent systems that allowed the separation of these extracts. Extracts from other media were developed using chloroform: methanol: 25% NH_3 (42.5:7:0.5) (Johdo et al. 1991).

Bioautograghy

Following TLC bioautography was used to detect active compounds on TLC strips. TLC strips were laid on the surface of a Mueller Hinton agar plate inoculated with *M. luteus* for about 2-10 hrs at 4^0 C to allow compound(s) on the strip to leach from the TLC strip into the agar. The strips were removed and the plates were incubated at 37^0 C overnight.

High Pressure Liquid Chromatography (HPLC)

HPLC was employed in the purification of the inhibitory compound. Active fractions from silica gel columns were pooled, dried, resuspended in 0.5ml of water, filtered using a 0.45um filter, and loaded/injected into an HPLC set up with a Waters 7.8 mm x 300 mm Novapak HR C-18 hydrophobic column as the stationary phase and degassed distilled H_2O and methanol as the mobile phases or eluents. Due to the polar nature of the inhibitory compound, a 2% methanol program was used in which a linear gradient system is produced that would allow compounds contained in the injected sample to separate and elute as the methanol concentration increases from 2% to 100% in the mobile phase. The presence of compounds in the sample were detected with the aid of a UV light (254 nm). Ninety fractions were collected

and tested for inhibitory activity. Fractions containing inhibitory compound were compared to the chromatogram.

Ion Exchange Chromatography

Normal phase chromatography was carried out using Alltech's HEMA IEC BIO 1000 DEAU HPLC column as the stationary phase. This hydrophilic column allows the binding and separation of hydrophilic compounds. Active silica gel column fractions were pooled, dried, and resuspended in water. A volume of 0.5 ml of this was injected into the HPLC. A gradient elution was employed using 0.05 M ammonium acetate (A) and 0.5 M ammonium acetate (B), pH 5.0, that is, 100% of A for 10 minutes, 100% A to 100% B in 10 minutes and then 100% of B for 10 minutes with the flow rate at 1 ml/minute. Thirty fractions were collected and tested for activity.

CHAPTER 3

RESULTS AND DISCUSSION

Strain COUK1

Strain COUK1 appeared as a contaminant on a Mueller Hinton agar plate in the form of

tiny colonies producing zones of inhibition against a culture of *R. erythropolis,* which was used

as a test strain in a disk diffusion assay (Figure 4). The contaminant appeared while testing

chromatographic fractions of extracts from *Rhodococcus* Strain MTM3W5.2 against *R.*

erythropolis for inhibitory activity*.*

Figure 4. Contaminant (strain COUK1) discovered on a plate. Red arrow shows a tiny dark colony with a zone of inhibition against the indicator organism, *Rhodococcus erythropolis*.

Identification of Strain COUK1

At the moment this strain was found, it wasn't known as *Streptomyces*; therefore, a

colony was streaked on MH agar to produce isolated colonies. One of the resulting colonies was

streaked out again to ensure a pure culture; 16s rRNA sequencing was then carried out on one

of the isolated colonies.

The 16S rRNA gene amplified via PCR resulted in an approximately 1.3 kilobasepairs (kb)

fragment (Figure 5). The amplified DNA was purified and sequenced. About 1299 bp of the

sequence was put into an online database (http://rdp.cme.msu.edu) and compared to other

bacterial isolates. The sequencing result shows that COUK1 belongs to the phylum

Actinobacteria, the family *Streptomycetaceae* and the genus *Streptomyces* (Figure 6). Because

species identification cannot be determined by 16S rRNA data alone, phylogeny as well as

cultural and biochemical properties were employed to narrow down to a possible species.

Figure 5. PCR amplified 16S rRNA gene. Lane 1: Molecular weight marker i.e. λDNA cut with *HindIII*. Lane 2: amplified DNA fragment of ~1.3 kb from a single colony of COUK1.

Williams et al. (1983) designed a numerical taxonomic system based on phenotypic

traits in which *Streptomyces* species with similar traits were clustered around a particular

species (within the group) in a bid to reduce the large number of described *Streptomyces*

species because many of these species were found to be synonymous. For example,

Streptomyces flavoviridis and *Streptomyces glaucescens* were both treated as *S. glaucescens*

and assigned to cluster 28 or the *S. glaucescens* group.

domain Bacteria (20)
  phylum "Actinobacteria" (20)
    class Actinobacteria (20)
      subclass Actinobacteridae (20)
        order Actinomycetales (20)
          suborder Streptomycineae (20)
            family Streptomycetaceae (20)
              genus Streptomyces (20)

ID				Description
S000581546	not_calculated	1.000	1333	Streptomyces cinnamonensis; JCM 4019; CSSP390; AY999746
S000615209	not_calculated	1.000	1266	Streptomyces cinnamonensis; CBS 411.63; DQ227422
S000651899	not_calculated	1.000	1361	Streptomyces virginiae (T); NBRC 12827; AB184175
S000652431	not_calculated	1.000	1364	Streptomyces cinnamonensis (T); NBRC 15873; AB184707
S000652521	not_calculated	1.000	1358	Streptomyces virginiae; NBRC 3729; AB184797
S000768905	not_calculated	1.000	1433	Streptomyces sp. FH-2006; DQ989584
S000966566	not_calculated	1.000	1276	Streptomyces sp. MH18; EU182847
S000968484	not_calculated	1.000	1351	Streptomyces virginiae; XSD-128; EU285473
S001093257	not_calculated	1.000	1369	Streptomyces lavendulae subsp. lavendulae; NBRC 13709; AB184469
S001154779	not_calculated	1.000	1330	Streptomyces virginiae; HBUM174861; EU841575
S001224399	not_calculated	1.000	1369	uncultured Streptomyces sp.; 2517; FJ429560
S001224400	not_calculated	1.000	1369	uncultured Streptomyces sp.; 2537; FJ429561
S001351240	not_calculated	1.000	1351	Streptomyces lavendulae; cfcc3092; FJ792548
S001351247	not_calculated	1.000	1348	Streptomyces virginiae; cfcc3118; FJ792555
S001351248	not_calculated	1.000	1350	Streptomyces virginiae; cfcc3126; FJ792556
S001417494	not_calculated	1.000	1343	Streptomyces virginiae; cfcc3077; FJ883741
S001613983	not_calculated	1.000	1417	Streptomyces sp. SXY10; GU045527
S001745129	not_calculated	1.000	1382	Streptomyces flaveus; T111; GU084180
S002352538	not_calculated	1.000	1310	Streptomyces sp. zx-10-5; HQ611052
S002408794	not_calculated	1.000	1331	Streptomyces sp. MTQ9; HQ143606

Figure 6. 16S rRNA sequencing results confirmed the genus of the unknown isolate is *Streptomyces*

Similarly, some of the species listed in the 16S sequencing result (Figure 6) such as

Streptomyces virginiae and *Streptomyces lavendulae* have been described as members of the

Streptomyces lavendulae group or cluster 61 and may be treated as *S. lavendulae* (Williams et al. 1983).

Phylogenetic Tree

A neighbor-joining tree was employed to show phylogenetic relationships between strain COUK1 and other *Streptomyces* neighbor (Figure 7).
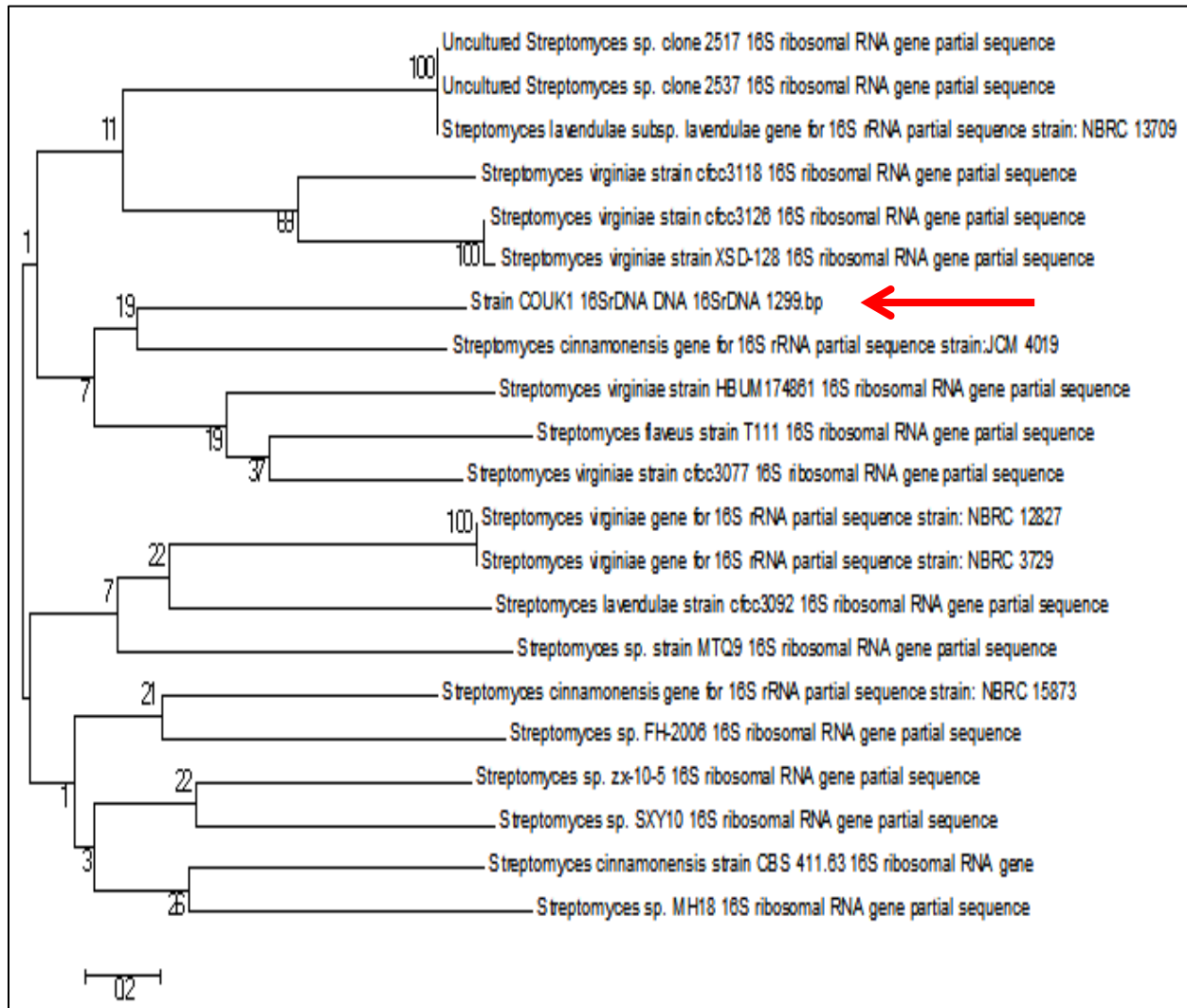

Figure 7. Neighbor joining tree based on 16S rRNA sequences showing phylogenetic relationship between strain COUK1 (red arrow) and other *Streptomyces* neighbors

The 16S gene sequence of strain COUK1 was found to pair up with (match) that of

Streptomyces cinnamonensis strain JCM 4019 (Figure 7). It's important to note that a partial

sequence of the 16S rRNA gene of strain of COUK1 was used and the closest matches were to

the partial sequences. Also, different *Streptomyces* species may have nearly identical 16S

sequences. The low bootstrap values (that is values < 50) observed at some nodes indicates

little support for branching patterns. These factors may affect the accuracy of the phylogeny.

COUK1 Phenotypic Characteristics

Strain COUK1 appears as Gram positive filaments in a standard Gram stain reaction

(Figure 8B). We suspected the contaminant was *Streptomyces* as they are known to form

branching filaments like fungi. This was later confirmed by 16S rRNA sequencing. Based on cell

morphology it may be classified as Retinaculum-Apertum i.e. *Streptomyces* whose

hyphae/filaments appear as open loops or hooks (Pridham et al. 1958). When cultured on MH

agar it produces a very dark pigmentation (Figure 8A). Other cultural characteristics such as

color of aerial and vegetative hyphae as well as pigmentation were observed on ISP media and
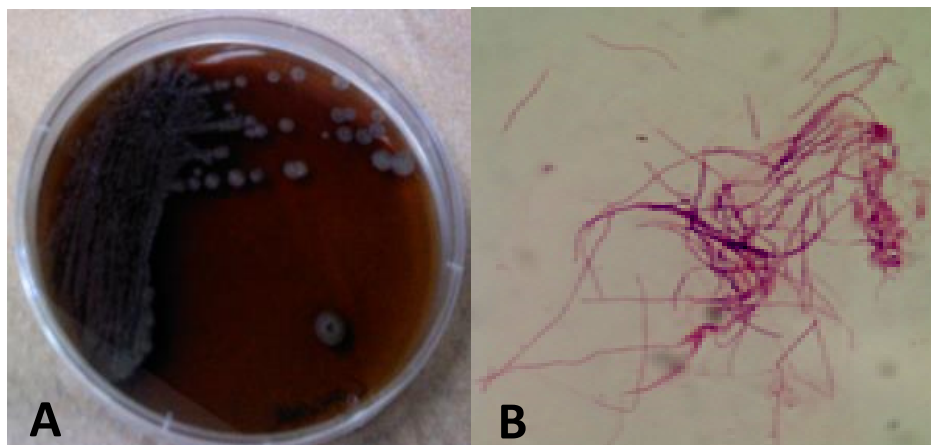
nutrient agar (Figure 9)

Figure 8. **(A)**; Colonies of strain COUK1 producing dark pigmentation on Mueller Hinton
Agar. **(B)**; Microscopic examination of Strain COUK1 appearing as gram positive filaments.

Cultural Characterization of Strain COUK1

Cultural characteristics of strain COUK1 were studied using the method described by

Shirling and Gottlieb (1966). Morphological and cultural properties were observed on Nutrient
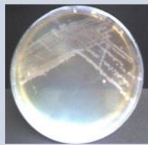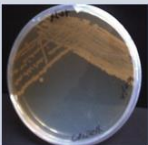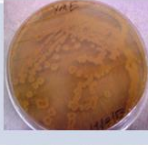
agar and ISP media (Figure 9).
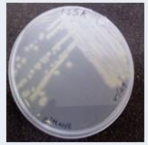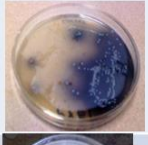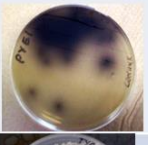
Media	Aerial mycelium		Vegetative mycelium		Soluble pigment
Nutrient agar	a		Brown		Yellowish Brown
Tryptone yeast extract broth (ISP1)	N.A.		N.A.		Dark Brown
Yeast malt extract agar (ISP2)	White/a		Brown		Yellowish brown
Inorganic Salt Starch Agar (ISP4)	White/a		White/a		None
Glycerol asparagine agar (ISP5)	White		White		None
Peptone-yeast extract iron agar (ISP6)	White		Black		Black
Tyrosine agar (ISP7)	a		White		Pink

Figure 9. Pigmentation of aerial and vegetative mycelium of strain COUK1 on different growth media. a indicates gray, N.A. indicates not applicable.

These properties were compared to those of already known *Streptomyces* species for

the purpose of identifying this isolate to the species level. Pigmentation on ISP media is one of

the main criteria used in identifying and classifying *Streptomyces*. The presence of black

pigmentation on peptone-yeast extract iron agar (ISP6) suggests the production of melanin by

strain COUK1. Melanins are a diverse group of pigments produced in living organisms. They are products of oxidative polymerization of phenolic substances and thus form a group of biopolymers (Plonka and Gabracka 2006). Types of melanins include eumelanins (black or brown), pheomelanins (yellow to red), and allomelanins (various colored dihydroxynaphthalene(DHN)-melanins) (Plonka and Gabracka 2006). The production of melanin has been linked to enzymes such as tyrosinases and polyketide synthetases (PKSs) that are associated with secondary metabolism and pigmentation in bacteria (Plonka and Gabracka 2006). Melanins are known to play a role in protecting an organism from UV radiation and could also serve as a virulence factor by protecting organisms from superoxide anions during phagocytosis. They can also function as electron carriers/acceptors making it possible to produce energy in a way similar to oxidative phosphorylation but under anaerobic conditions (Plonka and Gabracka 2006). *Streptomyces* species belonging to the *Streptomyces lavendulae* group or cluster 61 are known to produce melanin in peptone yeast extract iron agar (Williams et al. 1983). Pink pigmentation on Tyrosine agar (ISP7) was also observed.

The presence of these pigments also indicates secondary metabolism is active in this strain. Many pigments, like antibiotics, are produced by enzymes such as polyketide synthetases (PKSs) and nonribosomal peptide synthetases (NRPSs) (Bentley et al. 2002). However, more than one *Streptomyces* sp. might show similar pigmentation properties and production may vary with environmental factors, thus these properties may not be very reliable as a means for species identification.

Sugar/Carbon Use Pattern

The ability of strain COUK1 to use different carbohydrates for growth can be used to identify different species by culturing it on media containing different sugars. These include glucose, arabinose, sucrose, fructose, rhamnose, xylose, inositol, and mannitol. Growth or colony formation in the presence of a particular sugar indicates a positive reaction (Table 2). Sanchez-Marroquin (1958) observed the carbon use pattern of 5 strains of *Streptomyces lavendulae*, 2 strains of *Streptomyces virginiae,* and 3 strains of *Streptomyces cinnamonensis* (all of which belong to the *Streptomyces lavendulae* group), and the variation of sugars used among strains was noted. The carbon use pattern of these strains was compared to that of stain COUK1 (Table 2).

Table 2. Carbon use pattern of 3 species of the *S. lavendulae* group compared to that of strain COUK1.

Carbohydrate Source	Strain COUK1	S. cinnamonensis (3 strains)	S. virginiae (2 strains)	S. lavendulae (5 stains)
Negative control (No sugar)	-[a]			
Glucose	+[a]	+	+	+
Arabinose	-	-	-	-
Rhamnose	-	-	-	- to +/-
Xylose	+/-[a]	- to +	-	- to +/-
Fructose	-	- to +	-	- to +/-
Sucrose	+	+/- to +	- to +/-	- to +/-
Mannitol	+/-	- to +/-	- to +/-	- to +/-
Inositol	+/-	-	-	-

a: - indicates no growth, + indicates good growth, +/- indicates partial/poor growth. 'to' indicates variation among strains examined. Blank cells mean result was not published by source or experiment was not conducted (Sanchez-Marroquin 1958; Bergey and Holt 1977).

Based on its pattern of sugars used for growth, strain COUK1 appears most similar with strains of *S. cinnamonensis* except it partially uses inositol (Table 2). Bergey and Holt (1977) also observed the carbon use pattern for members of the *S. lavendulae* group and had a result similar to that of Sanchez-Marroquin (1958) (Table 2).

<center>Antibiotic Production Studies</center>

Antibiotic production was initially studied using MH agar, Rich Medium (RM) agar, and Tryptic soy broth in an attempt to find suitable media for growth and antibiotic production. Later we wanted to see if the ISP media primarily used in species classification would also be suitable for antibiotic production or have effect on expression with the hope that growing COUK1 on different types of media would induce the production of different compounds, possibly with different inhibitory activity. Thus, cultures grown on these media were extracted and the extracts tested for inhibitory activity.

Extract from ISP4 was found to show inhibitory activity against all 3 test strains, that is, *E. coli*, *M. luteus,* and *R. erythropolis* and it had a stronger inhibitory activity than extracts from other media as indicated by very wide zones of inhibition (Figure 10). This strong antibacterial activity suggests the possible ability of this strain (when grown on ISP4) to produce a different inhibitory compound or overexpress what it produces compared to other media and as a result the analysis of ISP4 extracts was prioritized. Extract from the broth culture, inorganic salts starch broth, also showed similar antibacterial activity (Figure 10). Initially agar cultures were extracted with ethyl acetate and methanol. But ethyl acetate extracts showed no activity (data not shown). Thus, all agar cultures were extracted with methanol.

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Media	Extracts and solvent Used:	Activity			TLC and solvent system used
Broth		*E. coli*	*M. luteus*	*R. erythropolis*	
Tryptone yeast extract broth (ISP1)	Spent broth				
	Ethyl-acetate extract of spent broth				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
	Methanol extract of cell pellet				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
	XAD fraction from spent broth				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
Tryptic soy broth	Spent broth				
	Ethyl-acetate extract of spent broth				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
	Ethyl-acetate extract of cell pellet				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
Inorganic salt starch broth (ISP4)	Spent broth				
	Methanol extract of cell pellet				Butanol: A. acid: Water (6:4:1)
Agar					
Rich Medium (RM) agar	Methanol agar extract				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
Mueller Hinton (MH) agar	Methanol agar extract				CHCL3 : CH3OH :25%NH3 (42.5:7:0.5)
Inorganic Salt Starch Agar (ISP4)	Methanol agar extract				Butanol: A. acid: Water (6:4:1)

Figure 10. Table showing different extraction methods, inhibitory activity of extracts from different media and TLC analysis of the extracts

For broth cultures, spent broths (liquid portion of broth cultures recovered via centrifugation) as well as extracts from spent broth and cell pellet were tested for activity. Also methanol extracts of cell pellets showed a wider spectrum of activity compared to the ethyl acetate extracts. Spent broth from tryptone yeast extract broth was found to show activity against *E. coli*, *M. luteus,* and *R. erythopolis*. However, ethyl acetate extract of the spent broth only showed activity against *M. luteus* (Figure 10). This suggests that ethyl acetate is leaving

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some inhibitory compound behind in the spent broth as different compounds will exhibit different polarity and would come out with an organic solvent of similar polarity in a solvent extraction procedure. This result indicates the possible presence of more than one inhibitory compound. Extracts from different media were also analyzed via Thin Layer Chromatography (TLC) to allow for separation of crude extracts into detectable spots.

TLC and Bioautography

Using TLC and bioautography, it may be possible to isolate and even purify antibiotic-like compounds based on the separation of visible spots on TLC plates. Crude extracts from COUK1 grown on Inorganic salts starch agar (ISP4) were separated by TLC and the spots on TLC strips were tested for inhibitory activity by laying the TLC strips on top of a Mueller Hinton agar plate inoculated with a test strain for about 10 hours to allow the compounds to leach into the agar from the strip. The TLC strips were removed and plates were incubated (Figure 11). Plates were observed for a clear zone of inhibition which represents the possible presence of inhibitory compounds. The Butanol: Acetic acid solvent systems moved only some of the active compound(s) away from the baseline (where the crude extract was spotted) leaving most of these compounds behind at the baseline (Figure 11).

The Propanol: Acetic acid: water solvent system moved all the active compound(s) away from the baseline but the band resolution or compound separation was very poor on the TLC strips when observed under UV light (see Figure 11).

Solvent system	Developed TLC Strip was cut into two and laid over plate inoculated with *M. luteus*
Butanol: Acetic Acid (1:1)	
Butanol: Acetic Acid: Water (6:4:1)	
Propanol: Acetic Acid: Water (5:1:3)	

Figure 11. Detection of active compound(s) in crude extracts from inorganic salts starch agar on TLC strips via Bioautography. Red arrows indicate the baseline where extract was spotted; blue arrows indicate other active spots (isolated compound(s)).

Purification of Inhibitory Compound from Cultures Grown on ISP4

Silica Gel Column Chromatography

Twenty plates grown with strain COUK1 were harvested and extracted. The pooled

extract was subjected to gel filtration through a silica gel column eluted with water due to the

hydrophilic nature of the compounds, as previous attempts to elute the column with

methanol:water solvent systems or propanol:water solvent systems were not very successful.

Individual fractions collected were tested for activity. Fractions containing inhibitory

compound(s) were obtained in column fractions 11 to 22. (Figure 12). These fractions were pooled together, spun to dryness, and resuspended in water for further purification by High Pressure Liquid Chromatography (HPLC).
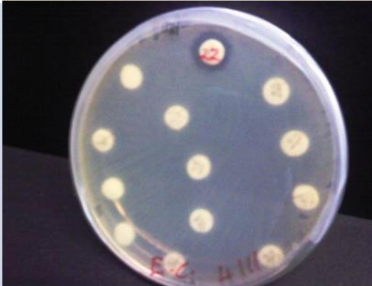
Test Organisms	Inhibitory activity of silica gel column fractions	
E. coli		
M. luteus		
R. erythropolis		

Figure 12. Inhibitory activity of silica gel column fractions

Silica gel column fractions were screened against 3 different indicator organisms with the hope of seeing a difference in activity among column fractions that would suggest the presence of different inhibitory compounds. Fractions 11-22 showed similar inhibitory activity against all 3 test organisms.

<u>High Pressure Liquid Chromatography</u>

Silica gel column fractions (11-22) showing activity were pooled, dried, and resuspended

in 1 ml of distilled water. This was injected into a C-18 hydrophobic column and eluted using a
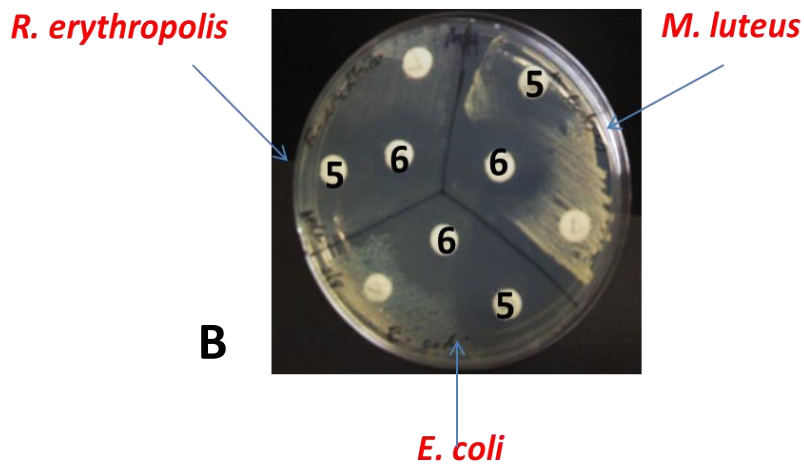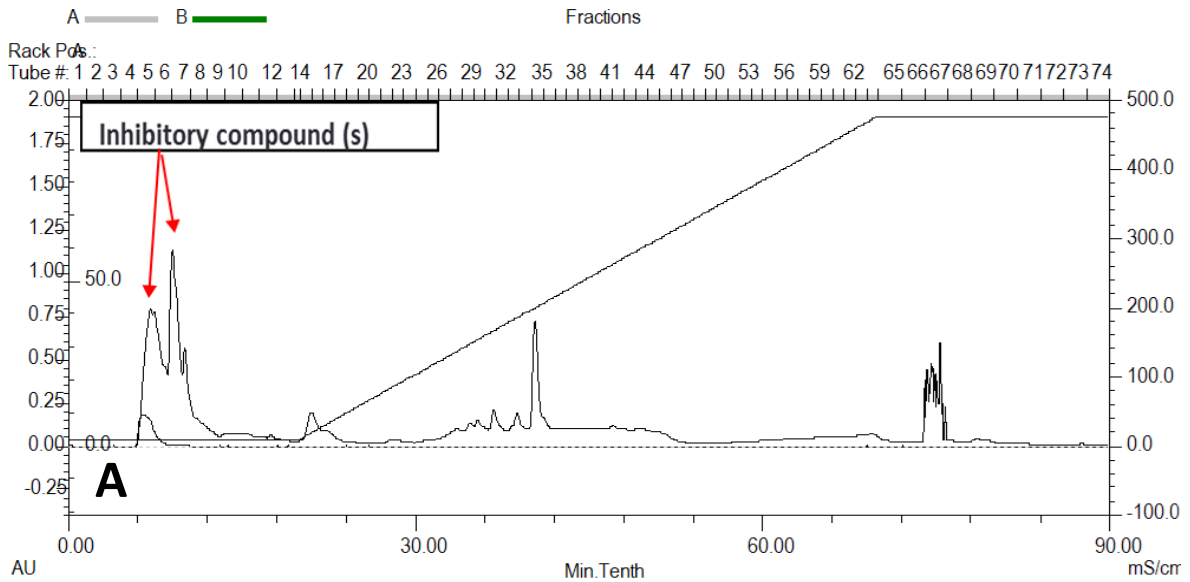
2% methanol program (Figure 13).

Figure 13. **(A)**; HPLC-C18 analysis of pooled active fractions from a silica gel column. Inhibitory compound(s) contained in fractions 5 and 6 corresponding to the two unseparated peaks Indicated by red arrows. **(B)**; Inhibitory activity of HPLC fractions 5 and 6

The compound(s) in the extract are expected to elute as methanol content increases from 2% to 100% in the mobile phase. Fractions were tested for activity against *M. luteus, E. coli,* and *R. erythropolis*. Active compound(s) came off early, with 2 unseparated peaks corresponding to fractions 5 and 6 (Figure 13).

The HPLC chromatogram (Figure 13) suggests that the inhibitory compound is hydrophilic in nature since it's not binding to the C-18 hydrophobic column.

Ion Exchange Chromatography (HPLC)

Due to the hydrophilic nature of the inhibitory compound(s), a normal phase chromatography was carried out using HEMA IEC BIO 1000 DEAU HPLC column. 0.05 M ammonium acetate and 0.5 M ammonium acetate (pH 5.0) was used for gradient elution. The 30 fractions collected were tested for activity. Inhibitory activity was found in fractions 6-9 and in fractions 27 and 28. Better peak separation and retention of the inhibitory compound was observed with ion exchange chromatography compared to HPLC-C18 analysis (Figure 14).

Ion exchange chromatography appeared to produce 2 separate peaks. The first peak (Peak 1) at fractions 6-9 showed inhibitory activity against all 3 indicator bacteria. The second peak (Peak 2) at fractions 27 and 28 showed inhibitory activity only against *M. luteus*. This suggests there are 2 different compounds (Figure 14).

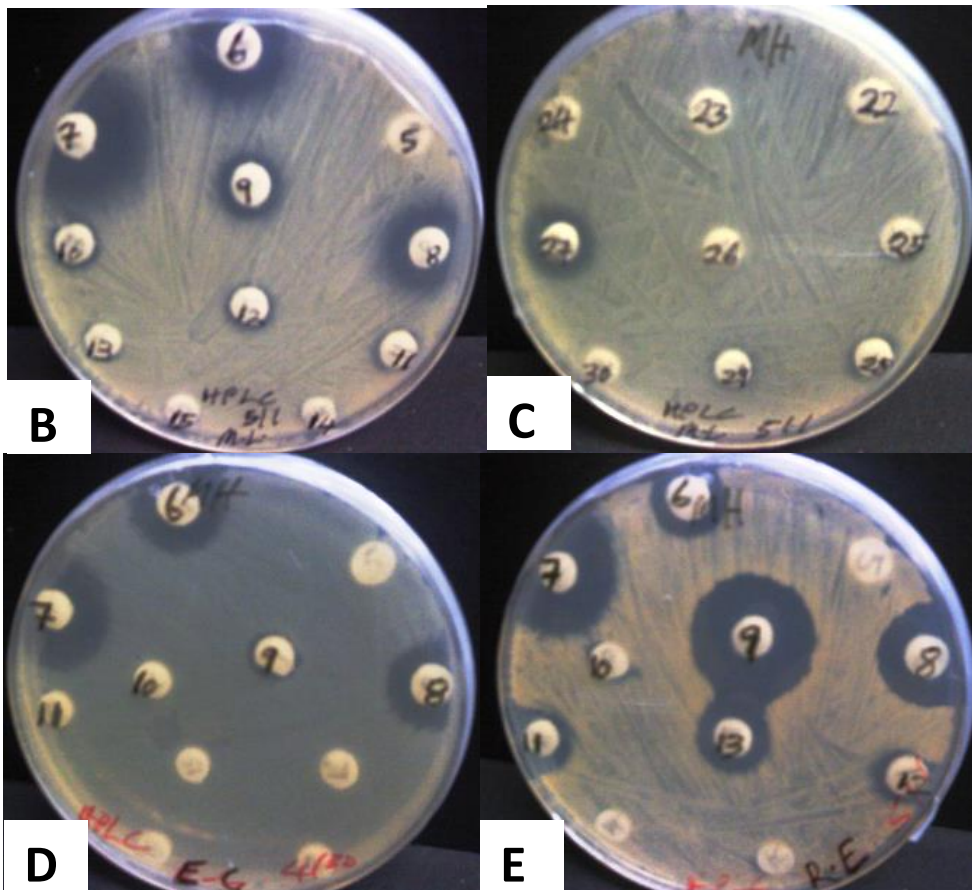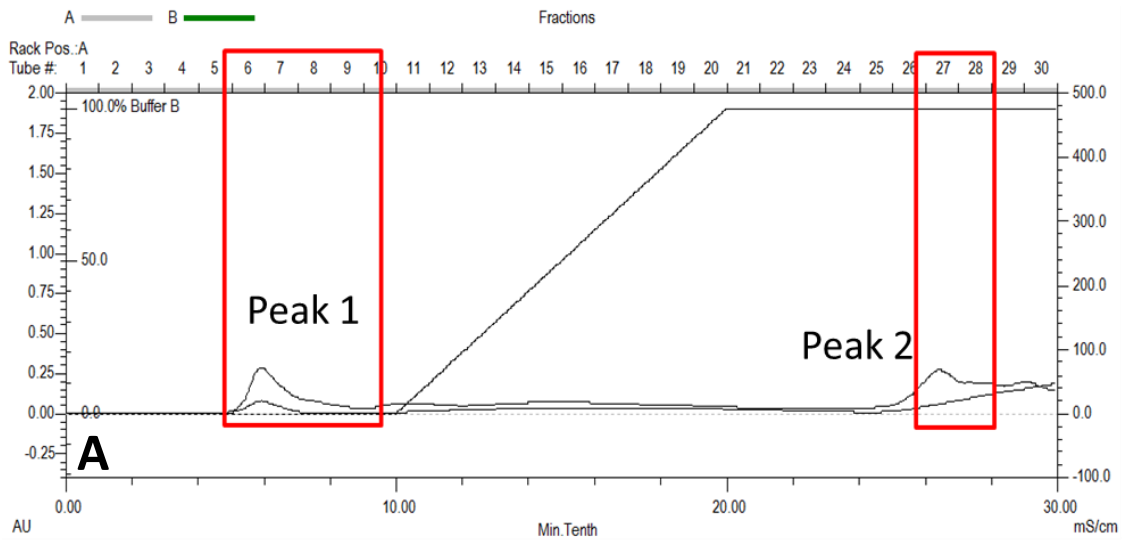Figure 14. **(A)**; HPLC chromatogram showing a peak corresponding to fractions 6-9 and a peak at 27 and 28. Red boxes indicate peaks with inhibitory activity **(B)**; Inhibitory activity of fractions 6-9 against *M. luteus*. (**C**); Inhibitory activity of fractions 27 and 28 against *M. luteus.* **(D)**; Inhibitory activity of fractions 6-9 against *E. coli.* **(E)**; Inhibitory activity of fractions 6-9 against *R. erythropolis*

Fractions 6-9 (Peak 1) were pooled together and dried down to approximately 1 ml, fractions 27 and 28 (Peak 2) were treated likewise. Peak 1 and 2 were then checked for possible inhibitory activity against some other human pathogens (Figure 15).

Bacterial Strains (Gram reaction)	Peak 1 (Fractions 6-9)		Peak 2 (Fractions 27&28)	
Citrobacter freundii (-)		4mm		0mm
Salmonella typhi (-)		4mm		0mm
Klebsiella pneumoniae (-)		12mm		0mm
Shigella dysenteriae(-)		6mm		0mm
Enterobacter aerogenes (-)		6mm		0mm
Escherichia coli (-)		14mm		0mm
Staphylococcus saprophyticus (+)		16mm		6mm
Bacillus subtillis (+)		8mm		0mm
Micrococcus luteus (+)		14mm		8mm
Rhodococcus erythropolis (+)		16mm		0mm

Figure 15. Inhibitory activity of Peak 1 and 2 against some human pathogens

Pooled fractions 6-9 (Peak 1) showed inhibitory activity against *Citrobacter freundii*, *Enterobacter aerogenes*, *Shigella dysenteriae*, *Salmonella typhi*, *Klebsiella pneumonia*, *Escherichia coli*, *Bacillus subtillis*, *Staphylococcus saprophyticus,* and *Rhodococcus erythropolis* (Figure 15) but was not active against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Shigella sonnei* (Data not shown). Pooled fractions 27 and 28 (Peak 2) showed inhibitory activity only against *M. luteus* and *S. saprophyticus* (Figure 15).

This result (Figure 15) suggests that the inhibitory compound at the first peak (Peak 1) may have a broad spectrum of activity against gram positive and gram negative bacteria pathogens compared to the inhibitory compound at peak 2 that has only shown inhibitory activity against *M. luteus* and *S. saprophyticus*.

The activity of our isolated compounds was compared to the activity of known compounds from some members of *S. lavendulae* group (Table 3). Virginiamycin, a macrolide antibitiotic (made up of virginiamycin S1 and virginiamycin M), has been reported from *S. virginiae* and it usually inhibits Gram positive bacteria. Most Gram negative bacteria are not susceptible with the exception of *Brachyspira hyodesentariae,* a Gram negative pathogen that causes swine dysentery (Cocito 1979; Molinero et al. 1989) (Table 3). Streptothricin, an aminoglycoside from *S. lavendulae,* is known to inhibit different Gram positive and Gram negative bacteria as well as mycobacterium. It also has antifungal properties (Waksman et al. 1951). Two quinone antibiotics, Saframycin (Aria et al. 1985) and Mitomycin C (Mao et al. 1999), have also been reported from *S. lavendulae* and are mainly used in cancer treatment.

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But mitomycin c also has bactericidal effects on Gram positive and Gram negative pathogens

(Ueda et al. 1983; Lorian 2005) (Table 3).

Table 3. Activity of known bioactive compounds from some members of *S. lavendulae* group
compared to that of strain COUK1

Producer	Bioactive compounds	Activity
S. cinnamonensis	Monensin (Lowicki and Huczynski 2013)	**Antibacterial**; Inhibit only gram positive bacteria; *Micrococcus, Bacillus* and *Staphylococcus,* **Antiprotozoan**: *treats coccidiosis,* inhibit plasmodium
S. virginiae	Virginiamycin (Cocito 1979; Molinero et al. 1989)	**Antibacterial**; Inhibits gram positive bacteria *Bacillus, Staphylococcus* as well as *Brachyspira hyodesentariae, a gram negative* bacteria
S. lavendulae	Streptothricin (Waksman et al. 1951)	**Antibacterial;** *B. sub, S. aureus, E. coli*, Mycobacteria. **Antifungal:** *Candida, Aspergillus*
	Saframycin (Aria et al. 1985)	**Antitumor**
	Mitomycin C (Ueda et al. 1983; Mao et al. 1999; Lorian 2005)	**Antitumor,** **Antibacterial**; *Staphylococcus, Streptococcus, Klebsiella, Proteus, Pseudomonas* and *E. coli*
Strain COUK1	Yet to be identified	**Antibacterial;** *M. luteus, E. coli, R. erythropolis, C. freundii, E. aerogenes, S. dysenteriae, S. typhi, K. pneumoniae, B. subtillis, S. saprophyticus*

Monensin, a polyether ionophorous (form complexes with monovalent cations)

antibiotic from *S. cinnamonensis,* has been reported to inhibit only Gram positive bacteria. It is

also reported to inhibit *Plasmodium falciparum* (Malaria parasite) and can also be used to treat

coccidiosis (Lowicki and Huczynski 2013) (Table 3). However, strain COUK1 (found similar to *S.

cinnamonensis* (Figure 7; Table 2)) produces a compound(s) (peak 1, Figure 15) active against

both Gram positive and Gram negative bacteria, an antibacterial activity similar to those of streptothricin and mitomycin c. Thus, COUK1 could be a unique strain of *S. cinnamonensis*. The inhibitory compound at peak 1 from COUK1 has not been screened against protozoa, fungi, and cancer cell lines, thus, its bioactive potential might not be fully known.

Coculture

Cocultivating 2 or more organisms together better mimics natural ecological conditions where microorganisms coexist in microbial communities. Many biosynthetic genes are not expressed under routine laboratory conditions. The competition experienced during cocultivation has been shown to induce the expression of biosynthetic genes that are silent in pure culture conditions (Marmann et al. 2014). To see what effect competition would have on strain COUK1's secondary metabolism, it was cocultivated with *R. erythropolis* (Figure 16).

Medium	Extracts	Activity		
		E. coli	*M. luteus*	*R. erythopolis*
Tryptone yeast extract broth	COUK1 Spent broth (pure)	8 mm	10 mm	6 mm
	COUK1 + *R. erythropolis* Spent broth (co-culture)	6 mm	12 mm	12 mm
	Methanol extract of COUK1 cell pellets (pure)	6 mm	8 mm	10 mm
	Methanol extract of COUK1 + *R. erythropolis* cell pellets (co-culture)	6 mm	10 mm	10 mm

Figure 16. Comparison of inhibitory activity of extracts from pure and coculture

Extracts from the coculture were tested for inhibitory activity and the activity was compared to that from pure (COUK1) culture. The spent broth from the coculture produced a slightly bigger zone of inhibition against *M. luteus* and *R. erythropolis* compared to that from the pure culture. There was no significant difference in the activity of methanol extract of cell pellet from pure or coculture (Figure 16).

CHAPTER 4

CONCLUSION

Streptomyces are known to be prolific sources of novel secondary metabolites with

diverse biological activities such as antibacterial, antifungal, and other pharmacologically active

substances. Genomic sequencing revealed an additional 30 gene clusters or genes for putative

secondary metabolites in *S. griseus* apart from the already known gene clusters for

streptomycin, grixazone, and other known secondary metabolites from this organism (Ohnishi

et al. 2008). Some actinomycetes produce more than one antibiotic substance (Waksman

1946). Antibiotics, including actinorhodin, undecylprodigiosin, calcium dependent antibiotic,

and methylenomycin A, have been reported from *Streptomyces coelicolor* A3(2) (Hobbs et al.

1992).

The definition of species in the *Streptomyces* taxonomy has not been resolved due to

the variety of morphological, cultural, and biochemical properties observed at the intra and

inter species level (Anderson and Wellington 2001). After the discovery of antibiotics from

Streptomyces in the 1940s, the screen for novel compound increased resulting in the need for

patenting novel bioactive compounds. This in turn resulted in over-classification of

Streptomyces. Described *Streptomyces* species increased from 40 to about 3000. Many of these

species were considered synonyms as they were proposed based on trivial differences in

morphological and cultural characteristics (Williams et al. 1983).

In 1964 the International *Streptomyces* Project (ISP) was established to come up with

standard criteria for species determination to reduce the number of poorly described

synonymous species. They described criteria that took into consideration properties like spore

chain morphology, color of aerial and vegetative hyphae, production of soluble pigments,

production of melanin, and use of carbon sources. More than 450 species were redescribed

with these criteria. The ISP was unable to establish an identification scheme but rather provide

standard methods by which identification can be achieved (Anderson and Wellington 2001).

Williams et al. (1983) employed phenetic characters (which include morphology, pigmentation,

antimicrobial activity, biochemical tests, degradative tests, antibiotic resistance, growth tests,

use of carbon, and nitrogen sources) in designing a numerical taxonomic approach that led to

reduction in the number *Streptomyces* species. This approach allowed the simultaneous

evaluation of a large number of phenotypic traits. About 400 *Streptomyces* strains were

clustered based on similarities obtained from phenetic tests. At a 77.5% simple matching

coefficient (S_{SM}) (a measure of distance or similarity), 19 major, 40 minor, and 18 single strain

clusters were described with most of the minor clusters consisting of fewer than 5 strains. For

example, Cluster 13 consists of *S. aureofaciens* and *S. roseofulvus,* both considered to be *S.*

aureofaciens. Major clusters varied in size from 6 to 71 strains. Each of these clusters was

treated as a single species despite the diversity observed within some clusters (to help resolve

the problem of overclassification/overspeciation of the genus) and is thus regarded as a species

group (Anderson and Wellington 2001).

Application of 16S rRNA sequence analysis to *Streptomyces* phylogeny has helped study

relationships at the genus, species, and strains level. Comparison of 16S rRNA sequences of the

representatives of the major cluster groups defined by Williams et al. (1983) supports the

groups' phenotype based taxonomy (Anderson and Wellington 2001). Determining a

correlation between genotypic and phenotypic characteristics will result in a better

understanding of *Streptomycetes* taxonomy. Despite the increasing applications of molecular

techniques to *Streptomycetes* taxonomy, many of the clusters defined using numerical

taxonomy are retained. However there's a need to emphasize which traits can be used as

certain phenotypes such as antibiotic production and antibiotic resistance are not suitable as

they might result from horizontal gene transfer events (Anderson and Wellington 2001).

Strain COUK1 was discovered as a contaminant. Results from 16S rRNA analysis and

other phenotypic/biochemical test (morphology, pigmentation, and sugar use pattern) revealed

it is a member of the *Streptomyces lavendulae* group. Our result revealed that strain COUK1

produces inhibitory compound(s) in broth and agar. The production of antibiotics by

Streptomyces in solid cultures is known to coincide with the development of the aerial hyphae.

The bald (*bld*) genes involved in formation of aerial hyphae have also been linked to antibiotics

production (Kieser et al. 2000). In liquid culture antibiotic production is linked to stationary

phase and it is assumed to result from nutrient limitation (Bibb 2005). This shows that the

production of antibiotics by *Streptomyces* is growth phase dependent. Crude extracts recovered

from solid and liquid cultures were tested for inhibitory activity and spotted on TLC strips for

separation into detectable spots. In most cases TLC analysis did not produce clear detectable

spots or bands that correspond to inhibitory activity (Figures 10 and 11).

We hypothesized that *Streptomyces* strain COUK1 might be producing more than one

type of inhibitory compound based on differences in antibacterial activity of different culture

extracts as well as chromatographic analysis of these extracts. There's evidence that ethyl-

acetate is leaving some inhibitory compound behind in spent broth during solvent extraction as spent broth from tryptone yeast extract broth culture showed a wider spectrum of inhibitory activity compared to the ethyl-acetate extract. This suggests the presence of 2 or more types of inhibitory compounds on the basis of polarity.

The analysis of extracts from inorganic salts starch agar (ISP4) was prioritized because it showed the strongest inhibitory activity compared to extracts from other media. Carbon sources are one of the main factors involved in the regulation of secondary metabolism (Sanchez et al. 2010). Several sugars are commonly used as carbon sources for growth and secondary metabolite production, but some of them are preferred by *Streptomyces* and this usually associated with catabolite repression (Sanchez et al. 2010). Starch and glycerol were found to increase the production of antibiotics in batch cultures of *S. violatus* compared to glucose and other carbon sources (Hassan et al. 2004). Also culturing *Streptomyces* on a chemically defined medium could put it under physiological stress and slow down its growth, promoting secondary metabolism (Demain and Fang 1995). All these factors might explain the reason why COUK1 expresses a strong inhibitory compound(s) in a minimal/chemically defined medium like ISP4.

The crude extracts from ISP4 were purified and separated into constituent compounds using different chromatographic analysis. The observation of 2 different peaks (with different retention time i.e. one observed early and the other late (Figure 14A)) with inhibitory activity following ion exchange chromatographic (HPLC) analysis of ISP4 extracts also corroborates the hypothesis that COUK1 produces more than 1 type of inhibitory compound because the first

peak showed a wide spectrum of activity against both Gram positive and Gram negative

bacteria whereas the second peak showed activity against only 2 Gram positive bacteria; *M.*

luteus and *S. saprophyticus* (Figure 15).

Mass spectrometry and Nuclear Magnetic Resonance will be required to determine the

elemental composition and structures of the inhibitory compounds at peak 1 and 2 (Figure 14).

However, in terms of antibacterial activity the compound from COUK1 at peak 1 might be

identical to streptothricin (an aminoglycoside) and mitomycin c (a quinone antibiotic) from *S.*

lavendulae (Table 3). Bacteria resistant to streptothricin and mitomycin c might also be used in

screening the compound at peak 1 from strain COUK1.

Standard methods or chromatographic techniques used in isolating and identifying

known broad spectrum compounds such as streptothricin and mitomycin c from S*. lavendulae*

cultures can be applied to crude extracts from COUK1 cultures and the results can be compared

to standard results of the known compounds for possible similarity. Another way to find out if

the compound at peak 1 has been reported from members of *S. lavendulae* group would be to

identify and sequence the gene involved in the synthesis. This can then be compared to genes

responsible for the synthesis of compounds like streptothricin, mitomycin c and other broad

spectrum antibiotics from members of *S. lavendulae* group.

To see what effect competition would have on antibiotic production, strain COUK1 was

cocultured with *R. erythropolis* (another actinobacteria). Spent broth from the coculture

showed slightly stronger activity against *M. luteus* and *R. erythropolis* compared to spent broth

from COUK1's pure culture. It's been found that coculturing forces direct interaction between

organisms and could result in increased production of known compounds or stimulate the expression of silent genes involved in the synthesis of novel secondary metabolites (Ola et al. 2013; Marmann et al. 2014). For example, pestalone, a new antibiotic was discovered from a coculture of the marine fungus *Pestalonia sp*. with an unknown alpha-proteobacterium, CNJ-328 (Ola et al. 2013). Similarly, *Streptomyces endus* was found to produce the new antibiotic alchivemycin A when cocultured with mycolic acid-producing bacterium, *Tsukarella pulmonis* (Marmann et al. 2014). It's been shown that intimate cell-to-cell interaction between cocultivated organisms is required to modulate the genes of the secondary metabolite producing strain (Marmann et al. 2014).

There was no significant difference in inhibitory activity of coculture extracts of COUK1 and *R. erythropolis* compared to COUK1's pure culture extracts probably because *R. erythropolis* dies 3 or 4 days after introducing strain COUK1 to the culture due to COUK1's inhibitory activity and might not have stayed long enough to stimulate COUK1's biosynthetic ability. The influence of competition on the antibiotic producing ability of strain COUK1 needs to be further investigated by cocultivating it with other bacteria.

Several factors have been linked to the regulation of antibiotic production. These include but not limited to nutrients source (carbon source, nitrogen source, phosphorus source) and amount, growth rate, and phase, as well as antagonism/competition. The mechanism of regulation of a secondary metabolism is complex, but the gene clusters involved are known to be manipulated or steered by different physiological and environmental conditions (Hobbs et al. 1992; Bibb 2005).

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