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Discovery and Characterization of an Antibiotic from the Soil Bacterium *Bacillus* sp.

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Discovery and characterization of an antibiotic from the soil bacterium *Bacillus* sp.

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ABSTRACT

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Many important antibiotics have become nearly obsolete due to the rise of antibiotic resistant pathogens. *Rhodococcus*, an actinomycete related to the prolific antibiotic producing genus *Streptomyces*, harbors over 30 genes for secondary metabolism that could be involved in antibiotic production. Several antibiotics have already been reported for *Rhodococcus*, suggesting the genus may be a good source for new inhibitory compounds. Fifty-four soil bacteria were isolated using enrichment culture techniques (including 37 *Rhodococcus*) and screened for antibiotic producers. BTHX2, a species of *Bacillus* was found to have activity against *Micrococcus luteus* and *Rhodococcus erythropolis*. BTHX2 has a 16S rDNA sequence 97% homologous to *Bacillus licheniformis*, and may be a new strain of *B. licheniformis*. The inhibitory substance produced by BTHX2 was found to have a spectrum of activity against a broad Gram-positive bacteria and some fungus and may have cytolytic activity.

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CHAPTER 1

INTRODUCTION

The Problem of Antibiotic Resistance

Antibiotic resistance has been on the rise for many years. Shortly after its introduction in the 1940s, microbial resistance to penicillin was reported (Infectious Diseases Society of America [IDSA], 2004). However, the problem of resistance was not widespread until years later. In fact, during the mid 1950s the problem of infectious diseases was considered "solved" (Spellberg *et al.* 2008). However, during the 1970s and 1980s antibiotic resistance among pathogens became widespread (Demain and Sanchez 2009). In 2004 it was estimated that 70% of pathogens were resistant to at least one type of antibiotic (Demain and Sanchez 2009), and that number has likely increased in more recent years. In that same year the IDSA put out a call to action against the development of resistance in a report garnered toward the general public and politicians. This report outlined the magnitude of antibiotic resistance and suggested legislative solutions to congress (IDSA 2004), some of which have been enacted (Spellberg *et al.* 2008). The problem of antibiotic resistance is most prevalent where antibiotic use is most prevalent – in hospitals, especially with nosocomial infections (Rice 2008, Boucher *et al.* 2009), and especially in intensive care units in countries with no usage restrictions (Owens 2008). In 2008 it was suggested that certain pathogens responsible for a large portion of nosocomial infections receive federal funding for study (Rice 2008). These pathogens were given the abbreviation "ESKAPE", for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanni, Pseudomonas aeruginosa,* and *Enterobacter* species.

There are several reasons for the drastic development of antimicrobial resistance of late. One example would be pharmaceutical companies pulling out of antibiotic research and

development to pursue more lucrative drug avenues, such as the treatment of chronic diseases and life style conditions (Projan 2003). Second, the past trend of relying on semi-synthetic derivatives of old compounds has led to higher resistance rates. Although semi-synthetic derivatives of antibiotics have served well to combat resistance, this practice simply creates compounds for which there are already underlying resistance mechanisms. Third, the traditional low-throughput method of finding novel classes of antibiotics by random screening of soil microbes is not applicable to antibiotic discovery today. During the 1940s to 1950s novel compounds were easily isolated from 1 in 10 or 1 in 100 randomly screened soil samples; Daptomycin, which was marketed in 2003 and was one of the first novel classes of antibiotics introduced in several decades, was isolated in 1 in ten million randomly screened actinomycetes (Baltz 2007). Thus to find new antibiotics by random screening of soil bacteria, drastic (and laborious) high-throughput screening is required.

New Methods for the Discovery of Antimicrobial Compounds

Old methods of developing and/or screening for new antimicrobial compounds may have failed thus far, but new methods employing contemporary technology are leading to the discovery of novel compounds with biological activity. The most promising and powerful of these new methods is through genomic analysis. Fifteen years ago, the first complete sequencing of a bacterial genome was reported (Bode and Müller 2005). Today, there are nearly 1500 completely sequenced bacterial genomes, with a further 600 bacterial genome sequencing projects underway (Rossolini and Thaller 2010). The original thought was this sequence information would provide a plethora of novel targets for screening against synthetic chemical libraries (Payne *et al.* 2007). The screening of a synthetic library of over 500,000 chemical

compounds against ~ 150 genetically identified essential targets by GlaxoSmithKline resulted in only 16 inhibitory compounds, none of which made it to market (Payne *et al.* 2007). In contrast, the concept of microbial genome-mining for novel natural products with biological activity appears quite promising. During the sequencing of bacterial genomes it was found that organisms known to produce antibiotics could produce more compounds than had been previously reported, as ascertained by the discovery of numerous polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) gene clusters (Bode and Müller 2005, Gross 2009). The sequencing of *Streptomyces coelicolor* identified 20 gene clusters for secondary metabolism; only 4 compounds were originally reported from the organism (Bode and Müller 2005). In addition, the machinery for secondary metabolism was found in bacteria for which no secondary metabolites had ever been reported (Bode and Müller 2005).

The concept of genomic mining is to make use of these "orphan" biosynthetic gene clusters. The first step is to identify NRPSs or PKSs in a given organism of interest, using PCR. This is carried out with primers specific for conserved regions of these biosynthetic genes. For PKSs, this involves targeting specific domains of the synthetase, including ketoacylsynthase domains, acyltransferase domains, and adenylation domains (Gross 2009). Adenylation domains from NRPSs are also targeted for PCR (Gross 2009). Once a gene cluster is identified there are several methods to find the chemical compound it produces.

One method is to use bioinformatic prediction and screening (Gross 2009). This method attempts to predict the chemical composition of the product produced by the biosynthetic pathway based on the different domains of the PKS or NRPS (as ascertained by sequence data). Thallion Pharmaceuticals Incorporated identified 10 orphan biosynthetic pathways in the known vancomycin producer *Amycolatopsis orientalis* (Gross 2009). One PKS cluster was predicted to

produce a new glycosidic polyketide. Culture broth screening revealed a compound with activity comparable to vancomycin which had a novel mechanism of action (Gross 2009). Another method of finding natural products from orphan gene clusters is the genomisotropic approach, in which the microorganism is provided a substrate during fermentation that contains an isotope of carbon or nitrogen (Gross 2009). Bioinformatics also comes into play with this method, as the chemical structure of the compound in question is predicted by tracking the isotope during isolation of the compound. Genome sequencing of *Pseudomonas fluorescens* revealed an orphan NRPS that was predicted to produce a leucine-containing peptide (Gross 2009). Orfamide A was discovered by providing the bacterium [^{15}N] leucine during growth and was found to have activity against amphotercin B resistant *Candida albicans* (Gross 2009). There are several other methods for discovering compounds through genome-mining that have also met with success. These include heterologous expression of the orphan gene cluster in another bacterial host, gene inactivation studies (by insertional mutagenesis), and the 'one strain many active compounds' (OSMAC) approach, in which culture conditions are varied in an attempt to induce gene expression (Gross 2009).

Another powerful tool of the post-genomics era is combinatorial biosynthesis. Combinatorial biosynthesis, as defined by Floss (Floss 2006), is "the application of genetic engineering to modify biosynthetic pathways for natural products in order to produce new and altered structures using nature's biosynthetic machinery". The concept of combinatorial biosynthesis is based on knowledge of enzyme function, as well as the structure and activity of PKSs and NRPSs. In short, the modules of PKS or NRPS gene clusters are modified in an attempt to produce new compounds from known biosynthetic pathways. For the field of antibiotic discovery this has met with moderate success. The biosynthetic pathway of

erythromycin has been modified to contain more polyketide chains, and in one case complete re-engineering of the pathway lead to the production of the antiparasitic compound megalomicin (Bode and Müller 2005). However, the genetic engineering of PKS and NRPS pathways can be unpredictable, and often the predicted transformations do not occur or occur only at a very low frequency (Bode and Müller 2005, Li and Vederas 2009).

An additional potential source of untapped antibiotics could be microbes from marine habitats. This is especially true for marine actinomycetes, whose terrestrial cousins are well documented antibiotic producers (Liu *et al.* 2010). Indeed, genome-scanning of *Verrucosispora maris* (a deep sea actinomycete) revealed over 20 orphan biosynthetic gene clusters (Liu *et al.* 2010). In addition there are over 30 compounds in clinical trials for the treatment of cancer that were derived from marine microbes (Liu *et al.* 2010). Antibiotic compounds isolated from marine actinomycetes include Proximicin, Caboxamycin, and Abyssomicin (Liu *et al.* 2010). It is predicted that there are many more novel natural products to be found in marine microbes. However, although many marine and soil microbes (such as the actinomycetes) can be cultured with standard lab media, the vast majority are 'unculturable'. One way to overcome this obstacle is special enrichment culture techniques, such as the use of seawater as an ingredient in growth media (Liu *et al.* 2010). Another enrichment technique involves a unique system of co-culture in which a diffusion chamber allows the passage of small molecules from a community of bacteria that may act as cofactors for promoting growth (Liu *et al.* 2010). In this method microbes can grow in the diffusion chamber in the presence of other bacteria in the environment and still remain a 'pure' culture (Liu *et al.* 2010). In addition, application of metagenomics (acquisition of DNA from environmental samples, especially of unculturable microbes) is very useful, especially when scanning for PKSs or NRPSs. One research group constructed a library of

30,000 metagenomic clones from marine microbes, of which 16 exhibited biological activity (Liu *et al.* 2010). As more and more scientists take interest in this area of research, it is likely that marine microorganisms will become an important source of novel natural products.

The Genus *Rhodococcus*

Overview

Forty-five percent of medically important antibiotics have come from actinomycetes, and especially from the genus *Streptomyces*. Considering this, it would seem that actinomycete bacteria are an excellent place to discover more biologically active compounds. Indeed, there is an actinomycete genus related to *Streptomyces* that may be a source of new antibiotics: *Rhodococcus*. The genus *Rhodococcus* is composed of soil bacteria widely distributed in the environment. Rhodococci have been isolated from ground water, bore holes, feces, marine sediment, 'dry' earth, as flora (in animals, plants and insects), as pathogens (from plants and animals), and from contaminated soils (Bell *et al.* 1998). Rhocococci are Gram positive, aerobic, nocardioform organisms that begin life as a mycelium that later fractionates into rods or cocci (Bell *et al.* 1998). One distinctive feature of *Rhodococcus* is strong pigmentation; on standard lab media coloration can range anywhere from orange, red, pink, white, cream, yellow, or even colorless (fig. 1) (Goodfellow 1989). In addition the rhodococcal cell wall contains mycolic acids ranging from 30 – 54 carbon atoms in length, which is intermediate between *Corynebacterium* (which have shorter chains) and *Mycobacterium* (which have larger chains).

Figure 1: A plate showing pigmentation of various *Rhodococcus* isolates

Pathogens from *Rhodococcus*

There are 2 major pathogens in the genus *Rhodococcus*: *R. equi* and *R. fascians*. *Rhodococcus equi* has long been recognized as a horse pathogen but can also cause disease in a range of other animals (Bell *et al.* 1998, von Bargen and Haas 2009). The vector of transmission is thought to be inhalation of aerosolized dust particles contaminated with *R. equi* (von Bargen and Hass 2009). In the lung, *R. equi* causes the formation of chronic, puss-filled lung abscesses from which *R. equi* can disseminate to infect other tissues (von Bargen and Haas 2009). *Rhodococcus equi* can also infect immunocompromised humans, with the disease presentation seen in horses (von Bargen and Hass 2009). Although rare, human infection with *R. equi* is often fatal.

Rhodococcus fascians is a plant pathogen with a broad host range encompassing 39 plant families and 86 genera (Goethals *et al.* 2001). The bacterium causes loss of apical dominance and the growth of lateral shoots that develop into leafy galls (Bell *et al.* 1998). Although this pathogen has an extensive host range including crops such as tobacco, peas, and asparagus and

ornamental plants such as chrysanthemum, the economic impact is not thought to be substantial (Bell *et al.* 1998, Goethals *et al.* 2001).

Ecological Importance of *Rhodococcus*

Rhodococcus has the ability to degrade a wide range of unusual and recalcitrant organic pollutants in the soil (Carvalho *et al.* 2005). An excellent example is the degradation of polyaromatic hydrocarbons (PAHs) by *Rhodococcus.* First, species of *Rhodococcus* harbor many genes for oxygenases (Mcleod *et al.* 2006), which are able to break open the aromatic ring of PAHs, catalyzing the first step in the degradation of these compounds (Carvalho *et al.* 2005). Second, *Rhodococcus* can produce surfactants that bind to and break up hydrocarbons, making them more available for attack (Carvalho *et al.* 2005). Lastly, mycolic acids in the rhodococcal cell wall act as cellular surfactants, allowing *Rhodococcus* to bind to oil/water interfaces and also allow easier passage of hydrocarbons into the cell. In addition to bioremediation, *Rhodococcus* could be used to produce cleaner burning fuels. Before processing, fossil fuels contain sulfur and can be a major cause of acid rain when burned (de Carvalho *et al.* 2005). *Rhodococcus erythropolis* strain IGTS8 was found to desulfurize dibenzothiophene (DBT) and release inorganic sulfur (de Carvalho *et al.* 2005). Dibenzothiophene desulfurization can be applied to fossil fuels, and the sulfur is removed without affecting carbon-carbon bonds (an ability so far unique to *Rhodococcus*) and thus the caloric value of the fuel is preserved (de Carvalho *et al.* 2005).

Hydrolases have also been reported for *Rhodococcus* that can catalyze the hydrolysis of a number of substrates. Most notable of these is the nitrilase enzyme of *R. erythropolis* strain J1, which catalyzes the hydrolysis of acrylonitrile to acrylamide (de Carvalho *et al.* 2005). The

Nitto Chemistry Industry Company Ltd (Japan) uses this conversion process to produce ~ 30,000 tons of acrylamide each year, which is the most successful application of a bacterial enzyme in industry ever reported (Bell *et al.* 1998, de Carvalho *et al.* 2005).

In addition to the above described metabolism, various rhodococci have been isolated using a broad range of chemicals as the sole source of carbon and energy. These include (but are not limited to): Explosives (Seth-Smith *et al.* 2008), insecticides (Jung-Bok *et al.* 2008), phenols (de Carvalho *et al.* 2005), benzenes (de Carvalho *et al.* 2005), crude oils (Ohhata *et al.* 2007), arsenic containing compounds (Nakamiya *et al.* 2008), polychlorinated biphenyls (de Carvalho *et al.* 2005), certain pharmaceuticals (Gauthier *et al.* 2010), solvents and hydrocarbons (Civilini 2009), styrene (Tischler *et al.* 2009), and narcotics such as cocaine and heroin (Ascenzi *et al.* 2008, Cameron *et al.* 1994).

Genome Sequence of RHA1

In 2006 the genome of *Rhodococcus* species RHA1 was sequenced (McLeod *et al.* 2006). It was found that the genome consisted of 9.7 million base pairs, one of the largest bacterial genomes sequenced to date. The genome is composed of 4 linear elements: 3 plasmids and the chromosome. Of the 9,145 protein coding sequences found, oxygenases were particularly abundant (numbering 201 proteins, some of which were homologues to different oxygenases). In addition, 38.4% of the genes were coding for proteins of unknown function. Before this report no secondary metabolites had been characterized for *Rhodococcus*. Despite this fact, over 30 genes for secondary metabolism were found, including 24 NRPSs and 7 PKSs, which, as shown previously, could be involved in antibiotic production (as well as other secondary

metabolites like siderophores and pigments). In fact, several *Rhodococcus* species have been reported to produce antibiotics.

Antibiotics Produced by *Rhodococcus*

The first inhibitory compounds reported were the rhodopeptins, isolated from *Rhodococcus* sp. Mer-N1033 (Chiba *et al.* 1999). Strain Mer-N1033 was isolated from the Iwate Prefecture in Japan, in a soil sample from Mount Hayachine (Chiba *et al.* 1999). Rhodopeptins are cyclic tetrapeptides that contain a β-amino acid and 3 α-amino acids. All 5 compounds were reported to exhibit strong activity against *Candida albicans* and *Cryptococcus neoformans*. Another interesting set of antibiotics reported from *Rhodococcus* are the rhodostreptomycins. Kurosawa *et al.* (2008) were growing *Rhodococcus fascians* 307C0 in co-culture competition with *Streptomyces padanus* which resulted in clearance of *S. padanus* from the culture broth. Subsequent genome analysis revealed that *R. facians* 307C0 was harboring a large segment of *S. padanus* DNA. Further research yielded 2 compounds from *R. fascians* 307C0 – rhodostreptomycins A and B. The authors postulate that the biosynthetic genes for the compounds were acquired via horizontal gene transfer (HGT) from *S. padanus* during co-culture. Interestingly, the rhodostreptomycins are aminoglycoside compounds that differ greatly from the actinomycin antibiotics produced by *S. padanus*.

In an antibiotic screening campaign against *Mycobacterium smegmatis*, Iwatsuki *et al.* found *Rhodococcus jostii* K01-B0171 to exhibit inhibitory acivity (Iwatsuki *et al.* 2007). The research group was able to isolate 2 compounds from K01-B0171: lariatins A and B. These compounds are peptides consisting of 18 to 20 amino acids and have a lasso structure (figure 2). Lariatin B has a minimum inhibitory concentration (MIC) of 6.25μg/ml against *M. smegmatis.*

Lariatin A shows activity against *Mycobacterium tuberculosis* (MIC 0.39µg/ml) as well as *M. smegmatis* (MIC 3.13µg/ml). Finally, 3 different quinoline compounds have been isolated from 2 rhodococci in the form of aurachins. Aurachin RE was isolated from *R. erythropolis* JCM 6824 and is similar in structure to aurachin C type antibiotics (Kitagawa and Tamura 2008). Aurachin RE displays a broad spectrum of activity against Gram-positive organisms. More recently aurachins R and Q were isolated from *Rhodococcus* sp. Acta 2259 (Nachtigall *et al.* 2010). Acta 2259 was isolated from the Milcote Pilot Sewage Treatment Plant (Stratford-upon-Avon, United Kingdom) from activated sludge foam. Aurachin Q was found to be inactive, and aurachin R exhibited minor activity against *Staphylococcus epidermidis, Bacillus subtilis,* and *Propionibacterium acnes*.

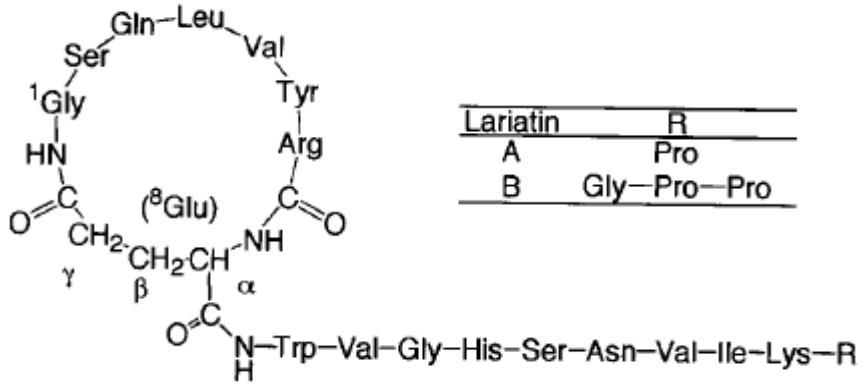

Figure 2: Structure and composition of the lariatins (Iwatsuki *et al.* 2007)

Enrichment Culture

These results suggest that additional bioactive compounds remain to be found in *Rhodococcus*. However the problem with applying random low-throughput methods of screening soil isolates for novel compounds was discussed above. One technique to get past that obstacle is to perform enrichment culturing to isolate *Rhodococcus*. The first step in enrichment culturing is to prepare a chemically defined minimal growth medium devoid of a required growth

factor, such as a source of carbon. The second step is to provide an unusual compound as the sole source of the required growth factor. In this case providing hexadecane as the sole source of carbon and energy is an excellent example. Hexadecane is a hydrocarbon compound that is similar to fuels such as gasoline and diesel, of which the biodegradation by *Rhodococcus* is well documented (Bell *et al.* 1998, de Carvalho *et al.* 2005, Ohhata *et al.* 2007). The assumption is that most bacteria in the soil will not be able to use hexadecane as the sole source of carbon and energy, and thus the growth medium has enriched for smaller populations of soil bacteria that *can* use hexadecane.

It is advantageous to perform enrichment culture techniques to isolate species of *Rhodococcus* for two reasons. First, as stated previously *Rhodococcus* is dispersed ubiquitously in the environment, especially in soil, and as such should be relatively easy to isolate from soil samples. Second, various species of *Rhodococcus* have been isolated growing on a wide range of unusual compounds (Cameron *et al.* 1994, Carvalho *et al.* 2005, Ascenzi *et al.* 2008, Jung-Bok *et al.* 2008, Seth-Smith *et al.* 2008, Civilini 2009, Tischler *et al.* 2009,de Gauthier *et al.* 2010). Thus it is more likely that *Rhodococcus* would be isolated using enrichment culturing than many other bacteria.

The Genus *Bacillus*

Overview

Besides actinomycete bacteria, another important source of antibiotics is the genus *Bacillus*. *Bacillus* is comprised of Gram-positive, rod-shaped soil bacteria that are widely spread in the environment, and different species vary greatly in the specific niches they inhabit, and include thermophilic, mesophilic, psychrophilic, halotolerant, halophilic, alkalinophilic,

neutrophilic, and acidophilic organisms (Logan and De Vos 2009). Species of *Bacillus* are also isolated from plants, animals, humans, and insects as flora and as pathogens (Logan and De Vos 2009). Members of the genus *Bacillus* are collectively referred to as "Gram-positive, aerobic spore-formers", yet several are known to be facultative anaerobes, and recently some species were discovered that are strict anaerobes (Logan and De Vos 2009). In addition, some species can be Gram-variable, Gram-negative, or even acid-fast at certain stages of their life cycle (Logan and De Vos 2009). Spore formation by *Bacillus* is a hallmark of the genus and is important during identification of species in relation to spore size, shape, and position within the cell (Logan and De Vos 2009). Bacterial endospores are hardy "over-wintering" structures that allow cells to survive harsh conditions, and are resistant to heat, chemicals, radiation, and dessication (Logan and De Vos 2009). These spore characteristics make *Bacillus* species very persistent wherever they are deposited, which includes almost every environment on earth, both land and sea (Logan and De Vos 2009).

Pathogens from *Bacillus*

Probably the most infamous pathogen of *Bacillus* is *B. anthracis*, causative agent of anthrax (Logan and De Vos 2009). Anthrax is primarily a disease of herbivores, and infection occurs after ingestion of *B. anthracis* spores from the environment. After ingestion, the pathogen gains access to the lymphatic system and the spleen, where it replicates. Within a few days *B. anthracis* causes septicemia in the afflicted animal, which is fatal. *B. anthracis* can also infect humans and is greatly feared as a bioterrorism agent (Logan and De Vos 2009). In humans infection can occur via inhalation, ingestion, or cutaneously, cutaneous transmission being most common (99% of cases). Cutaneous anthrax occurs in wounds and causes the

formation of skin lesions but does not disseminate to other tissues. Before antibiotic treatment anthrax was often fatal. However, *B. anthracis* shows little resistance to penicillin, and this drug can effectively treat infection.

Other prominent pathogens from *Bacillus* include *B. cereus* and *B. thuringiensis* (Logan and De Vos 2009). *B. cereus* causes two types of food poisoning: diarrheal, which is caused by an enterotoxin, and nauseal , which is caused by emetic toxin. *B. cereus* may also cause opportunistic infections, including endophthalmitis (eye infection), keratitis, and wound infection. *B. thruingiensis* is an insect pathogen, that produces parasporal bodies known as δ-endotoxins. The δ-endotoxins are lethal to insects and invertebrates.

Ecological Importance of *Bacillus*

The genus *Bacillus* has been of great importance to science for many years. Indeed, *B. subtilis* is a model Gram-positive organism in scientific studies due to its ease of isolation, cultivation, and manipulation in a laboratory setting (Logan and De Vos 2009). In addition *B. subtilis* was also the first Gram-positive organism to have its genome completely sequenced (Logan and De Vos 2009). Several species of *Bacillus* have been shown to degrade unusual substrates, including *p*-nitrophenol and 4-chlorobenzoic acid (Logan and De Vos 2009). One strain of *B. megaterium* with an engineered cytochrome P450 is known to degrade polyaromatic hydrocarbons (Logan and De Vos 2009). Some species of *Bacillus* may also be important in waste management as they have been shown to accumulate metal ions in their cell wall. *Bacillus* has also been isolated as flora from plants, both on outer surfaces and inner tissues. These organisms (termed epiphytes and endophytes) may be of importance in prevention of plant disease (Logan and De Vos 2009).

Antibiotics from *Bacillus*

The genus *Bacillus* has been known as an important source of antibiotic producers since the "golden age" of antibiotic discovery (Schaffer 1969). Just in the organism *B. subtilis*, 12 antibiotic compound have been isolated from different strains (Stein 2005). Many of the antibiotics from *Bacillus* are peptide compounds that are synthesized either ribosomally or non-ribosomally (by non-ribosomal peptide synthases) and are resistant to the action of proteases (Schaffer 1969, Nakano and Zuber 1990, Stein 2005). Many antibiotics produced ribosomally are lantibiotics, which contain the unusual amino acid lanthionine (Stein 2005). Type A lantibiotics are active by forming voltage-dependent pores in the cell membrane of bacteria (Stein 2005). Type B lantibiotics inhibit cell wall synthesis by binding to lipid II (Stein 2005). Non-ribosomally synthesized antibiotics from *Bacillus* are often lipopeptide compounds that exhibit surfactant-like qualities (Stein 2005). One of the best well-studied lipopeptides from *Bacillus* is surfactin, which is one of the most powerful surfactants known (Stein 2005). Surfactin can lower the surface tension of water from 72mN/m to 27mN/m and will also disrupt biological membranes (which is the source of its antimicrobial activity). Polyketide antibiotics are also produced by *Bacillus* such as difficidin, which has broad-spectrum activity against Gram-positives and Gram-negatives. *Bacillus* continues to be a good source of new antibiotics today, such as the recent discovery of a novel lantibiotic from *B. licheniformis* (Dischinger *et al.* 2009).

Current Research

This thesis describes the isolation and identification of many species of *Rhodococcus* (and other bacteria) from soil samples using two enrichment techniques: carbon source enrichment and M3 enrichment. Also described is the screening of the 54 identified soil isolates for inhibitory activity against 3 indicator strains: *Rhodococcus erythropolis* IGTS8, *Micrococcus luteus*, and *Escherichia coli*. The results of this screening process are also discussed. Unfortunately, none of the screened isolates showed inhibitory activity. However, a contaminant from one of the antibiotic screening assays was found to have inhibitory activity. This organism was identified as a species of *Bacillus*, and the substance it produces was found to exhibit activity against a wide range of Gram-positive organisms, as well as having antifungal and possibly cytolytic properties. The partial characterization of this organism and the compound it produces are described.

CHAPTER 2

MATERIALS AND METHODS

Soils

'Clean' soils were collected from several sites around the northeast Tennessee/ETSU region, including Winged Deer Park, the ETSU campus, the Johnson City area off campus, and from several locations in the neighboring town of Kingsport. In addition several 'clean' soils were donated from Alaska, Australia, and Aruba. 'Contaminated' soils were also collected from Embreeville, TN, Butler, TN, Johnson City, TN, and Kingsport, TN. In Embreeville, an abandoned Mobil gas station had a diesel storage tank that had been leaking diesel into the surrounding soil. In Butler there was a gas station with a similar situation, with the gasoline storage tank leaking gasoline into the surrounding soil. Soil samples from these sites were used for hexadecane enrichment. In Johnson City there were two sites of contaminated soils: Sinking Creek and a coal pile on campus. Sinking Creek had been contaminated with trichloroethylene (TCE) via runoff from an adjacent industry. Although this happened many years prior, soil was still collected for TCE enrichment. Soil was collected near the coal pile on campus for hexadecane enrichment. Similarly, soil was collected from around and underneath a coal pile in Kingsport for hexadecane enrichment. Soils were collected in plastic containers on site and stored at 4˚C until use.

Growth Media

There were several types of growth media used during this research:

A. Basal Salt Medium.

To prepare the basal salt medium, 3 different solutions need to be prepared: Na_2HPO_4 solution, KH_2PO_4 solution, and the agar solution. These solutions are autoclaved separately and are mixed after cooling in a 50 ˚C water bath. The preparation of these solutions is as follows:

1.) Na_2HPO_4 solution 5.11g in Na_2HPO_4 180 ml of dH_2O

2.) KH_2PO_4 solution 1.9 g KH_2PO_4 in 70 ml of dH_2O

3.) Agar solution 7.5 g Bacto Agar in 235 ml of dH_2O

4.) Autoclave separately, then cool in a 50 ˚C water bath before mixing

After mixing of the first 3 solutions, there are 3 other ingredients to add:

1.) NH_4Cl stock solution (20%) 5 ml

2.) $MgCl_2$ stock solution (4%) 5 ml

3.) Hutner's minerals 5 ml

These 3solutions should be prepared, autoclaved, and ready for use before the preparation of the first 3 solutions.

Hutner's Minerals

1.) dH_2O 95 ml

2.) Nitrilotriacetic acid 1 g

3.) $MgCl_2*6H_2O$ 2.44 g

4.) $CaCl_2*2H_2O$ 0.334 g

5.) $FeCl_2*4H_2O$ 8 mg

6.) $(NH_4)_6Mo_7O_{24}*4H_2O$ 0.9 mg

7.) Hutner's salts 5 ml

8.) Autoclave

Hutner's Salts

1.) dH_2O	100 ml
2.) EDTA*Na_2 salt	0.25 g
3.) $ZnCl_2$	0.52 g
4.) $FeCl_2*4H_2O$	0.36 g
5.) $MnCl_2*4H_2O$	0.20 g
6.) $CaCl_2*2H_2O$	0.03 g
7.) $Co(NO_3)_2*6H_2O$	0.25 g
8.) $Na_2B_4O_7*4H_2O$	0.02 g

9.) add a few drops of 1M HCl to stabilize the solution

10.) Autoclave

All ingredients are mixed and autoclaved in the same container. After mixing, a few drops of low concentration HCl should be added to stabilize the solution.

B. Bushnell – Haas (BH) medium

1.) dH_2O 500 ml

2.) KH_2PO_4 1 g

3.) K_2HPO_4 1 g

4.) NH_4NO_3 1 g

5.) $MgSO_4*7H_2O$ 0.2 g

6.) $FeCl_3$ 0.05 g

7.) $CaCl_2*2H_2O$ 0.02 g

8.) Agar 7.5 g

9.) Bring to a gentle boil, then autoclave

C. Rich Medium (RM)

1.) dH_2O 500 ml

2.) Glucose 5 g

3.) Nutrient Broth 4 g

4.) Yeast Extract 0.25 g

5.) Agar 7.5 g

6.) Autoclave

D. M3 medium

M3 (Rowbotham and Cross 1977) is the most difficult growth medium to make of all the others used. It consists of 5 different solutions:

Solution 1	0.446 g of KH_2PO_4 and 0.732 g of Na_2HPO_4 in 100 ml of dH_2O
Solution 2	0.10 g of KNO_3 and 0.29 g of NaCl in 100 ml of dH_2O
Solution 3	0.02 g of $CaCO_3$ in 100 ml of dH_2O
Solution 4	0.20 g of Na propionate in 100 ml of dH_2O
Solution 5	9 g of agar in 100 ml of dH_2O

All of these solutions need to be prepared and autoclaved separately and mixed after cooling in a 50 ˚C water bath. After mixing, there are a number of other ingredients to add to the medium. They are as follows:

1.) $MgSO_4*7H_2O$ stock solution (30 %)	165 µl
2.) $FeSO_4*7H_2O$ stock solution (0.001 %)	10 µl
3.) ZnCl stock solution (2.6 %)	3.5 µl
4.) $MnSO_4*4H_2O$ (0.01 %)	100 µl
5.) Thiamine HCl (0.4 %)	0.5 ml
6.) Cycloheximide	50 mg

Stock solutions of the first 5 ingredients should be prepared, autoclaved, and ready for use before preparation of M3 medium. Cycloheximide can be added in solid form directly after cooling and mixing of solutions 1 – 5.

Enrichment Culture

Two types of enrichment culturing were carried out: carbon source enrichment (for contaminated soils) and M3 enrichment (for 'clean' soils).

Carbon Source Enrichment Culture

Carbon enrichment culturing was carried out with a modified version of hydrocarbon enrichment by Peng *et al*. (Peng *et al.* 2006). Two different compounds were used: trichloroethylene (TCE) and hexadecane. Trichloroethylene is an industrial grade solvent that is highly toxic to living organisms. Hexadecane is a long chain hydrocarbon compound that is similar in structure to fossil fuels such as gasoline and diesel. Cultures were started by adding 10 ml of either BSM or BH medium to a small 50 ml Erlenmeyer flask. For TCE enrichment, BSM was used as the growth medium. For hexadecane enrichment, BH was used. Also, 1 gram of soil sample was added to the flask, along with 10 µl of TCE or hexadecane (giving the carbon source a final concentration of 0.1%). Reagent grade (~99% purity) carbon compound was added undiluted to the culture. Flasks were incubated for 7 days in a 30 ˚C water bath with shaking. At the end of 7 days flasks were removed from the water bath and the soil was allowed to settle to the bottom of the flask. Subculturing was carried out by drawing 1 ml from the settled culture and transferring to a 50 ml flask with 10 ml fresh growth medium and 0.1 % carbon source, which was then incubated for a further 7 days in a 30 ˚C water bath with shaking. This process was repeated 3 more times for a total of 4 subcultures, at which time 100 µl was transferred from the last subculture onto a plate composed of the same minimal medium, and streaked or spread out. Due to the volatile nature of both TCE and hexadecane, the carbon source was supplied on the plates by soaking a small square 2 cm X 2 cm piece of Whatman's

3mm chromatography paper in the compound. The paper was then placed on the lid of the petri dish such that when the plate was sealed the bacteria could use the gas phase of the compound as a source of carbon and energy. Isolated colonies that arose on the plates were transferred to RM plates for purification by several rounds of streak plating. Organisms on RM plates were Gram stained to check for Gram positive rods or cocci and to check for purity. Purity was determined usually by the presence of only 1 cell type in 8 optical fields at 100X magnification. However, as some rhodococci can fragment into both rods and cocci purity was also ascertained by observation of a single colony morphology on streak plates.

M3 Enrichment Culture

For M3 enrichment, a 1:10 dilution of the soil sample was prepared by placing 1 g of soil sample in 9 ml of sterile distilled water in a large tube. This suspension was then heat shocked rigorously by placing the tube in a 55 ˚C water bath for 6 minutes. The soil sample was then allowed to cool at room temperature, mixed by vortexing, and 100 µl spread onto M3 plates. M3 plates were incubated on the bench top at room temperature for 1 week or until colonies arose. *Rhodococcus* is most easily identified by the vibrant pigments displayed on plates: red, white, orange, yellow, cream, and even colorless variants have been observed. M3 plates were examined for colonies exhibiting these types of pigments. Pigmented colonies and mucoid colonies were streaked on to RM plates for purification and checked by Gram stain for Gram positive rods or cocci.

Identification of Soil Isolates

Once the purity of an isolate was confirmed, identification of the organism was carried out by colony PCR of its 16S rDNA gene. Table 1 shows the composition of each PCR reaction.

Table 1: List of reagents and quantities used for PCR of 16S rDNA

PCR reaction	
dH_2O	22 µl
10x PCR Buffer (Go Taq Flexi, Promega)	10 µl
25mM $MgCl_2$ (Promega)	3 µl
10mM deoxynucleotide triphosphate mix (Promega)	1 µl
10x Enhancer (Eppendorf)	10 µl
DNA template (single colony)	1 µl
20 µM Forward primer (63f or Bact 8f)[A]	1.25 µl
20 µM Reverse primer (1387r or Bact 1510r)[A]	1.25 µl
Taq polymerase (Go Taq Flexi, Promega)	0.5 µl (2.5 units)
Total volume of reaction	50 µl

A: Forward primer 63f was used in conjunction with 1387r (Marchesi *et al.* 1998), while Bact 8F (Rivas *et al.* 2001) was used in conjuction with Bact 1510R (Nagashima *et al.* 2003).

Colony PCR was carried out by picking a single colony from a fresh streak plate (2 to 3 days old, or until fresh colonies had formed). The colony was dispersed in 10 µl of dH_2O in a 1.5 ml Eppendorf tube by vigorously rotating the tip of the loop in the bottom of the tube. This cell suspension then served as the DNA template for the PCR reaction. The sequences of the primers are as follows: 63f (Marchesi *et al.* 1998) (5'-CAGGC CTAACACATGCAAGTC-3'); Bact 8f (Rivas *et al.* 2001)(5'-AGAGTTTGATCCTGGCTCAG-3'); 1387r (Marchesi *et al.* 1998) (5'-GGGCGGWGTGTACA AGGC-3'); Bact 1510R (Nagashima *et al.* 2003) (5'-CGGTTACCTTGTTACGACTT-3'). PCR reactions were cycled under the following conditions: 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) repeat steps 2 – 4 for 29 cycles, 5) 72 ˚C for 5 minutes. The PCR reactions were first checked on agarose gels, then purified using the GeneClean Turbo kit. Purified reactions

were sent to the University of Tennessee Department of Molecular Biology for sequencing. Sequence data was received as .seq and .ab1 files via email. The .ab1 chromatogram files were examined using the program Chromas. The DNA sequence was exported from Chromas to Microsoft Word, for manipulation. "Good" was defined using Chromas as nucleotides that had a single strong peak with no overlapping peaks. 'Bad' quality sequence was defined as more than one peak occupying a space for a single nucleotide or multiple nucleotides in a single peak. The code from the forward and reverse primers for the same organism was spliced together in Word in fasta format and submitted to Ribosomal Database Project (http://rdp.cme.msu.edu) for identification to the genus level. Sequence data were submitted under the "sequence match" query on this website. This procedure compares the sequence data to an online database of 16S rDNA, and will identify an organism from the Domain level down to the genus, and will give a list of possible species for the organism based on the percentage of homology to other sequences in the database.

Maintenance of Cultures

Isolates identified by 16S rDNA sequence were transferred from plates to RM slants and stored at 4 ˚C until use. *Rhodococcus erythropolis, Micrococcus luteus,* and *Escherichia coli* were used as indicator strains during screening for antibiotic activity. *Rhodococcus erythropolis* was maintained on RM plates. *Micrococcus luteus* was maintained on BHI plates; *Escherichia coli* was maintained on LB plates. All indicators were stored at 4 ˚C until use, and were transferred to fresh medium monthly.

Screening for Antibiotic Activity

Processing of microbial extracts was carried out according to the method of Garcia *et al.* (Garcia *et al.* 2009). Seed cultures were prepared from the isolates in 2.5 ml RM broth in test tubes and grown for 1 to 2 days. Next, 100 µl of the seed culture was used to inoculate a cotton-stoppered 250 ml Erlenmeyer flask containing 50 ml of RM broth and 1 g of XAD-16 Amberlite adsorbent resin (final concentration of 2%). The resin was weighed and transferred into the flasks aseptically right before inoculation. Cultures were grown for 8 days at 30 ˚C with shaking. After the culture period a turbidity reading was taken using a Klett colorimeter (red filter), then cultures were streaked out on RM plates to check for purity. The cultures were poured into oakridge tubes and centrifuged to collect the cells and resin. The supernatant was collected in a 50 ml polystyrene tube from which 1 ml was filtered into a small 1.5 ml eppendorf tube to be tested for antibiotic activity. Next, 7.5 ml acetone and 7.5 ml methanol (a 50:50 ratio) was added to the pellet in the oakridge tube, which was then vortexed to break up the pellet. This slurry was poured into a small beaker then extracted with a stir bar for 15 minutes. The extract was poured into an oakridge tube, and 7.5 ml of methanol + 7.5 ml of acetone were added to the resin + cells in the beaker for a second extraction with stirring. The second extract was poured into the same oakridge tube, which was then centrifuged to collect any leftover cells and/or resin. The extract was then passed through a syringe filter (0.45 µm) to remove any stubborn cell fragments or other artifacts and collected in a 50 ml polystyrene tube. Tubes containing 30 ml of extract were then balanced and placed in a Labcono Centrivap for evaporation to dryness (complete drying of the extracts typically took ~ 48 hours). After drying, 1 ml of methanol was added to the extracts and then vortexed to re-dissolve the dried pellets. To test for inhibitory activity, 25 µl of extract or supernatant was applied to a paper disk and

allowed to dry, before applying another 25 µl (bringing the total volume on the disk to 50 µl). Paper disks were created using a hole-punch on thick Whatman blot paper GB004, and sterilized by autoclaving. Disks were allowed to dry completely before use in the antibiotic activity assay.

Preparation of Indicator Plates for Antibiotic Assay

To prepare indicator strains for antibiotic screening, seed cultures were grown overnight in 2.5 ml MH broth in test tubes. The seed cultures were then vortexed to evenly disperse the cells in the medium, and dilutions were prepared in separate tubes containing 2.5 ml MH broth. Cultures were diluted by transferring 100 µl from the final culture into the fresh medium until the fresh medium had reached the same turbidity as a 0.5 McFarland Standard. Next, a sterile cotton swab was inserted into the dilution tube and rotated until air bubbles were no longer released. Excess fluid was removed by pressing the cotton swab on the side of the tube. Next, the cotton swab was used to inoculate an MH plate by starting at the plate edge spreading the inoculum over 1/3rd of the plate, rotating to the next uninoculated section, and repeating the process until the whole plate was covered. Finally, the swab was dragged around the perimeter of the plate to ensure full coverage. After the plates had dried, paper disks containing extract or supernatant were added, and the plates were incubated at the appropriate temperature (30 ˚C for 48 hours for *R. erythropolis*, 37 ˚C for all other organisms). After the incubation period, the plates were examined for zones of growth inhibition. For any isolates that exhibited a zone of growth inhibition the preparation of extracts and antibiotic assay was repeated to ensure consistency.

Large Scale Isolation of Inhibitory Compounds

To scale up antibiotic production 2.8 L Fernbach flasks containing 1 L of RM broth and 20 g of XAD-16 Amberlite resin were inoculated with 20 ml of a 100 ml overnight seed culture of BTHX2. These flasks were grown with shaking for 4 days at 30^{o} C. After 4 days, the cultures were streaked on RM plates to check for purity and a turbidity reading was taken with a Klett colorimeter. If the Klett reading was approximately 130, the culture broth was separated from the resin using a 212 µM sieve, and a 50 ml aliquot of the broth was saved in a 50 ml polystyrene tube for antibiotic testing. Next, resin from all 5 L (~ 100 g) was collected in a large 4 L beaker and extracted twice with 750 ml methanol and 750 ml acetone for 1 hour. The extract was then collected (without transferring the resin) in 250 ml centrifuge bottles. The extract was then centrifuged for 10 minutes at 4000 x g, and vacuum filtered using a 0.45µm HVHP type Millipore filter. The filtered extracts were transferred to a 2 L round bottom flask and evaporated to a small volume using a Büchi Rotavapor R-200. As the volume of the extract reduced, more extract was added to the flask until all 3 L was reduce to ~ 40 ml. The 40 ml of extract was divided into two 50 ml polystyrene tubes and evaporated to dryness with a Labcono CentriVap. The dried extract was brought up in 40 ml dH_2O, and 50 µl of supernatant from the cultures and extract from the resin was tested on disks with *M. luteus* as an indicator strain to ensure production and location of inhibitory activity (activity was always found to be in the extract). Next, the extract was run through an Amberlite XAD-16 column for partial purification, before passage through a Sephadex LH-20 column for further purification.

Amberlite XAD-16 Column Purification

The extracts derived from the methods used in this research contain a large amount of extraneous compounds in addition to the desired inhibitory compounds. To 'clean-up' the extract before passage through a Sephadex LH-20 column, it was passed through an XAD-16 column. XAD-16 resin was prepared by soaking 160 g in 1 L of ddH_2O overnight to allow the resin to soak up water. The next day all the dried resin at the top of the water was removed using a plastic 3 ml transfer pipet. The resin was packed into a 3 X 50 cm column to give a bed volume of 255 ml. To wash the resin, 3 bed volumes of methanol was passed through the column, which was then equilibrated with 4 bed volumes of ddH_2O. For partial purification, the entire volume of the extract (~ 40 ml) was poured directly into the column. Four bed volumes of ddH_2O was passed through the column (and saved in a clean beaker). Next, the extract was eluted from the resin with 650 ml methanol collected in 50 ml fractions. To determine which fractions contained inhibitory activity, 75 µl of each fraction was applied to paper disks, which were placed on an MH plate with *M. luteus* as an indicator strain. Those fractions showing inhibitory activity were evaporated to dryness in a Labcono Centrivap and dissolved in 10 ml methanol. The pooled fractions were then centrifuged at 9400 x g for 10 min to remove any precipitants, and the supernatant saved into a fresh 50 ml polystyrene tube.

Sephadex LH-20 Column Purification

Extract that had been partially 'cleaned' by passage through an XAD-16 column was further purified by passage through a sephadex LH-20 column that was prepared as described by Wright (2009). Briefly, a 5 ml aliquot of extract was poured directly onto the LH-20 resin, which separates compounds based on hydrophobicity and size. The column was run with

methanol as the mobile phase, and 47 fractions were collected in 175 drop aliquots using a Bio-Rad 2110 fraction collector. Next, 25 µl of each fraction was applied to a paper disk and placed on an MH plate with *M. luteus* as an indicator strain. Plates were incubated overnight, during which the fractions from the LH-20 column were stored at 4 ^{o}C. The plates were examined for inhibitory activity the next day. Those fractions that were positive were pooled in such a manner that precipitants that had formed overnight were left behind in the fraction tubes. The pooled fractions were then evaporated to dryness in a Labcono CentriVap and reconstituted in 2 ml of dH_2O. This partially purified extract was then used for HPLC and bioassays.

High Pressure Liquid Chromatography Purification

Part of the LH-20 purified extract was further purified using high pressure liquid chromatography (HPLC). A 2 ml aliquot of the extract was diluted with 2 ml of dH_2O and 9 ml of methanol, yielding a 70% methanol solution. This solution was then passed through a BioRad Biologic Duoflow HPLC using a Waters 7.8 mm x 300 mm Novapak HR C_{18} hydrophobic column as the stationary phase. Deaerated dH2O (buffer A) and deaerated methanol (buffer B) were used as the mobile phase. A total of 2 ml extract was loaded onto the column via 3 consecutive 0.7 ml injections. For each run, the UV detector was set to 254 nm, and a chromatogram file was produced showing absorbance of the various compounds in the extract. Because the inhibitory compound was unidentified, 100 µl of every other fraction of the 90 fractions collected for each run was tested for antibiotic activity, regardless of absorbance peaks. The antibiotic activity of the fractions was then compared to the chromatogram file to determine the gradient of methanol:water that provided the best separation of the inhibitory compound from impurities in the extract.

Amino Acid Analysis by Thin Layer Chromatography

Acid hydrolysis was carried out by mixing an equal volume of 6 M HCl with a small aliquot (200 µl) of HPLC purified material and autoclaved at 121^{o}C for 6 ½ hours. The acid hydrolyzed material was spotted onto the TLC plate using silica gel as the stationary phase along with amino acid standards. Standards of the 20 amino acids plus ornithine were prepared by mixing 1 mg of amino acid with 1 ml of ddH2O. The spots were allowed to dry completely before placing the TLC plate in a chamber with the solvent system propanol:water (70:30) (Storey 2005). The solvent was allowed to migrate up the plate until ~ 1 cm from the top, at which point the plate was removed from the chamber. The TLC plate was then sprayed with 0.25% ninhydrin, dried at room temperature, then sprayed with 0.1% n-cyanoguanidine, and developed at 55^{o}C until spots had formed (Storey 2005).

Characterization of the Inhibitory Material

Extract that had been passed through the LH-20 column twice was brought up in 2 ml of dH_2O, yielding 2.875 ml of extract. Eight hundred seventy-five microliters of the extract was saved for the purpose of characterizing the compound. To test its spectrum of activity, 25 µl of extract was applied to paper disks and allowed to air dry. The disks were then placed on plates containing the following laboratory indicator strains: *Klebsiella pneumoniae, Enterobacter aerogenes, Enterococcus faecalis, Streptococcus pyogenes, Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus mutans, Staphylococcus aureus, Staphylococcus saprophyticus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Pseudomonas denitrificans, Citrobacter freundii, Proteus vulgaris, Salmonella typhi, Salmonella arizoniae, Shigella dysenteriae, Shigella boydii, Shigella sonnei, Lactobacillus lactis, Bacillus subtilis,*

Bacillus megaterium, *Bacillus licheniformis*, *Neiserria subflava*, *Alcaligenes faecalis*, *Serratia marcesens*, *Corynebacterium diphtheria*, *Clostridium sporogenes*, *Mycobacterium smegmatis*, *Aspergillus niger*, *Penicillium notatum*, *Candida albicans*, and *Saccharomyces cervisiae*. Several bacterial species that were isolated during enrichment culturing were also used as indicator strains. *L. lactis*, *S. epidermidis*, and *S. mutans* seed cultures were grown on brain-heart infusion (BHI) broth and tested on BHI plates. All other organisms were grown in MH broth (for seed cultures) and tested on MH plates. Seed cultures were grown overnight and diluted to a 0.5 McFarland standard before inoculation onto plates using a sterile swab. Paper disks containing 25 µl of LH-20 column purified extract were placed in the middle of the plates after inoculation, and incubated overnight or until bacterial growth was clearly seen. Any plates containing zones of growth inhibition were photographed and the zone of inhibition measured and recorded.

Characterization of Isolate BTHX2

Characterization of the *Bacillus* organism found to produce inhibitory activity was carried out using various assays as described in the most recent edition of Bergey's manual of systematic bacteriology. These tests included Gram-staining, spore staining, fermentation of fructose, glucose, arabinose, trehalose, and xylose; the hydrolysis of starch, gelatin, and urea; use of citrate; growth at 4, 24, 37, and 55 ^{o}C; growth in 2, 5, 7, and 10% NaCl; and growth at 5, 6, 7, 8, 9, and 10 pH. Sugar fermentation was carried out using 4 ml purple broth base supplemented with 1 ml of a 10% solution of the appropriate sugar (mentioned above). The NaCl and pH plates were prepared using Bacto Nutrient Agar as the growth medium. Spore staining was carried out by covering the slide with malachite green dye and heating until the dye steamed for

1 minute. The malachite green was then washed off, and safranin used as a counter stain before observation with a microscope.

CHAPTER 3

RESULTS

Carbon Source Enrichment Culture

For Sinking Creek soil TCE was used as the only source of carbon in an enrichment culturing experiment. This yielded 5 Gram-positive isolates identified by 16S rDNA sequence analysis (table 2). The TCE enrichment gave a poor yield of the target bacterium: only 1 *Rhodococcus* along with 3 *Arthrobacter* and an *Aeromicrobium*. In contrast, hexadecane enrichment of hydrocarbon polluted soils gave an excellent yield of rhodococci: Out of 12 isolates, 11 were *Rhodococcus* and the remaining isolate (a member of the genus *Gordonia*) is a close relative to *Rhodococcus* (table 2).

Table 2: Isolates recovered from hexadecane and TCE carbon source enrichment.

Soil Source	Enrichment	Pigmentation	Genus[a]	Designation[b]
Sinking Creek	TCE	Cream	*Arthrobacter*	SCTEC2
Sinking Creek	TCE	Yellow	*Arthrobacter*	SCTEY2
Sinking Creek	TCE	Clear	*Aeromicrobium*	SCTEC3
Sinking Creek	TCE	Orange	*Rhodococcus*	SCTEO3
Sinking Creek	TCE	Yellow	*Arthrobacter*	SCTEY3
Old Coal Pile (ETSU)	Hexadecane	Pink	*Rhodococcus*	OCHXP1
Embreeville	Hexadecane	Orange	*Rhodococcus*	EMHXO1
Embreeville	Hexadecane	Light Orange	*Gordonia*	EMHXO2
Embreeville	Hexadecane	Cream/Orange	*Rhodococcus*	EMHXC1
Embreeville	Hexadecane	Cream/Orange	*Rhodococcus*	EMHXC2
Kingsport Coal	Hexadecane	Cream	*Rhodococcus*	KCHXC3
Kingsport Coal	Hexadecane	White	*Rhodococcus*	KCHXW3
Kingsport Basement	Hexadecane	White	*Rhodococcus*	BMHXW5
Kingsport Basement	Hexadecane	Mucoid	*Rhodococcus*	BMHXM5
Butler, TN	Hexadecane	cream	*Rhodococcus*	BTHXC2
Butler, TN	Hexadecane	cream	*Rhodococcus*	BTHXC6
Butler, TN	Hexadecane	Cream	*Rhodococcus*	BTHXC10

a: The genus of an isolate was derived by comparing the 16S rDNA sequence data to an online data base at http://rdp.cme.msu.edu. b: for designation: the first 2 letters pertain to the soil source, the second 2 letters are the enrichment used, the last letter is pigmentation, and the number is the order of isolation.

M3 Enrichment Culture

M3 enrichment culture proved to be a very successful method of isolating *Rhodococcus* from soil samples (figure 3). M3 enrichment using 6 local soils yielded 17 Gram-positive isolates. Eleven of these isolates were *Rhodococcus*, along with 3 *Gordonia* and 1 *Mycobacterium*, both of which are closely related to *Rhodococcus* (table 3). Other unrelated species include a *Cellulomonas* and an *Agromyces.*

In addition to local soils, several soils from exotic locations were used with M3 enrichment culturing, including soil from Africa, Alaska, and Aruba. M3 enrichment with these soils yielded 19 isolates, including 11 *Rhodococcus*, 3 *Arthrobacter*, 1 *Methylobacterium*, and 1 *Micrococcaea* (table 4).

Table 3: Isolates recovered by M3 enrichment of local soils

Soil Source	Enrichment	Pigmentation	Genus[a]	Designation[b]
Winged Deer Park	M3 (HEAT)	Dark Pink	*Rhodococcus*	WDM3P2
Winged Deer Park	M3 (HEAT)	Dark Orange	*Gordonia*	WDM3O11
Winged Deer Park	M3 (HEAT)	Light Pink	*Rhodococcus*	WDM3P13
Winged Deer Park	M3 (HEAT)	Cream/Pink	*Rhodococcus*	WDM3P18
Winged Deer Park	M3 (HEAT)	White	*Rhodococcus*	WDM310A
Winged Deer Park	M3 (HEAT)	White	*Rhodococcus*	WDM310A1
Winged Deer Park	M3 (HEAT)	Orange	*Gordonia*	WDM3O5
Winged Deer Park	M3 (HEAT)	Orange	*Rhodococcus*	WDM3O7A
Winged Deer Park	M3 (HEAT)	Yellow	*Cellulomonas*	WDM3Y6
Winged Deer Park	M3 (HEAT)	Orange	*Gordonia*	WDM3O10B
Kingsport Ditch	M3 (HEAT)	Cream	*Rhodococcus*	DSM3C1
Kingsport Ditch	M3 (HEAT)	Cream	*Rhodococcus*	DSM3C2
Basement Drain Exit	M3 (HEAT)	Cream/Yellow	*Rhodococcus*	BEM3C1
Basement Drain Exit	M3 (HEAT)	Yellow	*Agromyces*	BEM3Y1
Sinking Creek	M3 (HEAT)	Light Orange	*Rhodococcus*	SCM3O11
Butler, TN	M3 (HEAT)	White/Yellow	*Rhodococcus*	B1M3W4
Embreeville	M3 (HEAT)	Orange/Yellow	*Mycobacterium*	EM3O3

a: The genus of an isolate was derived by comparing the 16S rDNA sequence data to an online data base at http://rdp.cme.msu.edu. b: for designation: the first 2 letters pertain to the soil source, the second 2 letters are the enrichment used, the last letter is pigmentation, and the number is the order of isolation.

Figure 3: M3 plate showing pigmented colonies

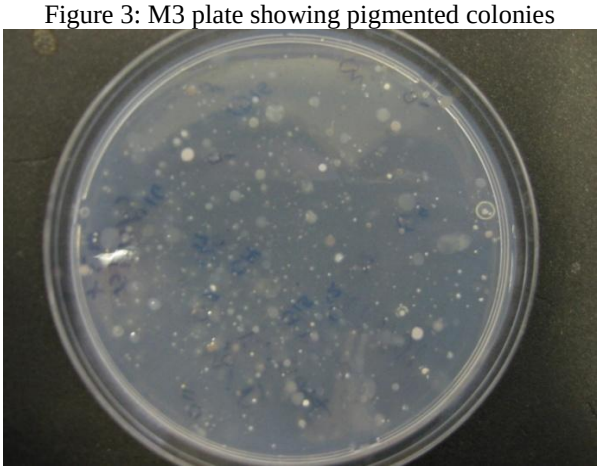

Table 4: Isolates recovered from M3 enrichment of exotic soils

Soil Source	Enrichment	Pigmentation	Genus[a]	Designation[b]
Aruba	M3 (HEAT)	Cream/White	Rhodococcus	ASM3W2
Africa, Mali	M3 (HEAT)	Red	Rhodococcus	A2M3R1
Africa, Mali	M3 (HEAT)	White	Micrococcaea	A1M3W6
Africa, Mali	M3 (HEAT)	White	Arthrobacter	A1M3W5
Africa, Mali	M3 (HEAT)	Cream/Clear	Rhodococcus	A2M3C2
Africa, Mali	M3 (HEAT)	Orange	Rhodococcus	A2M3O7
Africa, Mali	M3 (HEAT)	Red	Rhodococcus	A2M3R1
Africa, Mali	M3 (HEAT)	red	Methylobacterium	A1M3R4
Africa, Mali	M3 (HEAT)	Orange	Rhodococcus	A2M3O6
Alaskan Soil (cliffside)	M3 (HEAT)	Cream	Rhodococcus	ACM3C1
Alaskan Soil (cliffside)	M3 (HEAT)	Small White	Rhodococcus	ACM3W1
Alaskan Soil (cliffside)	M3 (HEAT)	Small White	Rhodococcus	ACM3W5
Alaskan Soil (cliffside)	M3 (HEAT)	Orange	Rhodococcus	ACM3W7
Alaskan Soil (cliffside)	M3 (HEAT)	Small Cream	Rhodococcus	ACM3C8
Alaskan Soil (cliffside)	M3 (HEAT)	Orange	Rhodococcus	ACM3O13
Alaskan Soil (cliffside)	M3 (HEAT)	White	Arthrobacter	ACM3W16
Alaskan Soil (cliffside)	M3 (HEAT)	Clear/Cream	Rhodococcus	ACM3W17D
Alaskan Soil (cliffside)	M3 (HEAT)	White	Rhodococcus	ACM3W20
Alaskan Soil (Glacier)	M3 (HEAT)	Yellow	Arthrobacter	AGM3Y4

a: The genus of an isolate was derived by comparing the 16S rDNA sequence data to an online data base at http://rdp.cme.msu.edu. b: for designation: the first 2 letters pertain to the soil source, the second two letters are the enrichment used, the last letter is pigmentation, and the number is the order of isolation.

Screening for Antibiotic Activity

All isolates identified by 16S rDNA sequence were screened for antibiotic activity (tables 5 and 6). EMHXC2, which was isolated from hexadecane enrichment, initially exhibited strong inhibitory activity against *M. luteus*. However, this activity was unable to be reproduced. Several attempts were made to 'induce' production of the inhibitory compound, including culturing with diluted RM broth (1/2 and 1/10 normal nutrient concentration), LB broth, BH with 0.1% hexadecane as the sole carbon source, BSM containing 10 µM phosphate, and extending the culturing time to 8 days. EMHXC2 exhibited weak inhibitory activity against *R. erythropolis* after culturing for 8 days. This activity was more pronounced when EMHXC2 was cultured in LB broth. The initial activity in the culture extract against *M. luteus* was finally attributed to

Table 5: Antibiotic screening of isolates derived from TCE/hexadecane enrichment.

Test Isolate	*M. luteus*	*E. coli*	*R. erythropolis*
SCTEC2	-	-	-
SCTEY2	-	-	-
SCTEC3	-	-	-
SCTEO3	-	-	-
SCTEY3	-	-	-
OCHP1	-	-	-
EMHXO1	-	-	-
EMHXO2	-	-	-
EMHXC1	-	-	-
EMHXC2	-	-	-[w]
KCHXC3	-	-	-
KCHXW3	-	-	-
BMHXW5	-	-	-
BMHXM5	-	-	-
BTHXC2	-	-	-
BTHXC6	-	-	-
BTHXC10	-	-	-

w: Weak growth observed around disk

Table 6: Antibiotic screening of isolates derived from M3 enrichment.

Test Isolate	Indicator Strain		
	M. luteus	*E. coli*	*R. erythropolis*
WDM3P2	-	-	-
WDM3O11	-	-	-
WDM3P13	-	-	-
WDM3P18	-	-	-
WDM310A	-	-	-
WDM310A1	-	-	-
WDM3O5	-	-	-
WDM3O7A	-	-	-
WDM3Y6	-	-	-
WDM3O10B	-	-	-
DSM3C1	-	-	-
DSM3C2	-	-	-
BEM3C1	-	-	-
BEM3Y1	-	-	-
SCM3O11	-	-	-
B1M3W4	-	-	-
EM3O3	-	-	-
ASM3W2	-	-	-[w]
A2M3R1	-	-	-
A1M3W6	-	-	-
A1M3W5	-	-	-
A2M3C2	-	-	-
A2M3O7	-	-	-
A2M3R1	-	-	-
A1M3R4	-	-	-
A2M3O6	-	-	-
ACM3C1	-	-	-
ACM3W1	-	-	-
ACM3W5	-	-	-
ACM3W7	-	-	-
ACM3C8	-	-	-
ACM3O13	-	-	-[w]
ACM3W16	-	-	-
ACM3W17D	-	-	-
ACM3W20	-	-	-
AGM3Y4	-	-	-

w: Weak bacterial growth observed around disk

contamination of the culture broth by another microorganism, and further research into EMHXC2 was abandoned. Interestingly, isolates ASM3W2 and ACM3O13 also displayed activity against *R. erythropolis* after 8 days of culture. However, the activity from these isolates was too weak to be of interest. Another organism isolated from hexadecane enrichment, BTHXC6, also initially exhibited activity against *M. luteus* and *R. erythropolis*. Analysis of the RM plate streaked from the culture broth revealed multiple colony phenotypes in addition to BTHXC6. BTHXC6 and the contaminant were both re-screened for antibiotic activity, and it was found that the contaminant was producing the inhibition against *M. luteus* and *R. erythropolis*. Subsequent streak plates isolated 2 different contaminants, designated BTHX1 and BTHX2. Isolate BTHX2 exhibited the stronger inhibitory activity, and thus was pursued for further study (figure 4).

Characterization of BTHX2

The 16S rDNA sequence data for BTHX2 showed 97% homology to *Bacillus licheniformis*. A series of phenotypic assays were then carried out to confirm the identity of BTHX2, including staining for the presence of endospores; fermentation of glucose, fructose, arabinose, trehalose, and xylose; use of urea, citrate, and hexadecane; hydrolysis of starch and gelatin; and growth at different temperature, salt concentrations, and in a pH range of 5 – 10. To determine the point of origin of the contaminant BTHX2, the RM slant containing BTHXC6 was streaked for purity. As BTHXC6 was isolated using hexadecane enrichment, the ability of BTHX2 to use hexadecane as a source of carbon and energy was also tested. The streak plate of

the BTHXC6 slant did not show any contamination. However, BTHX2 readily used hexadecane as the sole source of carbon and energy (table 7).

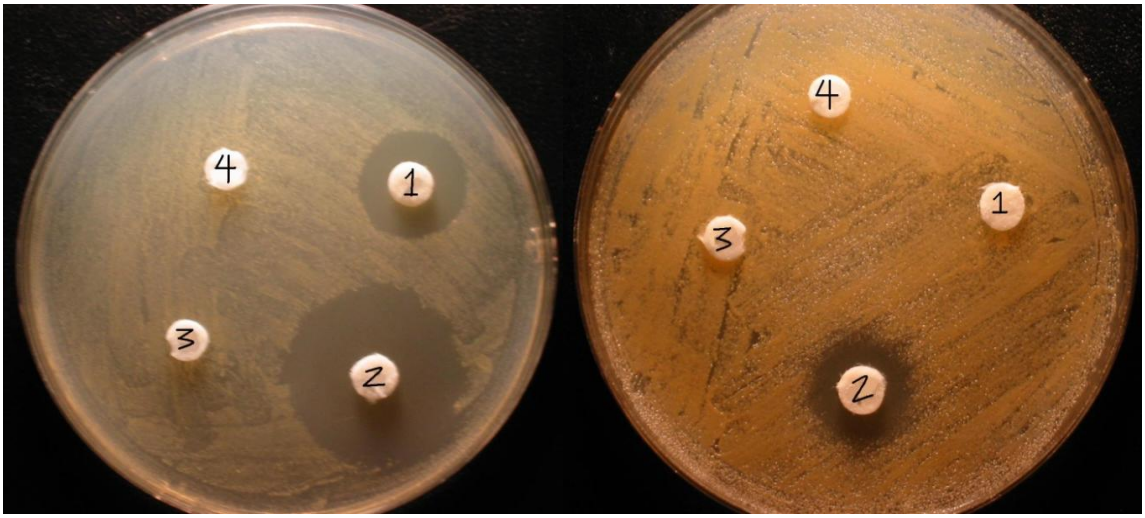

Figure 4: Inhibitory activity of BTHX1 and BTHX2. Left plate: *M. luteus.* Right plate: *R. erythropolis* IGTS8. Supernatant and cell/resin extract from BTHX1 and BTHX2. Each disk contains 50 µl of either cell and resin extract or supernatant from BTHX1 or BTHX2. Disk 1 contains extract from BTHX1, and disk 3 contains supernatant from BTHX1. Disk 2 contains extract from BTHX2 and disk 4 contains supernatant from BTHX2. Supernatants and extracts were prepared from 50 ml cultures of BTHX1 and BTHX2 grown for 4 days with resin.

Table 7: Phenotypic characteristics of BTHX2

Characteristic	**Result**	**Characteristic**	**Result**	**Characteristic**	**Result**
Acid from:		*Hydrolysis of:*		*Growth at:*	
Glucose	+	Starch	+	5% NaCl	+
Fructose	+	Gelatin	-	7% NaCl	+
Arabinose	+	*Growth at:*		10% NaCl	+
Trehalose	+	4 ^{o}C	-	pH 5	+
Xylose	-	25 ^{o}C	+	pH 6	+
Utilization of:		30 ^{o}C	+	pH 7	+
Citrate	-	37 ^{o}C	+	pH 8	+
Urea	+	55 ^{o}C	-	pH 9	+
Hexadecane	+	2% NaCl	+	pH 10	+

Thus, the origination of contaminant BTHX2 was unable to be established. BTHX2 produces endospores that are oval in shape, and appear to form near the center of the cell (figure 5). BTHX2 readily grew on Simmon's Citrate media, but did not produce an alkaline product, as the media did not change color. BTHX2 was able to hydrolyze starch and urea, but not gelatin (table

7). BTHX2 is a mesophile, as it did not grow at 4°C or 55°C (table 7). BTHX2 is also halotolerant, as it was able to grow in the presence of 10% NaCl (table 7). In addition, the growth of BTHX2 was unaffected by a pH range of 5 – 10, or the presence of oxygen (table 7). Interestingly, these phenotypic results rule out the possibility of BTHX2 being a strain of *B. licheniformis*, as BTHX2 does not produce acid from xylose and does not hydrolyze gelatin. Moreover, BTHX2 expresses different colony morphology when compared to *B. licheniformis* (figure 6). In fact, the phenotype of BTHX2 did not match any of the species described in Bergey's Manual of Systematic Bacteriology. Thus, at this time it can only be stated conclusively that BTHX2 is most genetically similar to *B. licheniformis* based off of 16S rDNA sequence data.

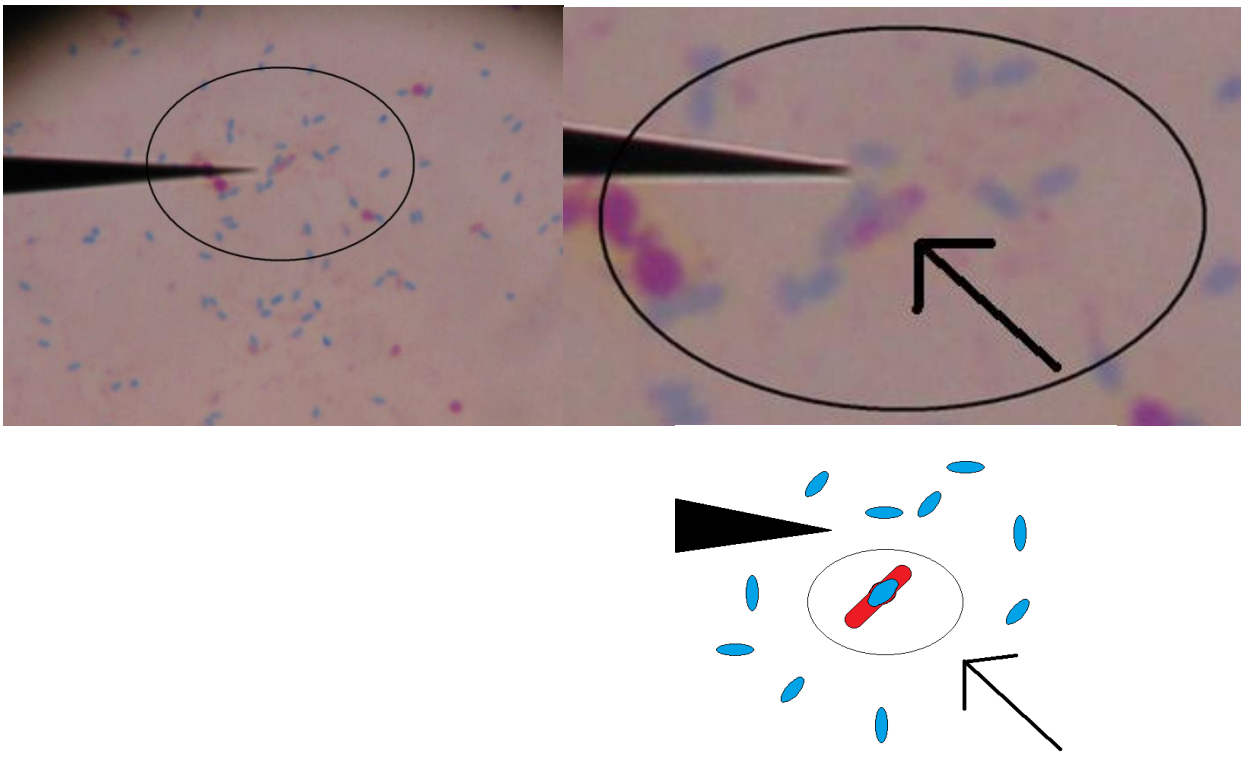

Figure 5: Spore stain of BTHX2. The endospores are stained blue, and vegetative cells are stained red. An endospore within a vegetative cell is circled. Left: larger view. Right: zoomed view of the endospore. Bottom: diagram of zoomed view of endospore. The endospore forming inside the cell is circled.

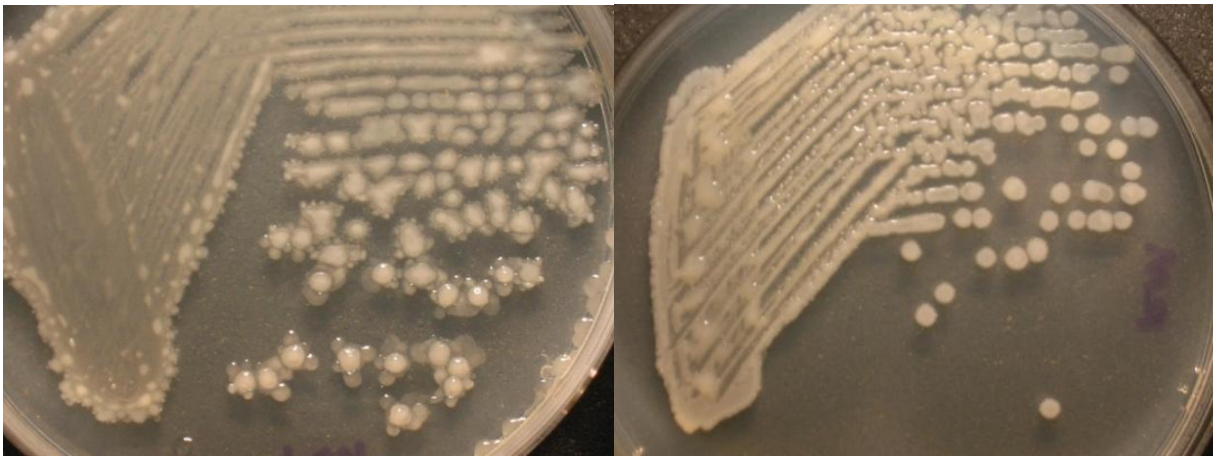
Figure 6: A comparison of BTHX2 (left) to *B. licheniformis* (right).

XAD-16 Column Purification

Another aim of this research was to characterize the inhibitory substance produced by BTHX2. First, BTHX2 was grown in 1 L batches (for a total of 5 L) and extracted as detailed in Materials and Methods, and brought up in 40 ml dH_2O. Next, the extract from the 5 L scale up was "cleaned" by passage through a column packed with "Amberlite" XAD-16. The evaporated, crude extract was reconstituted in 40 ml of dH2O and poured directly onto the column. To bind the compound to the resin, 1L of ddH2O was passed through the column, and collected in a 2L beaker. Next, the column was washed with 1L of ddH2O, and the material was eluted with methanol as the mobile phase. A total of 13 fractions were collected in 50 ml aliquots. The column was then washed with 1L of methanol, followed by 2L of ddH2O. All the steps of this purification, including washes, flowthrough, and the fractions were tested for antibiotic activity. Only fractions 3 – 8 showed inhibitory activity against *M. luteus* (figure 7). These fractions were evaporated to dryness and pooled using 10 ml methanol. This 'cleaned' extract was further purified using an LH-20 column.

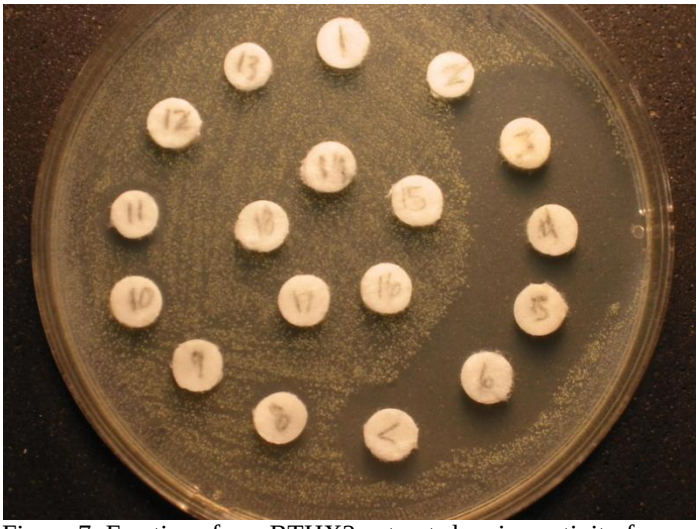

Figure 7: Fractions from BTHX2 extract showing activity from XAD-16 column purification.

LH-20 Column Purification

The XAD-16 purified extract was passed through an LH-20 column in 2 runs using 5 ml aliquots. The extract was poured directly onto the column, which was run using methanol as the mobile phase. For the 1st run, a total of 47 fractions were collected in 175 drop aliquots. Fractions 13 – 21 had activity against *M. luteus* (figure 8). For the 2nd run, a total of 45 fractions of the same volume were collected, with fractions 15 – 22 showing activity (figure 9). All the active fractions from both runs were pooled and evaporated to dryness, then reconstituted in 2 ml of dH2O, yielding a total of 2.8 ml of extract. An aliquot of 0.8 ml was saved for the purposes of testing the extract against a broad range of indicator strains. The remaining 2 ml was used for purification using HPLC.

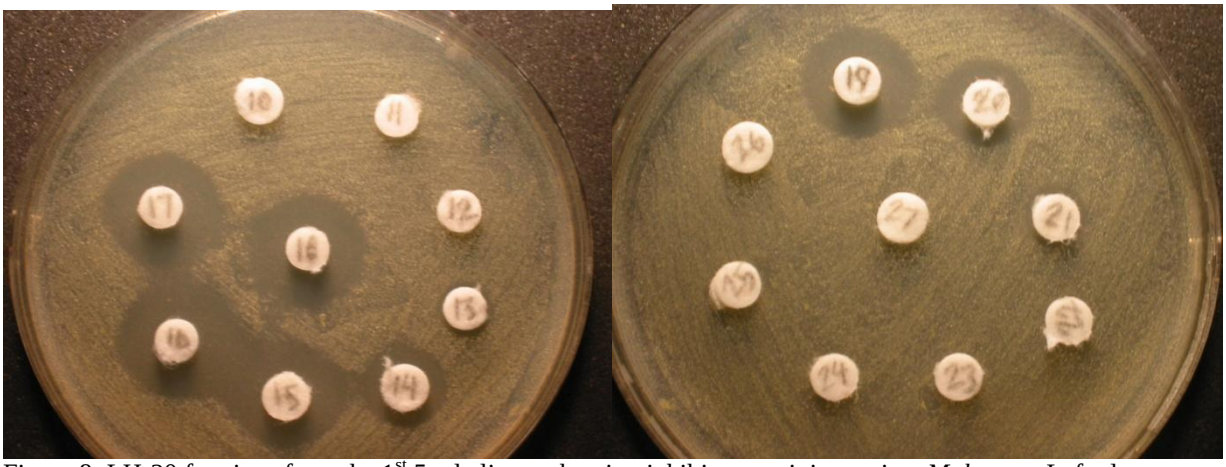

Figure 8: LH-20 fractions from the 1st 5 ml aliquot showing inhibitory activity against *M. luteus*. Left plate: fractions 10 – 18. Right plate: fractions 19 – 27.

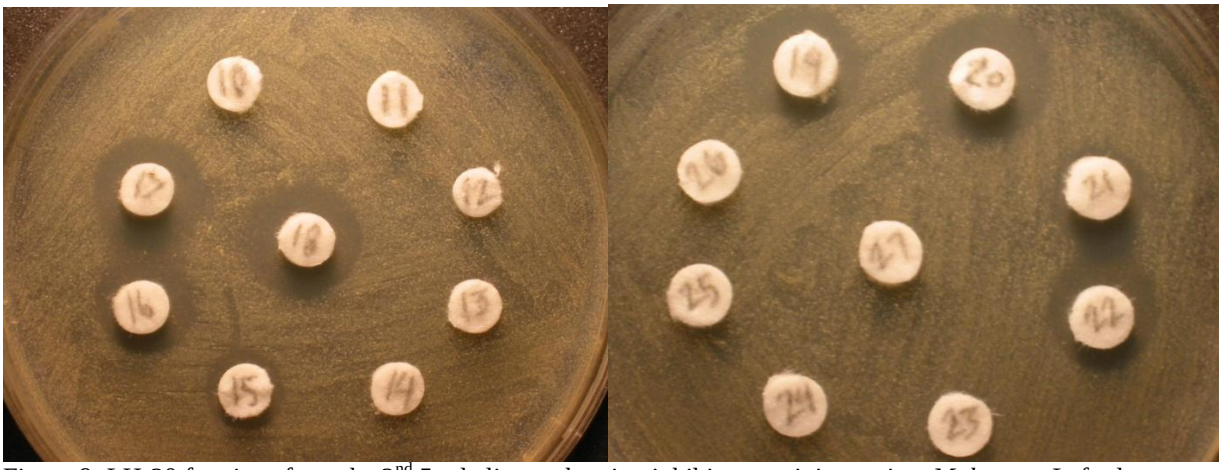

Figure 9: LH-20 fractions from the 2nd 5 ml aliquot showing inhibitory activity against *M. luteus*. Left plate: fractions 10 – 18. Right plate: fractions 19 – 27.

HPLC Purification

The extract from the LH-20 purification was extremely viscous and turbid. Centrifugation of the extract at 10,000 rpm for 30 minutes did not remove any precipitants. Therefore, the extract was diluted first with 2 ml of ddH2O and second with 9 ml of methanol, yielding a 70% methanol solution (13 ml total volume). The extract was then syringe filtered to remove any remaining precipitants and saved in a 15 ml polystyrene tube. To ensure the

inhibitory substance was not contained in the precipitants, the syringe filter was washed with 1 ml of methanol, and the precipitants were not found to have inhibitory activity. HPLC was used to purify the extract in 2 ml aliquots (as described in Materials and Methods). The first aliquot was purified using a linear gradient of 90% methanol to 100% methanol over 50 ml, which resulted in 2 large peaks on the chromatogram (figure 10). Fractions 23 – 29 exhibited activity against *M. luteus* (figure 11), which corresponded to 93 – 94% methanol (figure 10). To achieve better separation of the inhibitory substance from impurities, another HPLC program was run using a gradient of 85% methanol to 95% methanol over the course of 70 ml with a fresh aliquot of extract. This program resulted in 4 peaks, with the inhibitory compound eluting at 87% to 88% methanol (figure 12, figure 13). This program was used for the remainder of the extract, producing similar results with each run. Purification using HPLC is continuing.

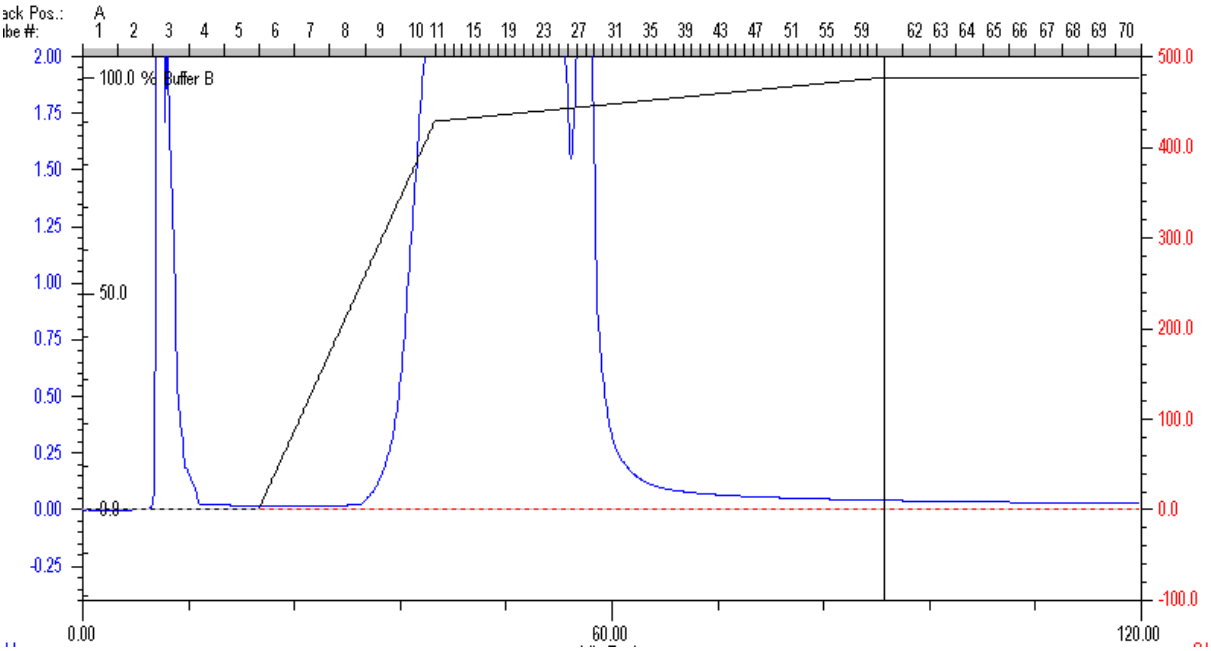

Figure 10: HPLC chromatogram using the first 2 ml aliquot of LH-20 purified extract.

Figure 11: Fractions showing activity from 1st HPLC run. Disks 12, 13, 14, and 15 represent fractions 23, 25, 27, and 29, respectively.

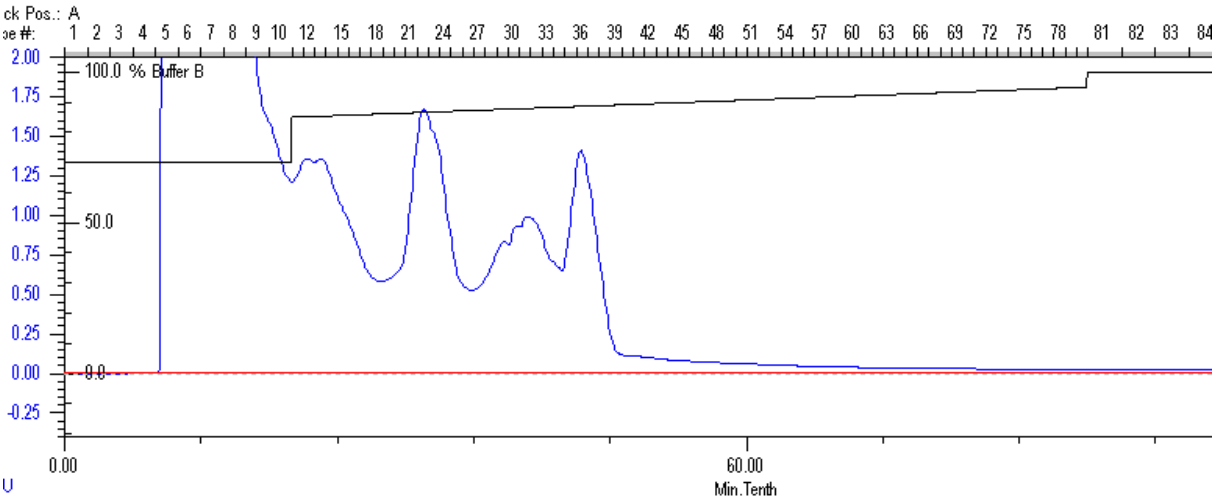

Figure 12: HPLC chromatogram using the second 2 ml aliquot of LH-20 purified extract.

Figure 13: Fractions showing activity 2nd HPLC run. The number on the disk is the same as the fraction.

Amino Acid Analysis by Thin Layer Chromatography

Many of the known antibiotics produced by *Bacillus* are peptide compounds. Thus, the amino acid content of the inhibitory material analyzed. To this end, the HPLC purified material was acid hydrolyzed, spotted on TLC plate along with the 20 standard amino acids plus ornithine, and placed in a TLC chamber containing the solvent system propanol:water (70:30). To visualize the amino acids, the TLC plate was developed by spraying first with ninhydrin and then with n-cyanoguanidine before incubation at 55^{o}C. Amino acid spots on the plate then were visible as red streaks. The migration of the amino acid standards up the plate were compared with the migration of the amino acids in the hydrolyzed sample. Amino acids matching the migration profile of the hydrolyzed sample were run on subsequent TLC plates to confirm the match. Using 70% propanol as the mobile phase, the acid hydrolyzed material appeared to contain 4 different amino acids (figure 14). The first two amino acid spots in the sample very clearly correspond to glutamic acid and valine. The third amino acid spot appeared to correspond to either leucine or phenylalanine, as both amino acids exhibited similar migration

profiles. The fourth amino acid in the sample migrated much higher up the TLC plate than did any of the standards, and the closest match that could be found was tryptophan. All 5 of these amino acids are common in antibiotics.

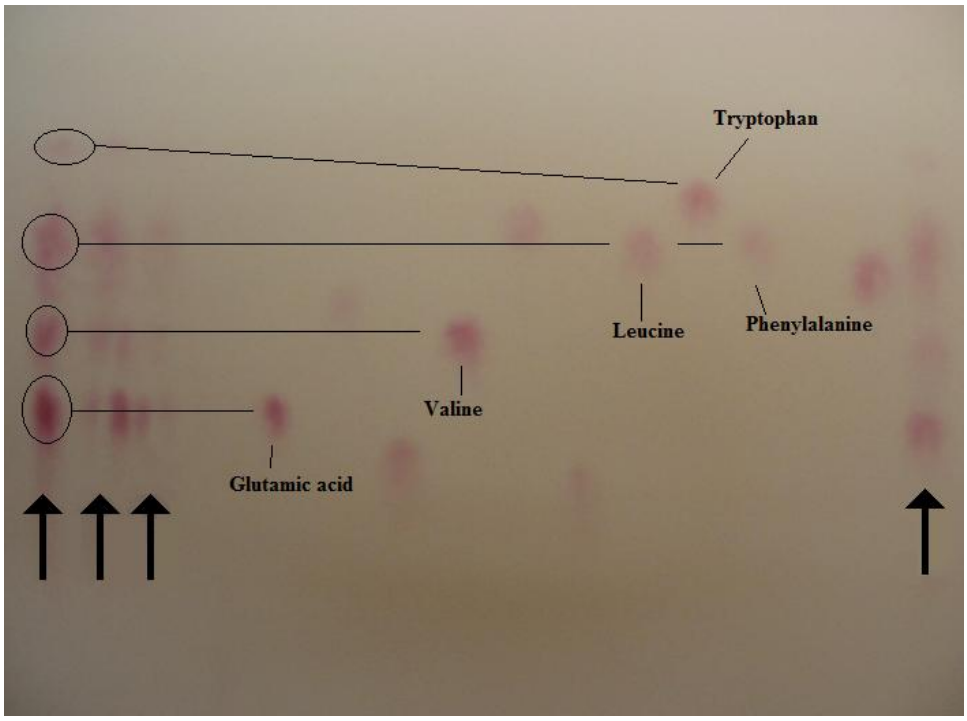

Figure 14: TLC plate of the acid hydrolyzed sample (arrows) and a narrow range of amino acid standards.

Spectrum of Activity of BTHX2

To test the spectrum of the LH-20 purified material, 25 µl aliquots of the 0.8 ml sample was applied to paper disks, and placed on MH plates with a broad selection of indicator strains (table 8). The material exhibited no activity against any of the Gram-negative organisms tested. However, the extract exhibited activity against most Gram-positive organisms. For members of the genus *Bacillus* activity was very weak, showing only a few millimeters of inhibition (figure 15 and table 8). This was also true for *S. aureus and S. epidermidis* (figure 15 and table 8). Much stronger activity was seen in the members of *Actinobacteria* tested, especially against *M. smegmatis* (29mm), *M. luteus* (29mm), *Gordonia* (37mm), *Rhodococcus* A2M3O7 (34mm), and

C. diphtheria (24mm). Other bacteria strongly inhibited by the material include all *Streptococcus* tested, *Staphylococcus saprophyticus*, and *Clostridium sporogenes*. The material also exhibited antifungal activity against the molds *A. niger* and *P. notatum*, but had no activity against the yeasts *C. albicans* and *S. cervisiae.* Interesting data were observed when testing the inhibitory material against *Streptococcus pneumoniae* on blood agar (figure 15i). The growth of *S. pneumonia* is not very visible, but a clear ring of red blood cell hemolysis is seen around the disk containing the inhibitory material, suggesting possible cytolytic activity.

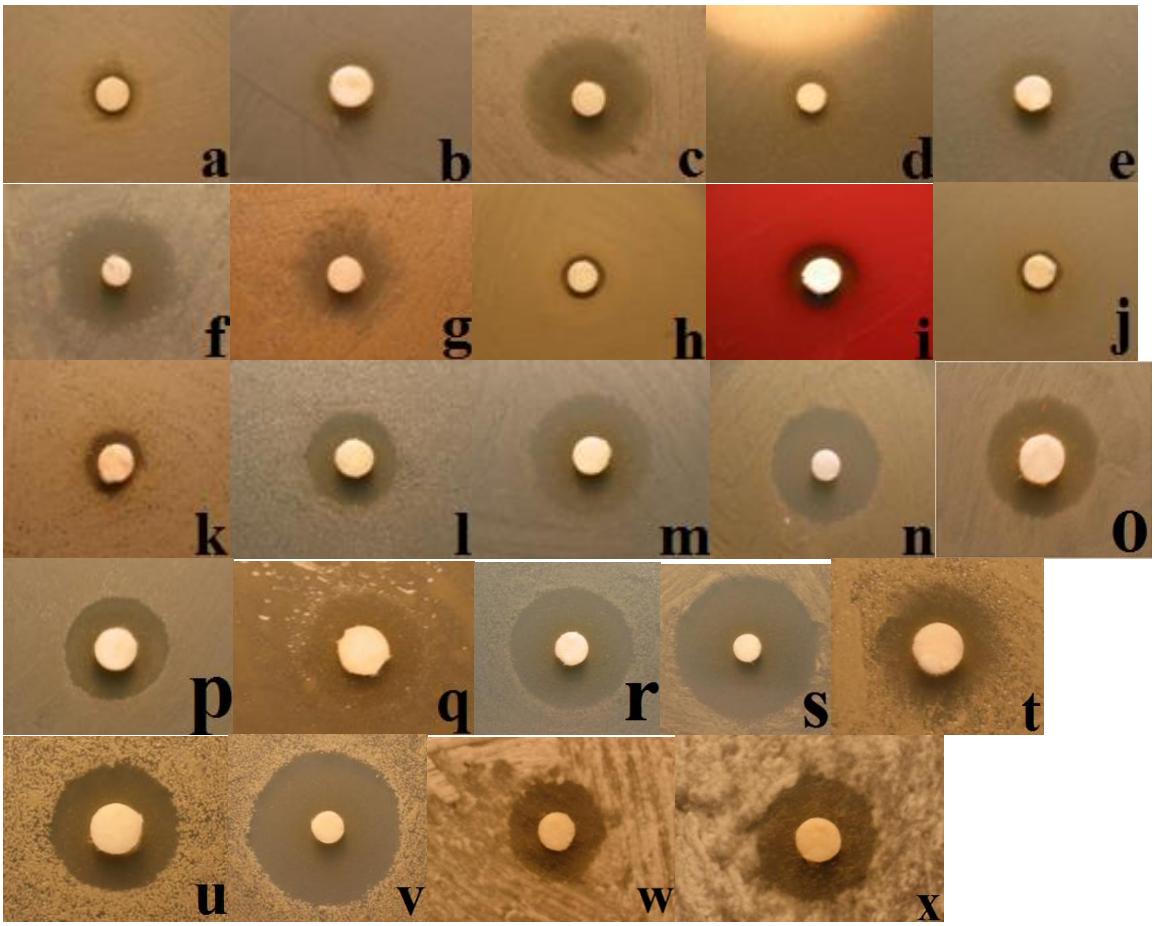

Figure 15: Inhibitory spectrum of BTHX2 partially purified extract. Disks containing 25 µl of LH-20 purified extract were placed on plates with indicator strains (a – x). A: *S. aureus*, zone size: 10 mm; B: *S. epidermidis*, zone size: 12 mm; C: *S. saprophyticus*, zone size: 28 mm; D: *Strep. agalactiae*, zone size: 15 mm; E: *L. lactis*, zone size: 13 mm; F: *M. smegmatis*, zone size: 29 mm; G: *R. erythropolis*, zone size: 15 mm; H: *B. cereus*, zone size: 13 mm; I: *Strep. pneumoniae* on a blood plate (note the zone of complete hemolysis around the disk), zone size 15 mm; J: *B. licheniformis*, zone size: 10 mm; K: *B. megaterium*, zone size: 11 mm; L: *M. roseus*, zone size:19 mm; M: *C. diphtheriae*, zone size: 24 mm; N: *M. luteus*, zone size: 29 mm; O: *S. mutans*, zone size: 18 mm; P: *S. pyogenes*, zone size: 17 mm; Q: *C. sporogenes*, zone size: 17 mm; R: *Aeromicrobium* SCTEC3, zone size: 27 mm; S: *Gordonia* WDM3O5, zone size: 37 mm; T: *Rhodococcus* ACM3W20, zone size: 17 mm; U: *Rhodococcus* A2M3R1, 18 mm; V: *Rhodococcus* A2M3O7, zone size: 34 mm, W: *A. niger*, zone size: 18 mm; X: *P. notatum*, zone size: 20 mm.

Table 8: Spectrum of activity of BTHX2 extract

Organism	Gram reaction	Activity	Zone size[a]
Bacillus cereus	+	+	13 mm
Bacillus licheniformis	+	+	10 mm
Bacillus megaterium	+	+	11 mm
Citrobacter freundii	-	-	
Corynebacterium diphtheriae	+	+	24 mm
Clostridium sporogenes	+	+	17 mm
Klebsiella pneumoniae	-	-	
Lactobacillus lactis	+	+	13 mm
Micrococcus luteus	+	+	29 mm
Micrococcus roseus	+	+	19 mm
Mycobacterium smegmatis	+	+	29 mm
Neiserria subflava	-	-	
Proteus vulgaris	-	-	
Pseudomonas aeruginosa	-	-	
Pseudomonas denitrificans	-	-	
Salmonella arizoniae	-	-	
Salmonella typhi	-	-	
Shigella boydii	-	-	
Shigella dysenteriae	-	-	
Staphylococcus aureus	+	+	10 mm
Staphylococcus epidermidis	+	+	12 mm
Staphylococcus saprophyticus	+	+	28 mm
Streptococcus agalactiae	+	+	15 mm
Streptococcus pneumoniae	+	+	15 mm
Streptococcus pyogenes	+	+	17 mm
Streptococcus mutans	+	+	18 mm
Rhodococcus erythropolis	+	+	15 mm
***Rhodococcus* ACM3W20**	+	+	17 mm
***Rhodococcus* A2M3R1**	+	+	18 mm
***Rhodococcus* A2M3O7**	+	+	34 mm
***Gordonia* WDM3O5**	+	+	37 mm
***Aeromicrobium* SCTEC3**	+	+	27 mm
Aspergillus niger	Fungus	+	18 mm
Penicillium notatum	Fungus	+	20 mm
Candida albicans	Fungus	-	
Saccharomyces cervisiae	Fungus	-	

a: size of paper disk containing extract: 7 mm

CHAPTER 4

DISCUSSION

Species of the genus *Bacillus* have been recognized as prolific antibiotic producers since the 'golden age' of antibiotic discovery (Schaffer 1969). Many antibiotics produced by *Bacillus* species are low molecular weight cyclic polypeptide compounds synthesized either ribosomally or non-ribosomally (by non-ribosomal peptide synthases) (Schaffer 1969, Nakano and Zuber 1990, Stein 2005). Ribosomally synthesized peptides include lantibiotics such as nisin and subtilin, which are active against Gram-positive organisms (Stein 2005). Bacillomycins and mycosubtilin, which are hemolytic and anti-fungal are examples of non-ribosomal peptides produced by *Bacillus* (Stein 2005). Many of the peptide antibiotics produced by *Bacillus* contain unusual amino acids and are also resistant to proteases (Schaffer 1969). Two peptide antibiotics produced by *Bacillus* have seen widespread use: bacitracin and polymyxin B. Bacitracin is a cyclic dodecapeptide produced by both *B. subtilis* and *B. licheniformis*, and is active mainly against Gram-positive organisms by inhibiting cell wall synthesis (Tay *et al.* 2010). Bacitracin is one of the active ingredients in the topical agents Neosporin and Polysporin, and is also used in livestock feed (Tay *et al.* 2010). Although this antibiotic has seen 50 years of use, resistance to bacitracin is still rare (Tay *et al.* 2010). Polymyxin B is part of the polymyxin class of drugs, cationic peptides that act against Gram-negative organisms by increasing outermembrane permeability (Gales *et al.* 2001). In the past polymyxin B was one of the few antibiotics effective against *P. aeruginosa*. However, other less toxic compounds have been found, and polymyxin B is now used as a topical agent (Tay *et al.* 2001). Species of *Bacillus* also produce polyketide antibiotics such as difficidin, which has a broad spectrum of activity against Gram-positive and Gram-negative organisms (Zimmerman *et al.* 1986).

During screening of the 54 identified soil bacteria, BTHX2 – a contaminant from the culture broth of BTHXC6 – was found to have inhibitory activity against both *R. erythropolis* IGTS8 and *Micrococcus luteus*. BTHX2 16S rDNA is 97% homologous with *B. licheniformis*. Interestingly, BTHX2 differs phenotypically from *B. licheniformis,* as BTHX2 does not hydrolyze gelatin or ferment xylose. Further phenotypic analysis shows BTHX2 to be facultative with regard to the presence of oxygen, mesophilic, and halotolerant, which is consistent with *B. licheniformis* (Logan and De Vos 2009). Coupled with 16S rDNA data, this suggests BTHX2 may be a new strain of *B. licheniformis*, or perhaps a new species that is very closely related to *B. licheniformis*. The inhibitory substance produced by BTHX2 was purified via column chromatography. Using HPLC analysis the substance was found to be very hydrophobic, as it was soluble only in a solution of 87 – 88% methanol. The HPLC purified material was analyzed for amino acid content and was found to contain four amino acids. These may correspond to glutamic acid, valine, leucine or phenylalanine, and tryptophan. The LH-20 purified material was tested against a range of microorganisms. The substance was not active against Gram-negative organisms but had broad spectrum activity against a broad spectrum of Gram-positive organisms. Weak inhibition was observed for species of *Bacillus*, *S. aureus*, and *S. epidermidis*. Against all other bacteria tested strong inhibition was observed, especially for *C. diphtheriae*, *M. luteus*, *M. smegmatis*, *S. saprophyticus*, *Rhodococcus* sp., *Gordonia* sp., and *Aeromicrobium*. The material was also has strong antifungal activity against the molds *A. niger* and *P. notatum*. Moreover, the material may exhibit cytolytic activity due to the zone of hemolysis observed on the blood plate containing *S. pneumoniae.* Future work with this substance should include further purification via HPLC and structural analysis, as well as optimization of culture conditions and identification of the genes responsible for production of

the inhibitory substance. It would also be interesting to further characterize the inhibitory spectrum of BTHX2 against a broader range of Gram-positive organisms and fungi. It would also be worthwhile to characterize the cytolytic activity of the material against eukaryotic cells, and to determine the species of BTHX2.

In 2008 the researchers Kitagawa and Tamura tested 80 strains of *Rhodococcus* for antibiotic activity, including many strains of *R. erythropolis* (Kitagawa and Tamura 2008a). They found that 14 *R. erythropolis* and 1 *R. globerulus* strain (roughly 19% of all the strains tested) produced inhibitory activity. These 15 organisms were further tested against 52 different indicator strains comprising α-, β-, and γ-*Proteobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes,* and *Actinobacteria*. The *Rhodococcus* strains were assigned to 3 groups according to the inhibitory activity of the antibiotics they produced. Group 1, comprised of 5 *R. erythropolis* strains, displayed a broad spectrum of activity against Gram-positive organisms. Group 2, comprised of 2 *R. erythropolis* strains and the *R. globerulus* displayed similar activity to group 1 in addition to activity against *Rhodococcus*. Group 3, comprised of 7 *R. erythropolis* strains had strong activity against *R. erythropolis* only. The compounds produced by groups 1 and 2 were extracted from the supernatants of their respective strains and partially characterized; the compound produced by group 3 could not be extracted from supernatant. Six months later the same researchers reported the characterization of aurachin RE from *R. erythropolis* strain JCM6824, which was one of the group 1 strains (Kitagawa and Tamura 2008b).

During this project 54 Gram-positive soil bacteria were isolated by enrichment culture, and 37 of these were identified as *Rhodococcus* via 16S rDNA sequence data. Kitagawa and Tamura found that roughly 19% of the rhodococci they tested produced inhibitory compounds. Thus it could be inferred that of the 36 *Rhodococcus* tested in this project, 6 to 7 would have

been expected to produce inhibitory activity. Unfortunately, none of the organisms isolated by enrichment culturing were found to produce inhibitory activity. Although some *Rhodococcus* were found to inhibit *R. erythropolis* IGTS8 after 8 days of culturing, this activity was extremely weak and therefore characterization of the activity was not pursued further. These findings were unexpected and discouraging, especially considering the success of other interested researchers in finding new inhibitory compounds produced by *Rhodococcus* (Iwatsuki *et al.* 2007, Kitagawa and Tamura 2008b, Kurosawa *et al.* 2008, Nachtigall *et al.* 2010). The work of these research groups suggests that *Rhodococcus* is a good source of antibiotics and that more compounds remain to be found from this genus. Therefore it is appropriate to consider what might have gone wrong, and what measures could be taken to overcome potential obstacles in the future.

One problem may be the use of only one type of growth media when conducting screening experiments. As discussed in Materials and Methods, all organisms were grown in 50 ml of RM broth to screen for antibiotic producers. In addition, culture conditions were not varied: all isolates were grown in a 30 ^{o}C water bath with shaking for 4 days. When searching for new antibiotics, other research groups have used the "One Strain Many Active Compounds" approach, where culture conditions are varied in an attempt to induce expression of the secondary metabolites. An excellent example would be *Aspergillus ochraceus* DSM7428 which is a known aspinonene producer (Bode and Müller 2005). This fungus was grown with complex and minimal media, and was grown with shaking and also without shaking (a static culture). Using these methods it was found that *A. ochraceus* produced 15 additional secondary metabolites (Bode and Müller 2005). Although none of these compounds were antibiotics, the results show the power of altering media and culture conditions in the quest for new natural products from microbes. In fact, one group of researchers undertook the OSMAC approach with

Streptomyces aizunensis NRRL B-11277 (McAlpine *et al.* 2005). *Streptomyces aizunensis* is a known producer of bicyclomycin. Genome scanning revealed 11 other biosynthetic gene clusters in addition to the cluster for bicyclomycin biosynthesis. One cluster was a large type 1 PKS, the product of which was predicted to be an amidohydroxycyclopentenone. The research group carried out fermentations in 50 different growth media to yield ECO-02301. Mass spectroscopy (MS) and nuclear magnetic resonance (NMR) results revealed that these researchers had bioinformatically predicted the structure of the compound. Although carrying out 50 different fermentations for 54 isolated organisms is far beyond the scope of a Masters level research, it shows that with patience, hard work and determination the OSMAC method can prove fruitful with the prior knowledge of an orphan biosynthetic pathway. Moreover, other researchers in this lab have found inhibitory activity from the *Rhodococcus* strains isolated during this project by attemping different screening techniques.

Heterologous expression could also be useful for tapping into the orphan biosynthetic gene clusters of *Rhodococcus*. Although *Rhodococcus* harbors the biosynthetic machinery for polyketides and non-ribosomal peptides, specific components may be missing, such as starter units for the pathway. Cloning the PKSs and NRPSs of *Rhodococcus* into a related organism known to produce antibiotics such as *Streptomyces* could provide an avenue of novel bioactive metabolites. Transfer of a PKS or NRPS has not yet been reported for *Rhodococcus* but has been performed successfully in other actinomycetes, such as *Streptomyces* (Bode and Müller 2005, Gross 2009). Moreover, rhodococcal PKSs and NRPSs could be expressed in other *Rhodococcus* strains reported to produce antibiotics (described previously). The rhodostreptomycins produced by *R. fascians* 307C0 are thought to be the product of horizontal gene transfer from *Streptomyces padanus* (Kurosawa *et al.* 2008). Interestingly, the

rhodostreptomycins are aminoglycoside compounds, and differ greatly from the actinomycin antibiotics produced by *S. padanus*. This suggests that the introduction of biosynthetic machinery from other *Streptomyces* (or other *Actinobacteria*) into *Rhodococcus* may bear fruit for the discovery of novel bioactive compounds.

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