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Characterization of the antimycobacterial effect of a pseudomonas-derived activity

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CHARACTERIZATION OF THE ANTIMYCOBACTERIAL EFFECT OF A *PSEUDOMONAS*-DERIVED ACTIVITY

Krupa Naran

WitsETD

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science.

Johannesburg, 2010

Have patience! In time even grass becomes milk.

- Charan Singh, mystic (1916-1990)

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Masters of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Krupa Naran)

14th day of October, 2010

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ABSTRACT

The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis, reinforces the need for novel antimycobacterial compounds. Secondary metabolites from various microorganisms have provided most antibacterials introduced clinically since 1935. Previously, a putative *Pseudomonas* isolate was identified that inhibited growth of the non-pathogenic *M. smegmatis* (MSM). Here, we demonstrate the stable isolation of the inhibitory compound(s) in crude extract, and present microbiological data characterizing the antimycobacterial effect. A parallel extraction was performed on an unrelated *Pseudomonas* isolate which failed to inhibit growth of MSM, thereby confirming that the inhibitory effect is limited to our strain, designated *Pseudomonas* αMB (anti-mycobacterial). Moreover, the crude extract inhibited growth of all Gram-positive organisms assayed, including other actinobacteria, but not the Gram-negative *E. coli,* suggesting the possibility of a Gram-restricted target range. As the cell wall constitutes the dominant target of natural-product antibacterials, we hypothesised that the active compound(s) might inhibit cell wall metabolism. However, preliminary data are inconclusive and the target of the extract remains to be elucidated, perhaps reflecting the presence of more than one active compound. Notably, the crude extract was shown by broth microdilution assay to inhibit growth of MTB at a concentration of 14-16 µg/ml, a value ten-fold higher than key frontline anti-TB agents tested. Therefore, although the identity of the constituent compound(s) and its mode of action are unknown, the apparent anti-MTB activity suggested by our preliminary experiments identifies the *Pseudomonas*-derived active agent(s) as a compelling candidate for further investigation as a potential lead compound(s) against a major human pathogen increasingly associated with drug resistance.

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List of Abbreviations

Abbreviation	Abbreviated Term
ADS	Albumin-Dextrose-Saline
AG	Aribinogalactan
ATP	Adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
Ca^{2+}	Calcium
CAR	Carbenicillin
cfu	Colony forming unit
DMSO	Dimethylsuphoxide
DNA	Deoxyribonucleic acid
DOTS	directly observed therapy, short course
EMB	Ethambutol
ERDR	Ethambutol resistance determining region
GS	Glucose salt
HIV	Human Immunodeficiency Virus
H^+	Hydrogen
INH	Isoniazid
K^+	Potassium
KAN	Kanamycin
LAM	Lipoarbinomannan
$MgCl_2$	Magnesium Chloride
MOA	Mechanism of action
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MIC_{EMB}	Minimum inhibitory concentration of ethambutol
MDR	Multidrug resistant
MSM	*Mycobacterium smegmatis*
MTB	*Mycobacterium tuberculosis*
NADH	Nicotinamide adenine dinucleotide, reduced

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OADC	Oleic acid, albumin-dextrose complex
PAS	Para-aminosalysilic acid
PCR	Polymerase chain reaction
PZA	Pyrazinamide
ROS	Reactive oxygen species
R	Resistance/resistant
RNA	Ribonucleic acid
RIF	Rifampicin
SDS	Sodium dodecyl sulphate
STM	Streptomycin
TB	Tuberculosis
VAN	Vancomycin
XDR	Extensively drug resistant
WHO	World Health Organization
ZOI	Zone of inhibition

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INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB), a gram-positive, facultative intracellular pathogen that successfully invades and persists within macrophages (34). Up to one third of the world's population is infected with this highly successful pathogen (145) and there are an estimated 8 million new TB cases and 2 million deaths worldwide each year. TB is the leading cause of death from an infectious agent worldwide (140). Several factors have contributed to the increase in incidence, including the Human Immunodeficiency Virus (HIV) pandemic, immigration, increased trade and globalization (145). HIV-associated TB has increased dramatically as the virus weakens the immune system, leading to the reactivation of latent TB. In addition, the threat of resistant strains, both multidrug resistant (MDR) and extensively drug resistant (XDR), have resulted in prolonged treatments due to the inefficiency of current antibiotics to eliminate persistent infection(8). Current efforts to control TB focus primarily on reduction of TB transmission yet 95% of infection exists in an asymptomatic latent form(144). This reinforces the need for more effective antimycobacterial therapeutics that are shorter-acting and active against non-replicating mycobacteria.

1.2 TB Chemotherapy

Following the discovery of penicillin in 1928, a significant number of antibiotics were discovered in the subsequent years, many of which were used for the treatment of gram positive infections (1). These included aminoglycosides, such as streptomycin, tetracyclines, chloramphenicol, neomycin, erythromycin, vancomycin, kanamycin and cephalosporin. However, since the 1960s, the rate of discovery of useful antibiotics has decreased dramatically (1). Since the introduction of streptomycin (STM) in 1946, the chemotherapy of TB has evolved over the years (67). However, shortly after its introduction as an effective chemotherapy, reports of streptomycin-resistant MTB appeared, later shown to be the result of streptomycin monotherapy (68). Therefore, the combination of streptomycin with para-aminosalysilic acid (PAS), also discovered in 1946, was

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shown to be more effective than treatment with either agent alone (68). In 1949, isoniazid (INH) became available and by 1955, the standard treatment for TB compromised the combination of STM, PAS and INH (67). The major breakthroughs in the treatment of TB came with the discovery of rifampicin (RIF) in the late 1960s and the rediscovery of the antimycobacterial activity of pyrazinamide (PZA) which made it possible to shorten the duration of treatment considerably (69). However, the drastic decline in antibiotic discovery since the 1960s has resulted in few developments in available therapies for the treatment of TB in particular (114), and the current TB therapeutic regimen is dependent on antibiotics discovered more than 40 years ago, emphasizing the need for novel antibacterials.

The recently implemented DOTS (*d*irectly *o*bserved *t*herapy *s*hort course) program relies on a complex regimen of administering several different drugs simultaneously. These include a two month long treatment with four drugs - either STM, INH, RIF and PZA or INH, RIF, PZA and ethambutol (EMB) - which is then followed by four months of INH and RIF (139). However, resistance has evolved to every antibiotic ever placed into clinical practice (101). Several problems are associated with the current anti-tuberculosis drugs that have led to the widespread emergence of drug resistance. These include the numerous side effects which are complicated by the long duration of therapy and , in turn, lead to patient non-compliance - a major contributor to the emergence of drug resistance (27).

1.2.1 Mechanisms of resistance

Unlike in other bacteria where horizontal gene transfer is frequent, MTB – owing in part to its isolation in the ecologically sterile macrophage niche – acquires drug-resistance exclusively by chromosomal mutations which alter either the drug target itself or the bacterial enzymes required for activating the prodrugs (112). Stress induced DNA damage is one mechanism thought to result in these mutations (15, 90). For example, there is some evidence to suggest that the rate of mutation may increase in an environmentally dependent manner through the upregulation of error-prone polymerases (17). Another mechanism by which multidrug resistance may be acquired is by mutagenesis through the production of reactive oxygen

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species (ROS), stimulated by sublethal concentrations of antibiotics (73). Resistance to INH occurs due to a single missense mutation in the *inhA* gene of MTB, the product of which is involved in fatty acid synthesis (133). Additionally, mutations in - or deletion of - the *katG* gene, encoding the catalase-peroxidase KatG, result in resistance to INH (70). RIF resistance is associated with missense mutations and short deletions within the *rpoB* gene that encodes the RNA polymerase β-subunit (107). Missense point mutations or deletions in the *pncA* gene result in PZA resistance by decreasing the pyrazinamidase activity (107, 115). EMB resistance is most often found in association with missense mutations at codon 306 of *embB* which encodes arabinosyl transferases (122).

MTB acquires MDR and XDR through a step-wise accumulation of chromosomal mutations, each of which confers resistance to individual drugs (92). In the early 1990s, several converging features led to the emergence of MDR-TB (112), which is defined as MTB that is resistant to at least INH and RIF, the two major front-line drug. The perceived threat of MDR-TB is enormous as TB control has been put in jeopardy and the cost of treating MDR-TB patients is extremely high relative to treatment for patients carrying drug-sensitive strains (43). In addition to MDR-TB, an increasing number of XDR-TB cases have been reported globally. XDR-TB is defined as resistance to at least INH and RIF, plus any fluoroquinolone, and at least one of the three injectable second-line drugs (amikacin, capreomycin, or kanamycin) (139). In South Africa, XDR-TB is considered endemic: a 2006 outbreak in the small town of Tugela Ferry, KwaZulu-Natal, highlighted the scale of the problem (51). This study reported that of the 221 patients diagnosed with MDR-TB, 53 of the cases were identified as XDR-TB (51, 118), and the mortality rate among patients co-infected with HIV was 98% (51, 92). Since the Tugela Ferry study, more than 250 cases of XDR-TB have been reported in South Africa, highlighting the need to identify novel drugs with novel mechanisms of action (MOA).

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1.3 Drug Targets

Antibiotics in clinical use target a limited number of cellular processes. There are four main targets for the majority of antibacterial drugs: protein synthesis, nucleic acid biosynthesis (DNA replication and RNA transcription), folate metabolism, and cell wall biosynthesis (Figure 1.1). In each of these, the antibiotics act selectively through the use of comparative biochemical differences between prokaryotic and eukaryotic machinery (136).

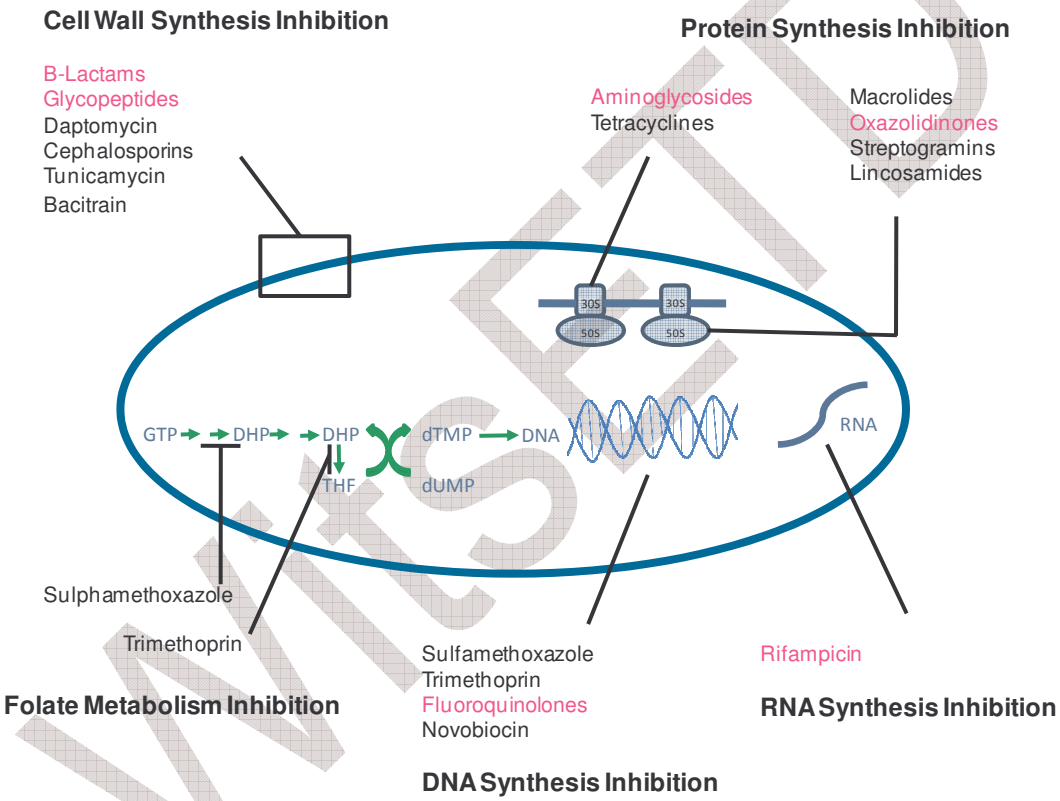


Figure 1.1: The Major Targets of Antibiotics (adapted from (119)). Antibiotic classes used for the treatment of tuberculosis are highlighted in pink. Some examples of front-line/ second-line TB drugs from each drug class include: Amikacin, Kanamycin (Aminoglycosides); Capreomycin, Vancomycin (Polypeptides); Ciprofloxacin, Ofloxacin (Fluoroquinolones); and Rifampicin.

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1.3.1 Inhibition of Protein Biosynthesis

Protein biosynthesis is essential for cellular function, and is catalyzed by ribosomes. Owing to the large number of molecular steps involved in initiation, elongation and termination of protein assembly, it is not surprising that there are many steps within the process that are targeted by various classes of protein-synthesis inhibitors (143). Important antibiotics that inhibit protein biosynthesis include the macrolides (such as erythromycin), the tetracyclines (chlortetracycline), and the aminoglycosides (streptomycin and its synthetic variant, kanamycin) (136). Many clinically significant mycobacteria are susceptible to macrolides, such as clarithromycin. These antibacterial drugs bind to the 50S ribosome subunit and act as physical 'plugs', blocking the exit of the peptide chain (95). However, several mycobacteria including MTB, *M. smegmatis* (MSM) and some members of the *M. fortuitum* group are intrinsically resistant to macrolides (95).

1.3.2 Inhibition of DNA and RNA Biosynthesis

DNA replication is an essential process for all organisms. A number of quinolone antibiotics have been shown to have activity against many mycobacterial species *in vitro* and this has been confirmed in animal models of infection (53). The synthetic fluoroquinolones exert their antibacterial effect by inhibiting DNA gyrase and DNA topoisomerase IV, enzymes that are essential for DNA replication and transcription (117). Transcription is an essential process for decoding genetic information from DNA to RNA. The antibiotic RIF inhibits RNA polymerase by binding to the β subunit of the enzyme (143).

1.3.3 Inhibition of Folate Biosynthesis

Sulfonamides are the longest-used antibacterial drugs and include sulfamethoxazole, which is used in combination with trimethoprim to confer a bactericidal effect. Folic acid is necessary for the production and maintenance of new cells. Each of the drugs inhibit a distinct step in folic acid metabolism (135). Sulfamethoxazole inhibits dihydropteroate synthase as it has a higher affinity for the enzyme than the natural substrate, *p*-aminobenzoic acid. Trimethoprim inhibits

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dihydrofolate reductase, a key enzyme in the folate biosynthetic pathway. Sulfonamides are primarily used to treat nontuberculous mycobacterial infections (mycobacteria other than MTB and *M. leprae*) (135).

1.3.4 Inhibition of Cell Wall Biosynthesis

The peptidoglycan layer of the bacterial cell wall provides mechanical strength and protects the organism from osmolysis. Peptidoglycan is a meshwork of covalently crosslinked strands of peptide and glycan. Transglycosidases crosslink the glycan strands, while transpeptidases crosslink the peptide strands (143). The β-lactam-containing penicillins and cephalosporins target the transglycosidase and transpeptidase domains of bifunctional enzymes. Transpeptidases (also called penicillin-binding proteins or PBPs) are inactivated by β-lactams through the acylation of the active sites. Therefore antibiotics such as penicillin prevent normal crosslinking of peptide chains in the peptidoglycan layer, resulting in a weakened cell wall, which is susceptible to lysis on changes in osmotic pressure (136).

In addition to β-lactams, the vancomycin family of glycopeptide antibiotics also targets the peptidoglycan layer of the bacterial cell wall. However the MOA is different from that of β-lactams, as vancomycin sequesters the peptide substrate, thereby preventing it from reacting with either the transpeptidases or the transglycosylases (136). This results in the failure of peptidoglycan cross-linkage, thereby rendering the cell wall susceptible to osmolysis.

The bacterial cell wall is a common target for the majority of natural-product antibacterial drugs. Many cyclic lipopeptides (CLPs) are compounds that are required in the metabolism of the producing bacteria and therefore remain bound to the cell when produced, while others play a role in pathogenesis and are released into the environment to kill or inhibit other bacterial cells (75). Daptomycin is a CLP antibiotic produced by *Streptomyces roseosporus*. Other CLP antibiotics related to daptomycin include calcium-dependent antibiotic (CDA) and A54145, which are secondary metabolites, produced by actinomycetes (6).

The proposed MOA of CLPs is the insertion of a number of monomers of the compounds into the lipid membrane in a Ca^{2+}-dependent manner. Insertion is facilitated by the amphipathic nature of the molecule (65). The monomers produce

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an ion channel that facilitates passive ion exchange of K^+, H^+ and Ca^{2+}, which ultimately results in cell death (59). A large influx of Ca^{2+} ions leads to the activation of intracellular signaling cascades associated with kinase-mediated phosphorylation of membrane proteins. The specific nature of the bacterium does mediate the activity of the CLP, for example syringomycin appears to be linked to the presence of teichoic acid in gram positive bacteria (12).

Other examples of peptide-based, natural-product antibacterial agents with novel MOAs are the structurally-related, bacteria-derived peptides bacitracin and polymixin, which were introduced clinically in 1942. These peptides interfere with peptidoglycan synthesis by inhibiting the transfer of cytoplasmically synthesized peptidoglycan precursors to bactoprenol pyrophosphate (62).

1.4 The Mycobacterial Cell Wall as a Drug Target

As previously described, the bacterial cell wall is ripe with potential targets for drugs, particularly those of bacterial origin. This is even more apparent with the mycobacteria owing to the complexity of their cell wall as well as the fact that it offers a significant barrier to entry to many compounds (18). The low permeability of the mycobacterial cell wall also offers resistance to chemical injury, dehydration and certain antibiotics (18). The cell wall of *Mycobacterium* spp. is structurally unique owing to the high percentage of mycolic acids. Mycolic acids are a class of complex fatty acids that consist of a hydroxyl group on the β-carbon and an aliphatic chain attached to the α-carbon of the fatty acid. These complex lipids make up approximately 40% of the dry weight of the cell and render the cell envelope extremely hydrophobic (71). The permeability of the cell wall is significantly enhanced by the disruption of this lipid layer, either by the inhibition of mycolic acid biosynthesis or by the interference of mycolic acid attachment to arabinogalactan (18).

The mycobacterial cell envelope is composed of three major components: the plasma membrane, the cell-wall core, and the extractable glycans, lipids and proteins (20). The cell wall core can be defined as the layer external to the plasma membrane and is termed the mycolyl aribinogalactan-peptidoglycan complex (MAPc) (20). This core is essential for viability of the cell as it is insoluble and

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remains intact when the cell wall is disrupted with certain solvents. Therefore, the MAPc is an ideal target for the development of novel drugs. Many currently available drugs inhibit the synthesis of various cell wall components. EMB is thought to inhibit the biosynthesis of the cell wall components arabinogalactan and lipoarabinomannan by inhibiting arabinosyl transferases (*embCAB*) which are involved in polymerizing arabinose into arabinan (111, 122). INH is activated by the catalase-peroxidase enzyme, KatG. The activated drug then reacts with a NADH radical to form a complex which binds tightly to ketoenylreductase, InhA, thereby preventing access of the natural substrate. This process inhibits mycolic acid synthesis (29). Some researchers have questioned the mycobacterial cell wall as a drug target for latent bacteria, as cell wall synthesis might not occur during the stationary phase (18). However, whether cell wall metabolic pathways (such as maintenance and re-modelling) are active during periods of non-replication remains to be established. Certainly, studies carried out on other bacteria have shown that cell wall turnover occurs during the stationary phase, reinforcing the potential relevance cell wall processes to non-replicating MTB (18). Thus, the complexity of the mycobacterial cell wall offers great potential as a drug target for the discovery of novel antimycobacterials, possibly with novel MOAs.

1.5 Novel Antibacterial Compounds: Secondary Metabolites

The inevitable development of resistance that follows the clinical introduction of antibiotics necessitates a constant supply of new compounds, ideally with novel MOAs. However, despite the mounting urgency for new antibiotics, only four new classes of antibiotics have been introduced since the early 1960's (48). There have been two lines of antibiotic discovery over the past 70 years: natural products and synthetic compounds (137). Most of the antibacterial drug classes known today are derived from natural products or natural product leads (96).

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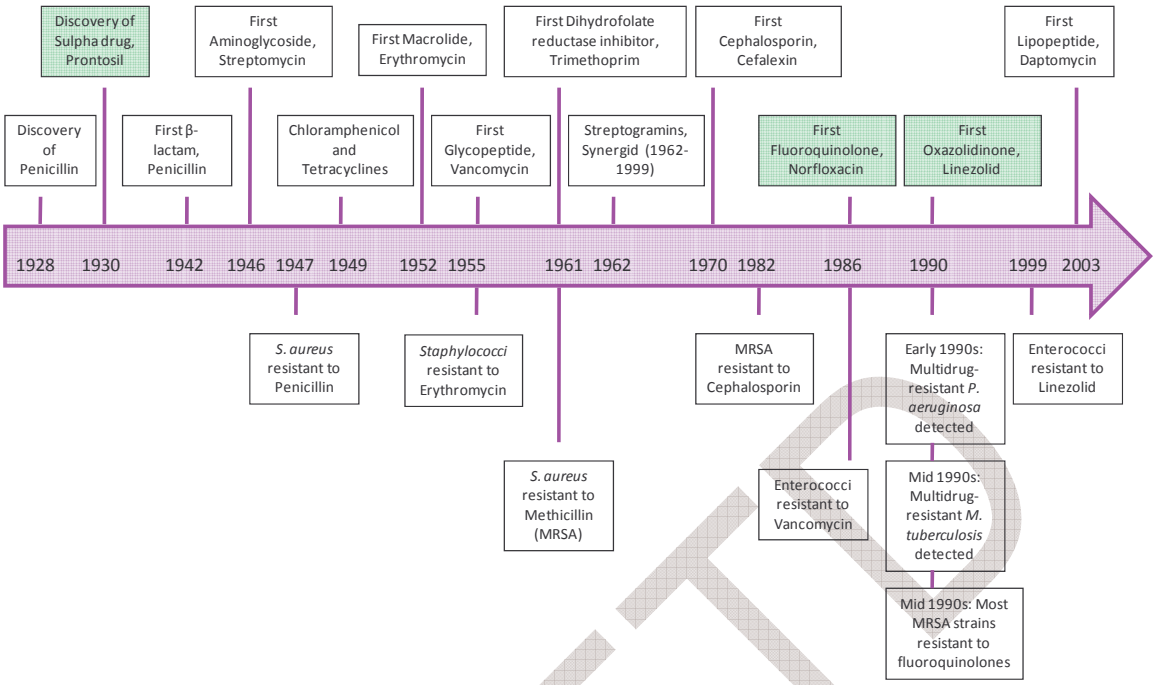


Figure 1.2: The clinical introduction of new classes of antibiotics and the subsequent development of resistance (Adapted from (1)). Antibiotics that are of synthetic origin are highlighted in green.

Recently, the availability of the complete genome sequence of MTB (30) has enabled the identification of essential proteins as novel targets for drug development. The success of the target-based approach relies on the quality of the target as well as the level of validation (5). Only a few validated targets have been revealed to date, namely RNA polymerase, DNA gyrase, NADH-dependent enoyl-(acyl-carrier-protein) reductase and ATP synthase. Current TB drugs, at various stages of clinical trials, founded on these validated targets include Rifapentine (Rifamycin drug class) and Gatifloxacin and Moxifloxacin (Fluoroquinolones) (104). Limitations of the target-based approach may, in part, be due to the formidable cell wall barrier of mycobacteria, but may also be due to unforeseen difficulties of this approach - highlighted by GlaxoSmithKline's general failure to identify new antimicrobials against *Helicobacter pylori* by this approach (101). In addition, the single-target approach is questionable, as a number of antibacterials have multiple targets (8). Whole-cell screening strategies offer an alternative to the target-based approach and allow for all essential targets to be screened simultaneously (8). In fact, the whole-cell screening approach has

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identified four of the compounds currently in clinical trials: SQ109, PA-824, TMC207 and OPC-67683 (5).

Secondary metabolites, produced by a variety of microorganisms, have proved a major source of antibacterial compounds introduced clinically since 1935. In fact, more than two-thirds of the antibiotics used today are microbial natural products or semisynthetic derivatives of these molecules (47). These compounds may confer upon the producing organism a competitive advantage in its ecological niche (119), and are identified through the random screening of environmental bacteria. These secondary metabolites are therefore a major source of natural product compounds and have not only served as leads that were chemically modified and developed as antibacterial agents, but have also provided novel chemical scaffolds for many drugs as well (119). Natural product antibiotics range from small molecular weight compounds (e.g., penicillins) to large peptides (e.g., teicoplanin). Of the three new antibacterial classes that have entered the market since 1970, two are natural-product derived (22): the oxazolidinones linezolid and mupirocin, and the CLP daptomycin. However, it is estimated that, through analyses of bacterial genome sequences, researchers have discovered only ~10% of the natural products produced by screened strains, and just ~1% of the molecules from the global consortium of known microbial producers. Even more interestingly, certain natural product antibiotics of the same class are produced by two or more microbial taxa (48).

1.6 The *Pseudomonas* spp. as a source for novel antimicrobial compounds

The Gram-negative, rod-shaped, aerobic bacteria of the *Pseudomonas* spp. are capable of producing a wide diversity of secondary metabolites with antibacterial activity (65). The metabolites produced by these bacteria include bacteriocins and CLPs. Bacteriocins are ribosomally produced peptide structures that have a narrow killing range, often eliminating or inhibiting cells of closely related species or killing different strains of the same families (25). These compounds are produced by a variety of Gram-positive and Gram-negative bacteria and act by disrupting the cell membrane of target cells, thereby resulting in cell death. CLPs

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are peptide-based compounds, with a framework consisting of a cyclic peptide ring attached to a fatty-acid residue, that are produced non-ribosomally by several different bacteria and have been shown to be promising antimicrobial compounds due to their structural diversity (75). These compounds have been tested against a range of non-pathogenic and pathogenic Gram-positive and Gram-negative bacteria as possible antimicrobial treatments. Several of these compounds have activity against the mycobacteria, both MTB and MSM, as well as other organisms such as *B. subtilis* (21, 52, 62, 105).

Previously, an isolate of the γ-proteobacterium, *Pseudomonas*, that exhibited antagonism towards MSM was identified (Department of Microbiology, School of Molecular and Cell Biology, University of the Witwatersrand). The primary objective in this study was to characterize the inhibitory effect of the *Pseudomonas* isolate. Since the vast majority of secondary metabolites target the bacterial cell wall, and given the intractable barrier offered by the mycobacterial cell wall, we hypothesized that the *Pseudomonas*-derived inhibitory compound(s) were exerted extracellularly, i.e. either at the cell wall or cell membrane of the mycobacteria cell. Also, given that the inhibitory compound(s) are of *Pseudomonas* origin, it was hypothesized that the inhibitory compound might be related to the CLP's.

Consequently, this study had the following aims:

1. To identify the inhibitory bacterial isolate through 16S rRNA sequence analysis.
2. To extract the inhibitory compound(s) from the bacterial isolate.
3. To determine the spectrum of target specificity.
4. To determine the MOA by which this *Pseudomonas*-derived extract inhibits bacterial growth.
5. Additionally, to utilize exposure to cell wall perturbing agents to aid the possible identification of the mycobacterial cell wall as a possible target of the inhibitory compound(s).
6. To compare the potency of the inhibitory compound(s) to other antimycobacterial drugs.
7. To determine the effect of the inhibitory compound(s) on (MTB).

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2.0 MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

The list of strains used in this study is contained in Table 2.1. All strains were stored at -70℃ in 66% glycerol, and recovered by streaking onto the appropriate media (See Appendix A), followed by incubation at 37℃.

Table 2.1: Bacterial strains used in this study

Species	Strain	Genotype	Reference/ Source
M. smegmatis	mc^2155	High frequency transformation mutant of *M. smegmatis* mc^26	Snapper *et al.* (1990)[121]
M. parafortuitum	IFM 0490		Y. Shibayama*
M. tuberculosis	H37Rv		MMRU Laboratory Strain
			Chris Sassetti Laboratory Strain
R. erythropolis	ATCC 4277		Y. Shibayama
R. equi			Y. Shibayama
R. fasciens			Y. Shibayama
R. rhodochrous	01		Y. Shibayama
R. rhodochrous	Ri8		Y. Shibayama
N. farcinica	IFM 10757		Y. Shibayama
N. farcinica	IFM 10779		Y. Shibayama
B. cereus	ATCC		K. Naicker*
B. subtilis	01	Wild Type	S. Deva*
S. aureus			Microbiology Department, School of Molecular and Cell Biology
S. epidermidis			Microbiology Department, School of Molecular and Cell Biology
E. coli	29		K. Naicker*
E. coli	32		K. Naicker*

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P. putida	317	Wild type	D. Lindsay
P. putida	αMB	Wild Type	K. Lukasa*

* Previous/ current post-graduate students within the School of Molecular and Cell Biology, University of the Witwatersrand.

2.2 Identification of the producer organism

2.2.1 DNA extraction

The colony boil method was used for the isolation of all mycobacterial and *Pseudomonas* genomic DNA. Cultures were streaked out onto 7H10 (mycobacteria) or LA (*Pseudomonas*) to obtain single, pure colonies. Colonies were picked and resuspended in 50 µL of 1x TE buffer. For reference, 10 µL was spotted onto a plate. The remaining 40 µL was boiled for 20 min at 100℃ followed by the addition of 40 µL of chloroform (99% Analysis Grade, Merck). The solution was mixed and allowed to rest at room temperature for 5 min followed by centrifugation at 13 000 rpm (Eppendorf Centrifuge 5415D, Merck) for 5 min. The supernatant was used as the DNA template during PCR reactions.

2.2.2 PCR and agarose gel electrophoresis

Amplification of 16S rDNA was performed, using the primer set Bac27F and U1392R (IDT, USA) (see Table 2.2). The PCR reactions were performed in an Eppendorf MasterCycler (Eppendorf International) and conditions were identical for all reactions. Primers (each to a final concentration of 0.5µM) were used in combination with 2x PCR Master Mix (Fermentas Life Sciences), according to the manufacturer's instructions, and yielded a product of approximately 1300 bp. The 1x concentrations of each component in the 2x PCR Master Mix is as follows: 0.025 units/µl *Taq* DNA Polymarase in reaction buffer; 2mM $MgCl_2$ and 200µM of each dNTP. PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 94℃ for 3 min; 35 cycles consisting of denaturation (94℃, 30s), annealing (60℃, 45s), extension (72℃, 1min), and a

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final extension at 72℃ for 7 min. All PCR products were analysed on agarose gels. For a 1% agarose gel, 0.4 g of agarose (Electrophoresis Grade, Invitrogen Life Technologies) was dissolved in 40 ml of 1x TAE buffer with 2 µl of Ethidium Bromide (final concentration = 0.5 µg.ml). Samples were loaded with DNA loading buffer (see Appendix B12) and resolved until dye front was three quarters the length of the gel. The product sizes were compared to the appropriate DNA markers (Roche Applied Science, Germany).

2.2.3 DNA sequencing

The NucleoSpin®ExtractII kit (Macherey-Nagel, Separations) was used to purify PCR products. The purified PCR product was sequenced (DNA Sequencing Facility, Stellenbosch University) and the resulting sequences were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (4) against 16S rDNA sequences from GenBank (GenBank database of the National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/). A homology tree of the isolates was constructed using the neighbour joining method.

Table 2.2: Primer sequences used to amplify the 16SrDNA locus

Primer	Sequence (5'- 3')	Size of Product (bp)
Bac27F	AGA GTT TGA TCC TGG CTC AG	**1300**
U1392R	ACG GCT ACC TTG TTA CGA CTT	

2.3 Ethyl acetate extraction of the inhibitory compound(s)

A 15 ml culture of *P. putida* αMB was grown in LB (see Appendix A2) to a colony forming unit/ml (cfu/ml) of 10^8. The culture was plated onto 100 plates of unsupplemented 7H10 (see Appendix B3) (100µl/ plate) and plates were incubated at 37˚C for 3 days. The cells from each plate were then scraped and added to ethyl acetate (99% Analysis Grade, Merck) to a final volume of 300 ml followed by storage at 4˚C for 7 days. During this one week storage period, the

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cells were frequently agitated to ensure maximum exposure to the ethyl acetate. The solution was then filtered, to remove the cells, and a rotavapor (Buchi RotaVapor, Switzerland) was used to concentrate the solution to a final volume of ≈10ml. The extract was immediately tested on an MSM indicator plate (as described below), and the remaining extract was stored at 4˚C, covered in aluminium foil as sensitivity to light was unknown.

2.4 Antagonism of the *Pseudomonas*-derived extract

2.4.1 Preparation of Indicator Plates

Indicator plates were made by seeding agar with the various indicator organisms (OD_{600} of 0.6- 0.8). All 7H10 indicator plates were supplemented with GS (10 ml GS per 1L 7H10. A 10% volume of the cultures was seeded into the appropriate cooled media (See Appendix A) which was then poured into Petri dishes at a standardized volume of 25ml/ plate. Following solidification of the media, indicator plates were stored at 4˚C.

2.4.2 Inhibition Assay

Agar plugs (diameter = 5 mm) were created and removed in the various indicator plates. A standardized volume of 15 µl/hole of either inhibitory extract in 100% Ethyl acetate or inhibitory extract in 50% ethanol was added to each hole. Each solvent was also tested on the indicator plates, as the appropriate control. The plates were incubated at 37˚C for 2 days and zones of inhibition (ZOIs) were recorded for comparative analyses.

2.5 Broth microdilution method

The broth microdilution method (32, 41) allows a range of antibiotic concentrations to be tested, on a single 96-well microtitre plate, to determine the minimum inhibitory concentration (MIC). Briefly, a 10 ml culture of MSM is grown to an OD_{600} of 1.0. The culture is then diluted to an OD_{600} of 0.1 in 7H9 media. In a 96-

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well microtitre plate, 50 µl of 7H9 media was added to all wells from Rows 2-12. In the first and last well of Row 1, 100 µl of 7H9 media was added as a control. The solvent (100 µl) in which the inhibitory extract is dissolved is added to the second and second last well of Row1. The inhibitory extract (100 µl) is then added to the remaining four wells in the centre of Row 1. A serial dilution was prepared, using a multichannel pipette, by transferring 50 µl of the liquid in Row 1 to Row 2 and aspirating to mix. 50 µl of the liquid in Row 2 was transferred to Row 3 and aspirated. The procedure was repeated until Row 12 was reached, where 50 µl of the liquid in Row 12 was discarded to bring the final volume in these wells to 50 µl. Finally, 50 µl of the diluted MSM culture was added to all the wells of Rows 2-12. The microtitre plate was sealed in its original plastic bag and incubated at 37°C for 72 Hrs. After the incubation period, Alamar blue (Molecular Probes®, USA) was added to each well (10% of the final volume in each well, thus, 10µl) and the plate was incubated at 37°C for 6 Hrs, after which observations were made. The lowest concentration of drug that inhibited more than 95% of the bacterial population was considered to be the MIC.

2.6 The effect of the inhibitory extract against ethambutol-resistant MSM mutants

2.6.1 Isolation of spontaneous ethambutol-resistant mutants

MSM was grown in 7H9/ OADC (see Appendix B4) supplemented with either 0.5 µg/ ml or 1.0 µg/ ml EMB. The cultures were spread onto 7H10 media supplemented with OADC, T80 (0.05%) and EMB at either of the following concentrations: 0.5 µg/ ml, 1.0 µg/ ml, 2.0 µg/ ml, 4.0 µg/ ml. 8.0 µg/ ml and 10 µg/ ml (78). If a plate had more than 20 colonies, an assumptive EMB^R mutant was picked and re-plated on EMB plates, at concentrations at which they were arose identified, to confirm resistance. Seven independent spontaneous mutants were isolated in this manner despite the numerous mutants that were isolated but not selected for further experimentation.

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2.6.2 Confirmation of mutation in *embB* gene

Genomic DNA was extracted by the colony boil method (as described above). Lety *et al.* (1997) showed that a single amino acid substitution in a highly conserved region among the mycobacterial EmbB proteins results in resistance to EMB (78). In MSM, this region is located at nucleotide position 898-909. Mokrousov *et al.* (2002) successfully amplified the region within the MTB *embB* gene, known to confer resistance to EMB (91). Therefore, the primers used by Mokrousov *et al.* (2002) were selected and the corresponding primers in the MSM *embB* gene were designed to amplify a highly conserved region, the Ethambutol Resistance Determining Region (ERDR), within the *embB* gene of MSM (Table 2.3) (91). The PCR reactions were performed in an Eppendorf MasterCycler (Eppendorf International) and conditions were identical for all reactions. Each 50 µL reaction consisted of 1x Phusion HF Reaction Buffer (with $MgCl_2$) (Finnzymes, Finnland), 20.5 µl dH_2O, 200 µM of each dNTP (New England Biolabs Inc., UK), 0.5 µM of each primer, 0.02 units/µl Phusion® DNA Polymerase and 6 µl of the genomic DNA. PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 98°C for 1 min; 30 cycles consisting of denaturation (98°C, 1 min), annealing (64°C, 30s), extension (72°C, 1min), and a final extension at 72°C for 7 min. The PCR products were resolved by agarose gel electrophoresis on a 1% agarose gel using a Gel Doc Imaging System (BioRad, USA) and product sizes were assessed in comparison to molecular weight marker VI (Roche Appled Science, Germany).The PCR products were purified using a PCR clean-up gel extraction kit, Nucleospin® Extract II (Macherey-Nagel, Germany). The purified products were sequenced (Inqaba Biotech, Inc, South Africa) and the resulting sequences were analyzed using SeqMan (DNAStar, Inc, USA).

Table 2.3: Primer sequences used to amplify the ERDR within the *embB* gene

Primer	Sequence (5'- 3')	Size of Product (bp)
embF	CGC CTG ATC CCG ACG CGC	254
embR*	TCG GAT CCG ATG CTG GCG TC	

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*A point mutation was introduced unintentionally during the design of primer embR. However this did not interfere with the identification of mutations associated with EMB resistance. The actual sequence is [5´-TCG GAT CCA GAT GCT GGC GTC-3´]. The use of this primer, together with embF, will yield a 255bp product.

2.6.3 Drug resistance assay

For further confirmation of resistance to EMB, a drug resistance assay was performed on the assumptive EMB^R mutants. A susceptible MSM mc^2155 strain has an MIC of 0.5µg/ml (3). Therefore the experiment was repeated, using a modification of the conventional agar proportion method. MSM mc^2155, used as a control, and all 7 mutants were grown to an OD_{600} of 1.2-1.3 (starting OD_{600} = 0.1). A standard dilution series of each culture was prepared (0 to -7), and spotted onto 7H10 media supplemented with 2, 10, 20, 30, 40, 50, or 60 µg/ml EMB. 7H10/ GS plates were used as a control.

2.6.4 Inhibition assay

To examine sensitivity of the EMB^R mutants to the inhibitory extract, indicator plates of each mutant was prepared and exposed to two independent inhibitory extracts. These were then compared to a wild-type MSM indicator plate that was also treated with the inhibitory extracts to determine if there is an increased sensitivity of these cell wall mutants to the extract. For comparative analyses, indicator plates were standardized by ensuring all mutants and wild-type MSM were grown to an OD_{600} of 0.65-0.75 (starting OD_{600} = 0.06). The volume in each indicator plate was also standardized (as described above).

2.7 Production of spontaneous MSM mutants, resistant to the *Pseudomonas*-derived inhibitory extract.

An exponential culture of MSM mc^2155 was serially plated onto 7H10 media containing different dilutions of the inhibitory extract. Three different dilutions were selected: 100 µl, 500 µl and 1 ml. 7H10 (GS) plates containing no extract were

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used as a control. Plates were incubated at 37℃ for 5 days after which possible mutants were isolated and cultured in 7H9 media. To confirm resistant mutants, indicator plates of each possible mutant were prepared and exposed to the inhibitory extract.

2.8 Comparative analysis of the potency of the inhibitory extract

2.8.1 DMSO as an alternative solvent for the inhibitory compound(s)

An extraction of the *Pseudomonas*-derived inhibitory compound(s) was performed in duplicate to obtain two independent extracts (called Extract 1 and Extract 2). The ethyl acetate from both extracts was completely evaporated off and the remaining residues were weighed. Extract 2 was stored at -20˚C for 5 weeks. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) as a solvent was examined on Extract 1, however significant amounts of insoluble deposits resulted, even as the volume of DMSO was increased. These insoluble deposits were removed and weighed; the mass of which was deducted from the initial mass of Extract 1. The soluble inhibitory compound(s) was dissolved in a final volume of 2 ml of DMSO and tested on a MSM indicator plate. Extract 2 was removed from storage after 5 weeks and subjected to the procedure as Extract 1. The insoluble deposits of Extract 1 were added to 1 ml of DMSO, to determine if precipitation had occurred due to saturation in the original 2 ml.

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3.0 RESULTS

3.1 Isolation of an unidentified organism which inhibits the growth of MSM

Previously, an unknown organism that appeared to be antagonistic to MSM was isolated in the laboratory of Dr. Steven Durbach, in the School of Molecular and Cell Biology at the University of the Witwatersrand. The organism was initially identified as a contaminant on an MSM indicator plate. Subsequently, the isolate was cultured and shown to result in growth inhibition as determined by well-defined clearings or zones of inhibition (ZOIs) in the agar (Fig. 3.1). The data are from a representative experiment that was performed each time a freezer stock of the unidentified organism was cultured.

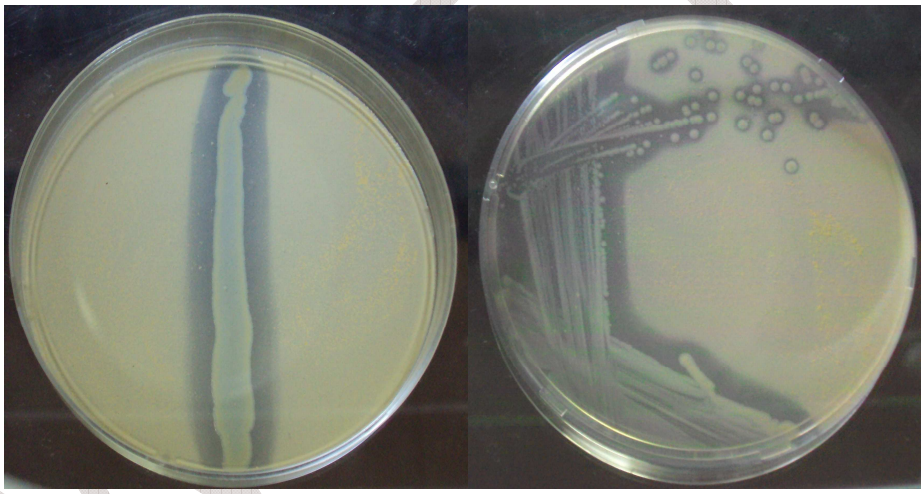


Figure 3.1: Inhibition of MSM by the unidentified isolate. MSM indicator plates illustrating the inhibitory effect of the unidentified isolate, as determined by ZOIs.

3.2 The inhibitory organism is identified as *Pseudomonas*

Having demonstrated inhibitory activity, it was necessary to identify the producing organism. The physical characteristics (round, yellow coloured colony), suggested that the producer organism was a bacterium. As this organism was originally isolated from a soil sample, the isolate was considered likely to belong to one of the following phyla: *Actinomycetes* (64), *Proteobacteria* or *Firmicutes* (47).

Numerous genera from each of these phyla, including *Streptomyces* (13, 64); *Pseudomonas* (64, 106) and *Bacillus* (64), are soil-dwelling organisms. Therefore the identity of the isolate was determined by sequencing the 16S rRNA locus, which is highly conserved among bacteria (76). DNA was extracted from the isolate and MSM mc^{2}155, as a control, followed by amplification of the 16S rRNA sequence using a universal primer set for eubacterial 16S rRNAs (138). The PCR products were resolved by agarose gel electrophoresis and a 1300bp PCR product identified for both the unknown isolate and the MSM control (Fig. 3.2). PCR of 16S rRNA was performed once since an amplicon of the expected size was identified, and because high concentrations of DNA were obtained for subsequent confirmatory sequencing reactions.

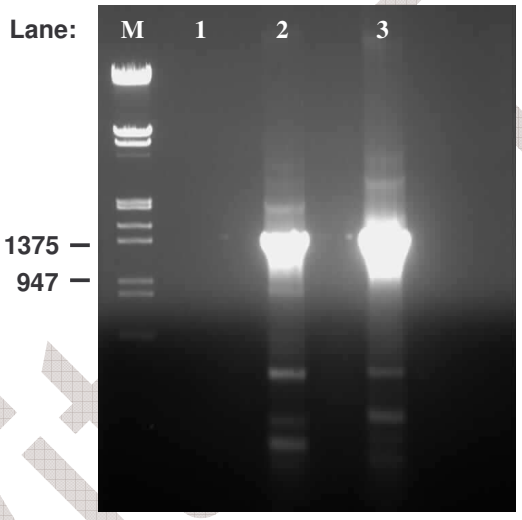


Figure 3.2: Amplification of the 16S rRNA region. Ethidium bromide stained 1% agarose gel containing 1300 bp amplification products obtained from the primers Bac27F and U1392R (Table 2.2). Lanes: M, DNA Molecular Weight Marker III; 1, no template DNA control; 2, MSM mc^{2}155 and 3, unidentified isolate.

The 1300 bp fragments from MSM and the unknown isolate were sequenced, and a 730bp portion of the sequenced PCR products compared to sequences lodged at the Genbank database (GenBank database of the National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/) by BLAST (4) analysis (see Appendix C2). The results indicated 99% sequence similarity of the isolate to the γ-proteobacteria class (Fig. 3.3.).

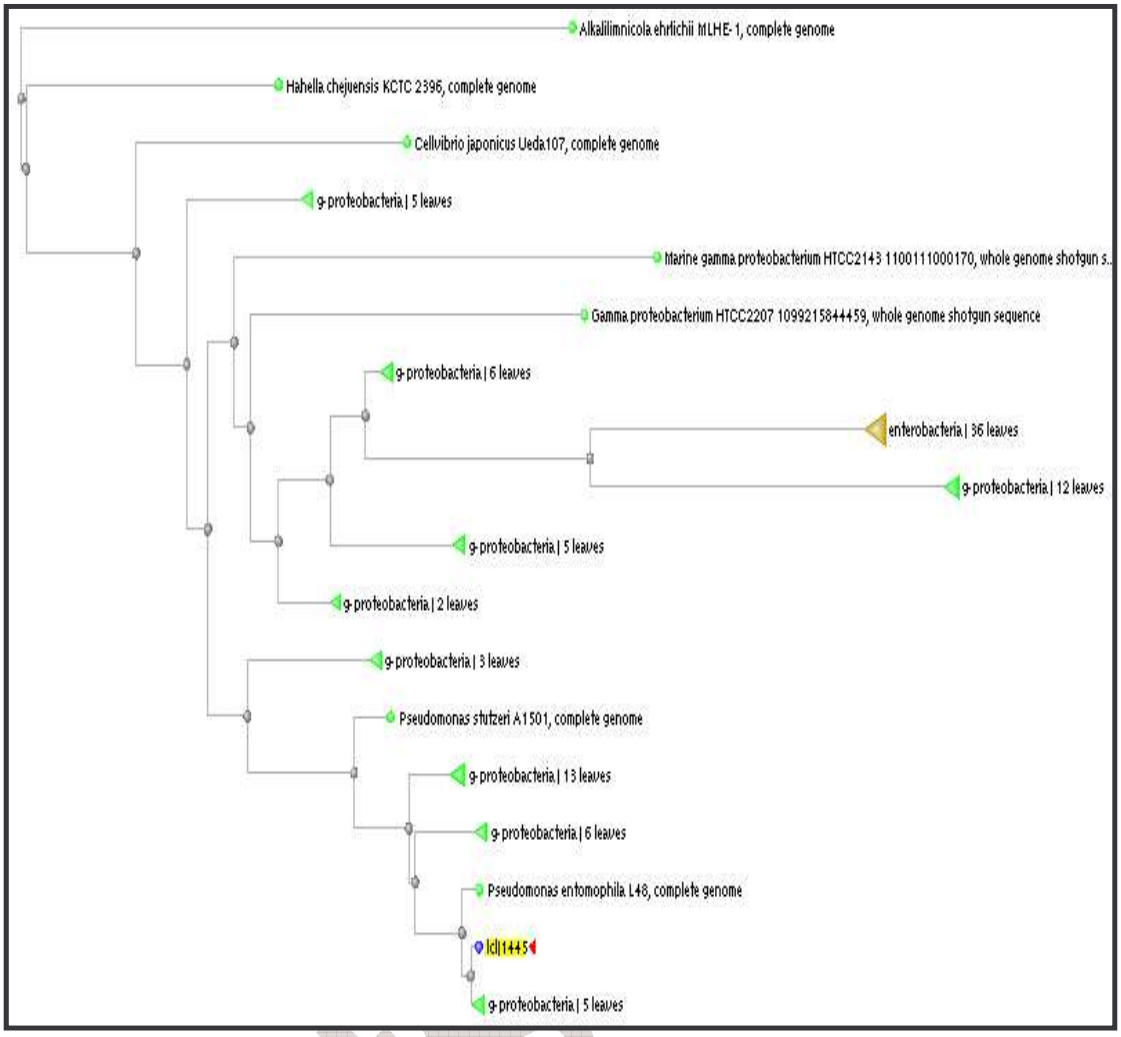


Figure 3.3: Phylogenetic tree comparing the unknown isolate to the domain Bacteria. Distance tree using BLAST pairwise alignments of 16S rRNA sequence. From this analysis, the unidentified isolate (highlighted in yellow) appears most closely related to γ-proteobacteria (http://www.ncbi.nlm.nih.gov/blast/tree_view/blast_tree_view.).

In order to identify the isolate at a species level, the 16S rRNA sequence of the isolate was then compared to sequences within the γ-proteobacteria class. The results indicated 99% sequence similarity to *P. putida* and *P. entomophila* and 98% sequence similarity to *P. mendocina.* Homology trees were constructed from the original query sequence (Fig. 3.4) using the Blast Tree View Widget (http://www.ncbi.nlm.nih.gov/blast/tree_view/blast_tree_view.).

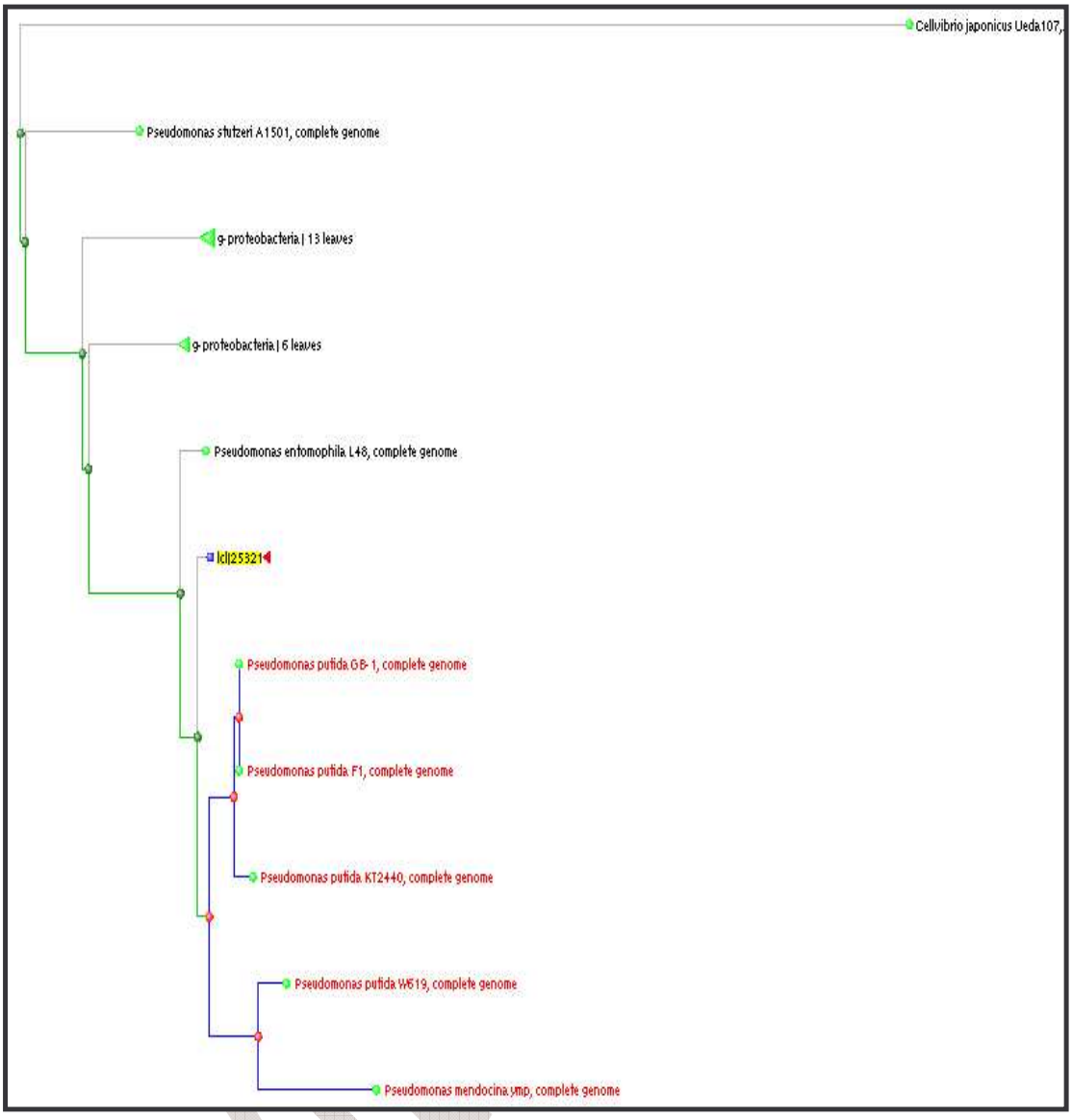


Figure 3.4: Phylogenetic tree comparing the unidentified isolate to the γ-proteobacteria family. Distance tree using BLAST pairwise alignments illustrating relationship of the unknown isolate (highlighted in yellow) to the Gamma subdivision of Proteobacteria: *Pseudomonadaceae*. (http://www.ncbi.nlm.nih.gov/blast/tree_view/blast_tree_view.).

The phylogentic trees are from a representative BLAST search that was performed in duplicate. On the basis of these results, the producing strain was designated *Pseudomonas* αMB to denote its demonstrated antimycobacterial effect.

3.3 The inhibitory compound(s) are extractable using ethyl acetate as a solvent

3.3.1 The extraction protocol

The observed ZOI (Fig. 3.1) suggested that *Pseudomonas* αMB was producing and secreting an inhibitory compound(s) into the growth medium. Therefore, in order to identify the nature of the inhibitory agent(s), a protocol was developed to extract and purify the inhibitory compound(s). The protocol adopted (Fig. 3.5) was initially developed by K. Naicker in the laboratory of Dr. Durbach and was based on methods developed for various antibiotic-producing bacteria (K. Naicker, MSc Dissertation, Wits, 2010) (94). Importantly, this protocol enabled the successful extraction of the inhibitory activity from the *Pseudomonas* αMB cells using ethyl acetate as a solvent. Antibiotic biosynthesis has been extensively characterized for the genus *Streptomyces* (9, 13, 66). In these organisms, secondary metabolism is growth-phase dependent; for example, antibiotic production is upregulated during the stationary phase (66). Although not a streptomycete, we reasoned that by applying similar methods to *Pseudomonas* αMB, we might enhance the production of the inhibitory compound(s). *P. putida* has a doubling time of 62 minutes when glucose is utilized as the carbon source (54). Therefore the protocol was developed to allow an exponential-phase culture of *Pseudomonas* to incubate for 3 days on solid medium, ensuring entry into the stationary phase. Most antibiotic biosynthetic pathways are initiated by suboptimal growth conditions, imposed by some nutritional limitation (9). While Luria-Bertani medium sustains optimal growth of *P.putida* (54) and *P. entomphila* (63), 7H10 was developed by Middlebrook and colleagues in 1958 specifically for growth of mycobacteria (87, 88). We postulated, therefore, that a degree of nutritional stress might be imposed on *Pseudomonas* αMB by growing the exponential-phase culture on solid 7H10 medium. As *Pseudomonas* spp. grow optimally at 25-30℃ (54, 63, 106), the *Pseudomonas* strain isolated in this study was cultured at 37℃ at all times to further increase the likelihood of inducing a stress response that might result in increased antibiotic production. In her original protocol, K. Naicker settled on ethyl acetate as the preferred solvent after testing various organic

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solvents (94), consistent with the observations of a number of published studies (10, 58).

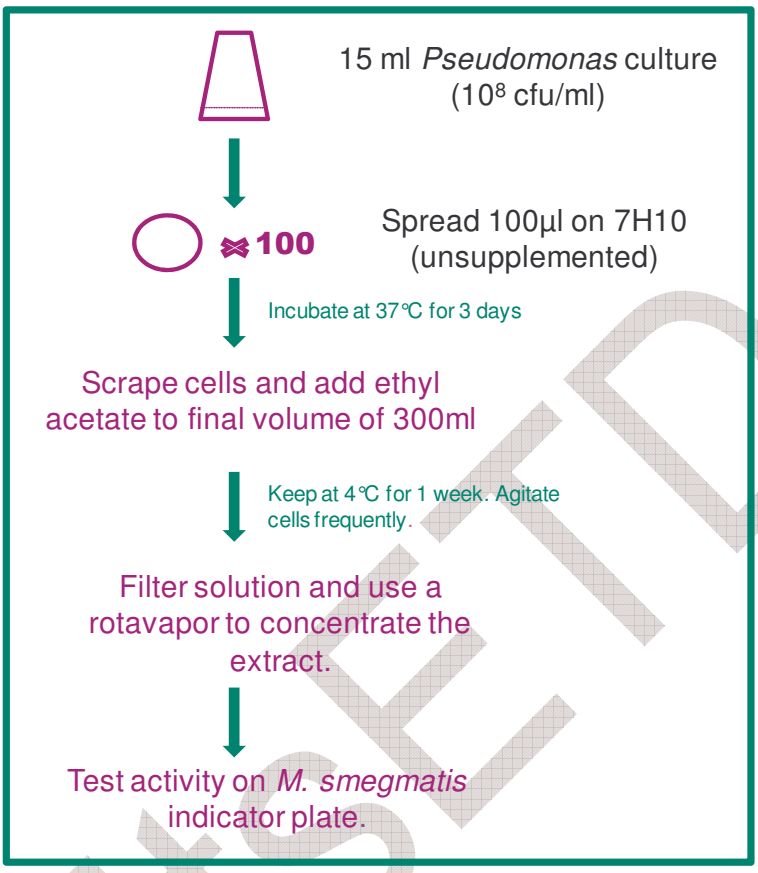


Figure 3.5: Schematic representation of the ethyl acetate extraction protocol. Briefly, an exponential culture of the *Pseudomonas* αMB isolate was spread across 100 7H10 plates, and incubated at 37℃ for 3 days. Cells were then scraped from the plates, added to ethyl acetate, and incubated for 1 week at 4℃. The resulting solution was then filtered and concentrated, and the potency of the extracted compound(s) confirmed on an MSM indicator plate.

During the one-week incubation period of *Pseudomonas* αMB in ethyl acetate, the cells appeared increasingly dehydrated and formed large clumps (Fig. 3.6A). Therefore, to ensure maximum exposure to the ethyl acetate, the culture was agitated frequently. At the end of the week the ethyl acetate solution was filtered and concentrated using a rotavapor, resulting in a yellow-coloured solution of approximately 4ml volume (Fig. 3.6B). The data are form a representative experiment that was performed each time the inhibitory compound(s) was extracted.

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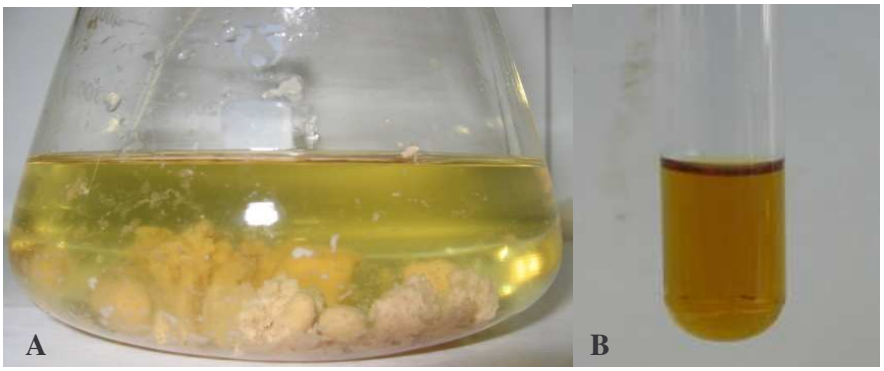


Figure 3.6: Ethyl acetate extraction of the inhibitory compound(s). (A) *Pseudomonas* αMB cells suspended in ethyl acetate. Notice the large cellular aggregates or clumps. (B) Filtration and concentration yields approximately 4 ml solution contain active inhibitory compound(s).

To ensure that the concentrated extract retained inhibitory activity, an aliquot of the 4ml solution was immediately tested on an MSM indicator plate before continuing further with any microbiological assays. Extractions were considered successful only when they yielded concentrated extract that was inhibitory to MSM mc^2155, as determined by ZOI measurement (Fig. 3.7). The data are form a representative experiment that was performed each time the inhibitory compound(s) was extracted.



Figure 3.7: The inhibitory effect of the *Pseudomonas*-derived extract. Anti-mycobacterial activity of two independent extractions from *Pseudomonas* αMB (B) and (C) were tested on an MSM indicator plate and compared with an ethyl acetate only control (A).

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3.3.2 Optimization of the extraction protocol

The extraction protocol described above was laborious, and required the processing of 100 plates for each extraction which yielded approximately 4 ml of the inhibitory compound(s). Therefore, we attempted to optimize the extraction procedure. Specifically, we wanted to determine whether the yield of the inhibitory extract could be increased, or if it was possible to isolate concentrated extract with increased inhibitory activity, thereby decreasing the quantity required to achieve the inhibitory effect. As noted above, for many organisms optimal antibiotic production occurs during the stationary phase (66). Therefore we examined the effect of a longer incubation period on the production of the inhibitory activity. To this end, 100 7H10 plates were again spread with *Pseudomonas* αMB. However, after the normal 3 day incubation, the batch was split: 50 plates were scraped and the inhibitory compound(s) were extracted as described (see section 3.3.1), whereas the remaining 50 plates were incubated for a further 7 days (total incubation period of 10 days) before processing according to the same ethyl acetate-based extraction protocol. The resulting extracts ("3 day" and "10 day") were tested on MSM indicator plates and their ZOIs compared (Fig. 3.8). The 7H10 plates inoculated with *Pseudomonas* αMB for an extra 7 days yielded concentrated extract with a significantly larger ZOI than was obtained from the normal 3 day incubation. These data established that the extraction protocol could be improved by extending incubation time. The data are from replicate experiments performed at least twice.

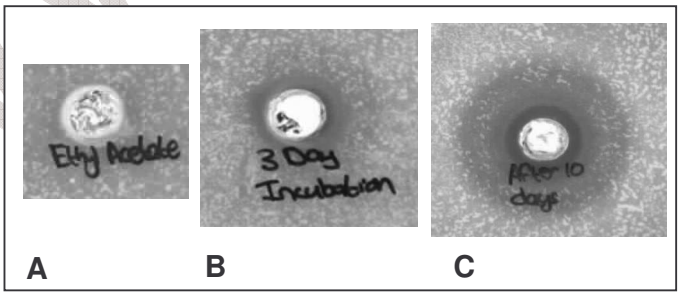


Figure 3.8: Increasing the incubation period of the *Pseudomonas* yields more potent inhibitory extract. Ethyl acetate control (A); inhibitory extract after a 3-day incubation period (B) and inhibitory extract after a 10-day incubation period (C).

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3.4 The inhibitory compound(s) is not extractable from the agar

To determine whether the inhibitory compound(s) were secreted into the agar during the incubation period and, if so, whether the compound(s) were extractable using ethyl acetate, a normal 100 plate extraction was performed. After scraping the cells, the agar was sliced up and suspended in ethyl acetate for 2 weeks at 4℃. The agar was then removed by filtration and the remaining ethyl acetate concentrated as per the standard protocol before testing for inhibitory activity. No improvement in inhibitory activity was observed (Fig. 3.9), suggesting that an insignificant amount of inhibitory compound(s) was secreted into the agar during the 3-day incubation period or, alternatively, that this protocol was inefficient at extracting the inhibitory compound(s) from the agar. The data are from replicate experiments performed at least twice. In any event, the failure to improve the yield significantly meant that this approach was abandoned; therefore, all future extractions did not include the agar medium.

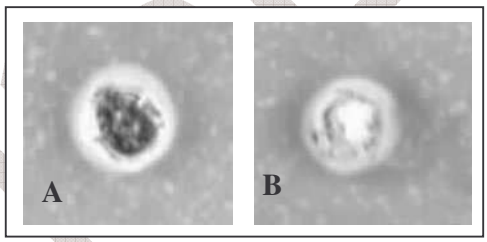


Figure 3.9: Extraction from agar failed to improve yield. Ethyl acetate control (A) and inhibitory compound(s) extracted from the agar (B).

3.5 The inhibitory compound(s) are active in liquid culture

To determine whether the *Pseudomonas*-derived extract was able to inhibit growth of MSM in liquid culture, we assayed inhibitory activity of the concentrated extract using the broth microdilution method (32, 41). The initial results were inconclusive as the 96-well microtitre plate was degraded by the ethyl acetate solvent. Therefore, 50% ethanol was examined as an alternative solvent. To this end, a 4 ml extract in ethyl acetate was split into two equal (2 ml) volumes, and the ethyl

acetate allowed to evaporate. The dried-down extracts were then resuspended in either 100% ethyl acetate or 50% ethanol. The extract was not completely soluble in 50% ethanol as moderate quantities of insoluble deposits were observed. However these insoluble deposits were retained in the 50% ethanol, and the extract was tested on an MSM indicator plate. Thereafter, inhibitory activity was assessed on solid medium. The extract was active against MSM whether dissolved in 100% ethyl acetate or 50% ethanol (Fig. 3.10), although ethyl acetate appeared to be a slightly better solvent on the basis of a more distinct, and slightly larger, ZOI. Nevertheless, these results suggested the utility of 50% ethanol as alternative solvent to ethyl acetate, a conclusion supported by the observation that the 50% ethanol only control did not inhibit MSM in solid medium. Importantly, these results implied a potentially significant role for the solvent in the efficacy of the inhibitory compound(s). The data are from a representative experiment that was performed in duplicate.

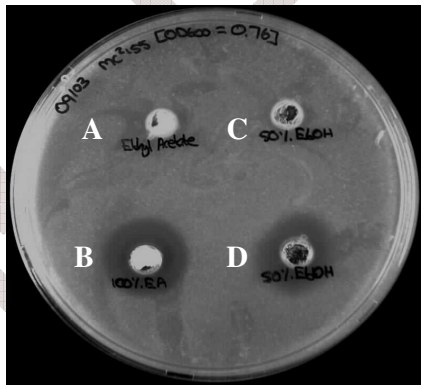


Figure 3.10: The inhibitory effect of the *Pseudomonas*-derived extract in different solvents. Comparison of inhibitory activity of (A) the 100% ethyl acetate control, (B) the extract dissolved in 100% ethyl acetate, (C) the 50% ethanol control, and (D) the inhibitory extract dissolved in 50% ethanol. Inhibition is observed when both 100% ethyl acetate and 50% ethanol are used as solvent, though the ZOI appears larger and more distinct in (B).

Having confirmed inhibition of MSM on solid medium, we assayed the potency in liquid culture of the extract dissolved in 50% ethanol. To this end, we applied the broth microdilution method (32, 41) which allows a range of antibiotic

concentrations to be tested, on a single 96-well microtitre plate, to determine the minimum inhibitory concentration (MIC). Briefly, this method involves the serial dilution of the extract across a 96-well microtitre plate containing MSM cells growing in 7H9 medium from a low starting inoculum. An equal volume of a diluted MSM culture (OD_{600} = 0.02) is added to each well, excluding the first column as this serves as a no-cells (or cell-free) control, and the plate is incubated at 37°C for 72 hrs (See section 2.5 of Materials and Methods). Growth of the bacterial cells in the microtitre plate is usually scored by recording the size of the pellet in each well. Therefore, in wells that contain high concentrations of the antibiotic, no bacterial pellets are observed. As the antibiotic is serially diluted (as one progresses down a single row) bacterial pellets are observed of increasing size; that is, pellet size correlates directly with antibiotic concentration and, therefore, inhibitory activity. In some cases, this method of scoring growth can be difficult as relative pellet size is not always easily discerned. Moreover, pellets are not always visible in photographs. Therefore, Alamar Blue, a resazurin-based oxidation-reduction dye (49), was added to each well for visualization purposes. This dye is a general indicator of cellular growth and/or viability: it is blue in its non-fluorescent, oxidized form (31) but, during cellular growth, the dye turns pink and fluoresces upon reduction. Therefore, growth can be measured with a fluorometer, spectrophotometer or determined by an easily visible colour change (31). In the study presented here, all microtitre plates were scored by visible colour change, and the MIC was defined as the concentration at which > 95% growth inhibition is observed (indicated by blue or deep purple). In Fig 3.11, Alamar Blue was added to half the microtitre plate (Rows 1-4) after 72 hrs of incubation, allowing relative bacillary growth to be visualized as a gradient extending from blue to pink across each row. From this assay, we confirmed growth inhibition of MSM in liquid 7H9 where wells contain up to a 1/16 dilution of the *Pseudomonas*-derived extract dissolved in 50% ethanol. The data are from a representative experiment that was performed in duplicate.

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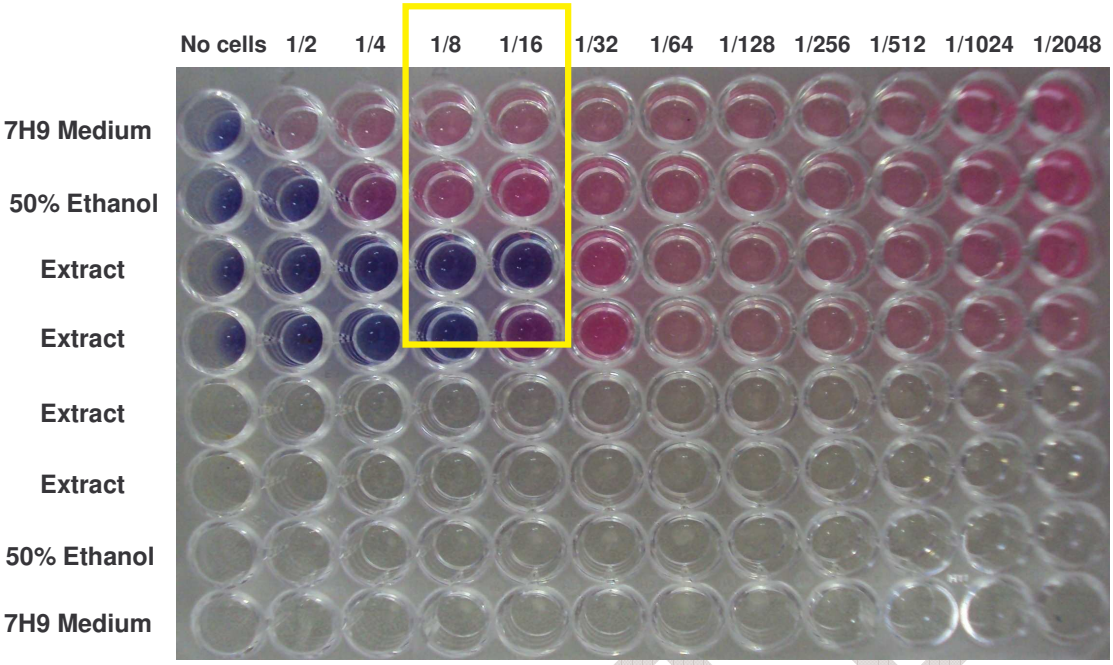


Figure 3.11: Broth microdilution assay of the inhibition of MSM by the *Pseudomonas*-derived extract dissolved in 50% ethanol. Image was taken after 6 hrs of incubation with Alamar Blue; pink indicates growth and blue indicates no growth. From this assay, the MIC falls in the range 1/8 – 1/16 dilution of the neat extract, and is indicated by the yellow box.

3.6 The inhibitory compound(s) is produced by *Pseudomonas* αMB exclusively

Having demonstrated the efficacy of the ethyl acetate-based extraction procedure to isolate cell-free, active inhibitory compound(s), we next wanted to eliminate two potential confounding factors: firstly, the possibility that the inhibitory compound(s) was produced by all *Pseudomonas* strains; and, secondly, the concern that the inhibitory effect resulted from the ethyl acetate extraction protocol itself, and was independent of the producer organism. To address these concerns, a parallel extraction was performed on *Pseudomonas* αMB and an alternative strain, *P. putida* 317. The *P. putida* 317 isolate was obtained from Dr. D. Lindsay in the School of Molecular and Cell Biology at the University of the Witwatersrand and was provided as a confirmed *Pseudomonas* isolate on the basis of 16S rRNA analysis (N. Fernandez, MSc Dissertation, Wits, 2010) (46). Extractions from both organisms, *Pseudomonas* αMB and *P. putida* 317, yielded a yellow-coloured liquid, which was tested against MSM on an indicator plate. In contrast to that isolated from *Pseudomonas* αMB, the extract derived from *P. putida* 317 failed to

inhibit growth of MSM (Fig. 3.12). The data are from a representative experiment that was performed in duplicate. This result confirmed that the inhibitory compound(s) was limited to *Pseudomonas* αMB and, importantly, demonstrated that the inhibitory effect was not as a result of the extraction protocol.

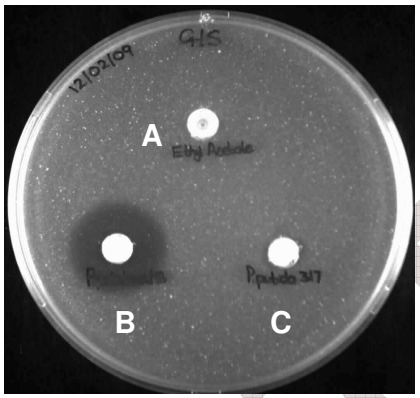


Figure 3.12: The inhibitory compound(s) is specifically produced by the *Pseudomonas* αMB strain. Ethyl acetate control (A), *Pseudomonas* αMB-derived extract (B) and *P. putida* 317-derived extract (C). Inhibitory effect as determined by ZOI.

3.7 Resistance to ethambutol does not confer cross-resistance to the *Pseudomonas*-derived inhibitory compound(s)

Cyclic lipopeptides (CLPs) are produced by several *Pseudomonas* species (38, 97) and have a diverse range of inhibitory activity against several human pathogenic organisms, including enveloped viruses, mycoplasmas and Gram-positive bacteria (105). Since the inhibitory organism clustered most closely with *Pseudomonas* species (Fig. 3.4), we speculated that the inhibitory compound(s) might be related to the CLPs. CLPs target teichoic and lipoteichoic acids of gram positive bacteria (12). In mycobacteria, arabinogalactans (AGs) and lipoarabinomannans (LAMs) are the structural equivalents of teichoic and lipoteichoic acids (123). Ethambutol (EMB) is a first-line antituberculosis drug that is thought to inhibit the biosynthesis of the cell wall components AG and LAM by targeting the arabinosyl transferases *embCAB* (11, 127). EMB differentially inhibits AG and LAM biosynthesis in wild-type and EMB-resistant (EMB^R) bacilli, indicating

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that AG and LAM are synthesized by different pathways (72, 89). To determine whether the *Peudomonas*-derived inhibitory compound(s) targets AG and/or LAM biosynthesis, spontaneous MSM EMB^R mutants were isolated and exposed to the inhibitory extract. The MIC of EMB ($MIC_{EMB)}$ for MSM is 0.25 µg/ml - 0.5 µg/ml (3, 78, 89) and was used as a guideline to isolate EMB^R mutants. Spontaneous EMB^R MSM mutants were isolated by exposing wild-type MSM in liquid culture to 0.5 µg/ml of EMB, and thereafter plating the EMB-exposed culture onto 7H10 containing various concentrations of EMB (0.25 µg/ml; 0.5 µg/ml; 1.0 µg/ml; 2.0 µg/ml; 4.0 µg/ml; 8.0 µg/ml and 10 µg/ml) as described previously (78, 89). The estimated mutation frequency to EMB-resistance was calculated to be between10^{-8} to 10^{-7}, consistent with previous reports (78). We then selected seven independent EMB^R mutants for further characterization(summarized in Table 3.1) EMB resistance is primarily associated with missense mutations within the EMB resistance determining region (ERDR) of the *embB* gene (3, 108, 122, 127).

Table 3.1: Characterization of EMB^R mutants

Mutant Number	EMB concentration at which mutant was isolated (µg/ mL)	Base change	Corresponding amino acid change	Maximum MIC (µg/ mL)
1	2	ATG909ATC	Met292Ile	10
2	4	ATC898ATG	Ile289Met	10 - 20
3	2	ATG909ATC	Met292Ile	10
4	4	ATC898ATG	Ile289Met	20 - 30
5	4	N/A	N/A	N/A
6	2	ATG909ATC	Met292Ile	10 - 20
7	2	ATG909ATC	Met292Ile	10

Spontaneous EMB^R MSM mutants were isolated by plating wild-type MSM on 7H10 containing various concentrations of EMB. Of the seven mutants that were selected for further characterization, four mutants had a Met292Ile amino acid change, while two mutants had an Ile289Met amino acid change. EMB^R in mutant 5 was not associated with an identifiable mutation in the ERDR, so this isolate was not selected for further characterization.

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To confirm that the identified mutants carried genetic mutations, a 254 bp region within the ERDR of the *embB* gene was amplified with the primers EmbF and EmbR (Fig. 3.13). PCR of the 254bp region was performed once since an amplicon of the expected size was identified, and because high concentrations of DNA were obtained for subsequent confirmatory sequencing reactions. From PCR sequencing, we identified six mutants with mutations in this region.

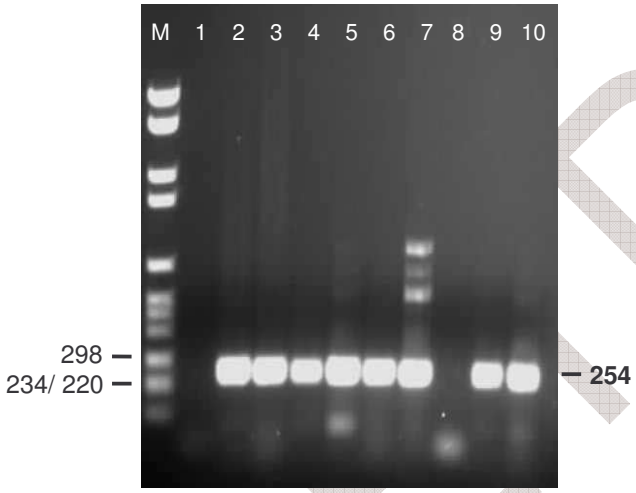


Figure 3.13: PCR amplification of a 254 bp region by the EmbF and EmbR primers. Lanes: M, Marker VI; Lane 1, no template DNA control; Lane 2 and Lane 3 MSM mc^2155; Lane 4-10, EMB^R mutants 1-7. Mutant 5 (Lane 8) was not successfully amplified.

Sequence analysis revealed that Mutants 2 and 4 had a point mutation at nucleotide 898 (C→ G), resulting in the amino acid change Ile289Met, while Mutants 1, 3, 6 and 7 had point mutations at nucleotide 909 (G→ C), resulting in the amino acid change Met292Ile (Fig. 3.14). Both transversion mutations are consistent with those described previously (78).

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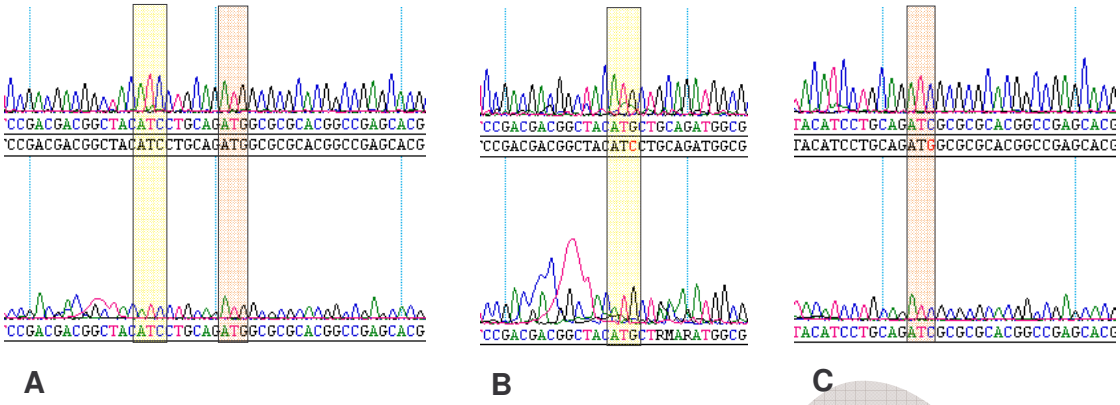


Figure 3.14: Single transversion mutations are associated with EMBR in MSM. DNA sequencing chromatograms showing the two types of point mutations identified in the ERDR of spontaneous EMB^R MSM mutants. The wild-type codons in EMB-susceptible isolates are ATC (Ile) and ATG (Met) (A), ATC→ ATG mutations found in Mutants 2 and 4 are highlighted in yellow (B) while ATG→ ATC mutations found in Mutants 1, 3, 6 and 7 are highlighted in orange (C).

Although associated with (and often diagnosed by) single mutations in target genes, the level of resistance to a specific drug is a function of multiple factors, including the presence of drug efflux pumps (45). Therefore, while the identified EMB^R mutants (Table 3.1) carried genetic mutations consistent with EMB^R, we wanted to determine whether these mutants were associated with different levels of EMB^R. The MIC_{EMB} was determined for seven mutants and one susceptible strain of MSM by spotting serial, log-fold diluted cultures onto 7H10 medium supplemented with the standard glucose/salt (GS) mix, as the control, and EMB at a range of concentrations between 0 and 60 µg/ml. The parental, EMB-susceptible mc^2155 strain had an MIC_{EMB} of 2 µg/ml as determined by this method, which compares favourably with reported values (3, 78, 89). All seven mutants isolated had an MIC_{EMB} of 10 µg/ ml or greater, a five-fold increase over the wild-type MIC (Fig. 3.15). The variability of the MICs between the mutants indicates that additional mutations could have been co-selected during growth on EMB resulting in mutants that exhibited higher MICs. The data are from a representative experiment that was performed in duplicate.

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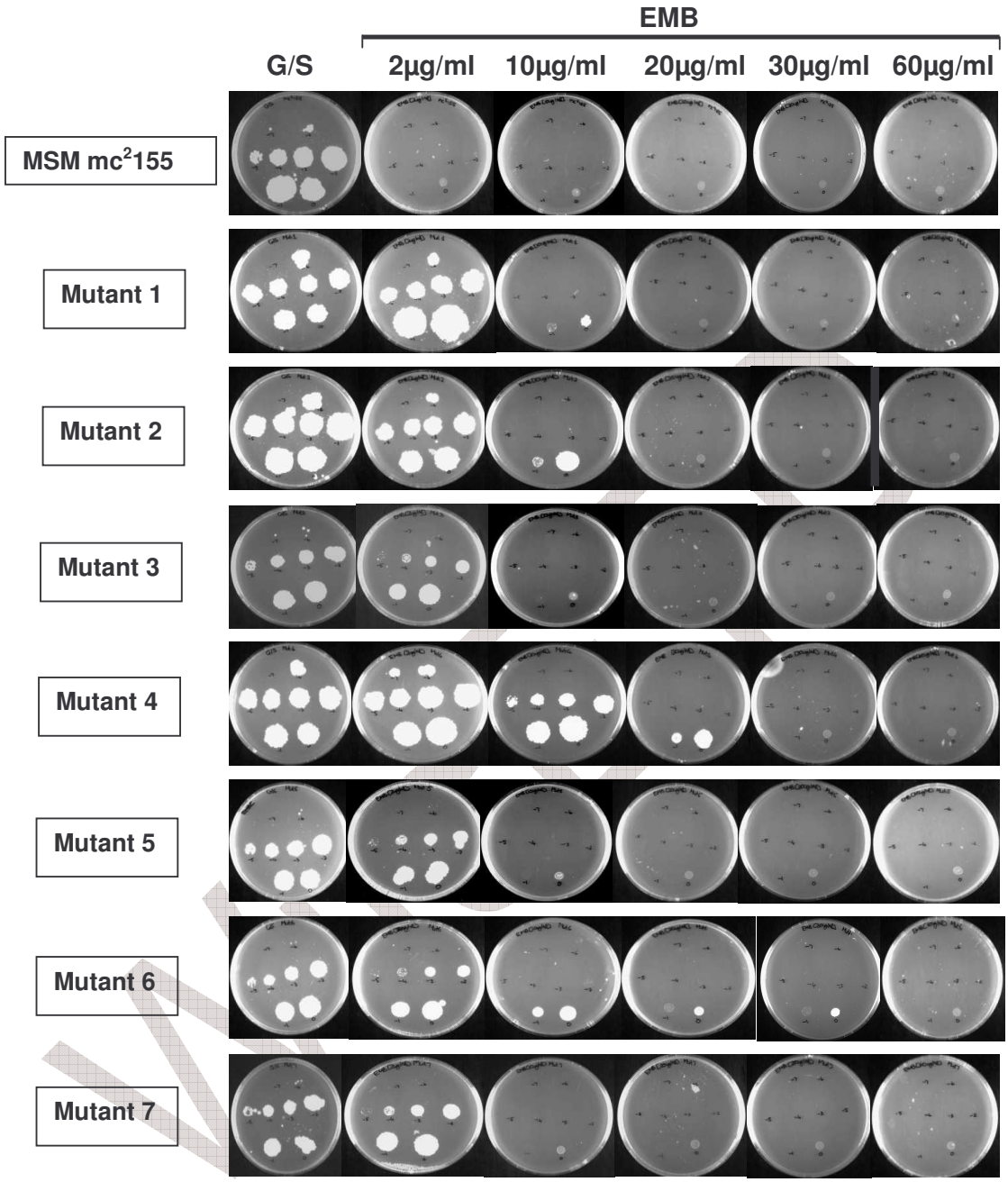


Fig 3.15: Drug resistance assay used to determine the MIC of EMB for each mutant strain. Column 1 illustrates the Glucose/Salt (GS) control, Column 2-6, EMB plates at the following concentrations respectively: 2, 10, 20, 30 and 60 µg/ml. The growth of MSM mc^2155 is inhibited at EMB (2 µg/ml), consistent with reported MIC_{EMB} values (3, 78, 89). By contrast, all seven mutants grew at this concentration.

To determine the effect of the *Pseudomonas*-derived extract on the EMB^R mutants, indicator plates containing each mutant were exposed to the inhibitory extract. For comparative analyses, indicator plates of wild-type MSM were also prepared; moreover, all indicator plates were standardized to include a similar number of wild-type or mutant cells (OD_{600} between 0.6 – 0.8), and each plate was prepared from the same volume of solid 7H10 agar (25 ml/plate). Each of the mutant and wild-type indicator plates was exposed to two independent extracts, as well as an ethyl acetate only control. Measurements of the ZOIs indicated that EMB^R mutants were not cross-resistant to the *Pseudomonas*-derived inhibitory compound(s). The data are from replicate experiments performed at least twice. Although the complex nature of EMB-mediated growth inhibition (11, 40, 72, 89, 124) precludes a simple interpretation of these results, the failure of EMB^R mutations to confer cross-resistance to the *Pseudomonas* extract eliminated this assay as a simple test for mechanism of action (MOA).

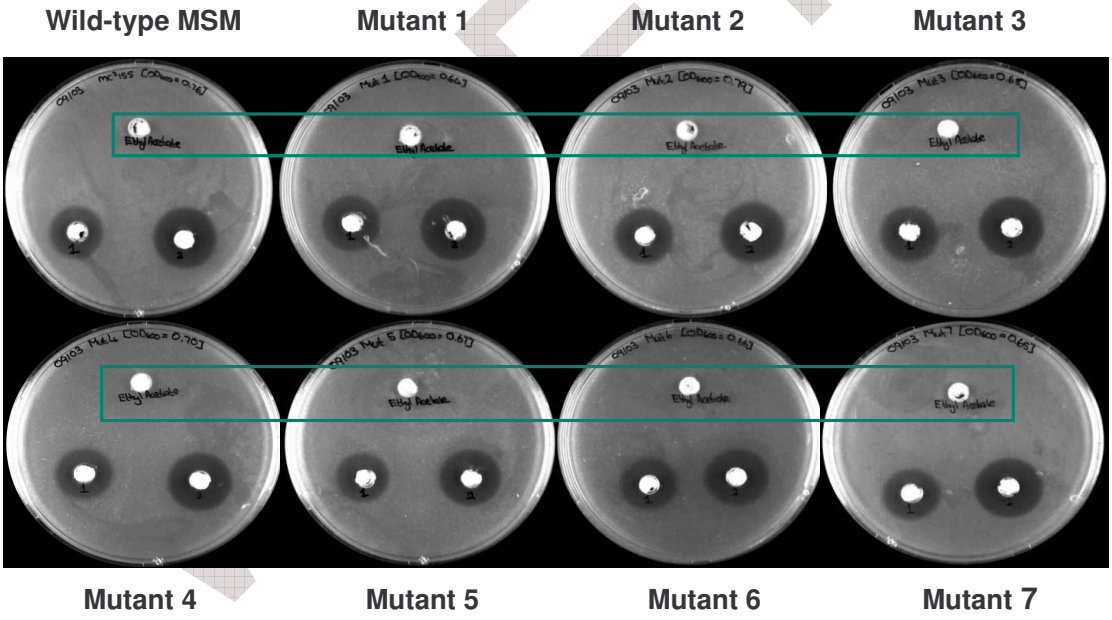


Figure 3.16*:* Resistance to EMB does not correlate with resistance to the *Pseudomonas*-derived extract. The ZOIs of the two independent extracts were measured (the diameter, mm, of the extract on the left of each plate is given first): wild-type MSM, 20 and 24; Mutant 1, 21 and 22; Mutant 3, 21 and 21; Mutant 4, 21 and 23; Mutant 5, 20 and 24; Mutant 6, 20 and 23; Mutant 7, 21 and 25. There was no significant increase in the sensitivity of these mutants to the inhibitory extract. The 100% ethyl acetate control is indicated by the green box.

3. 8 Tyloxapol and SDS increase susceptibility of MSM to the inhibitory compound(s)

Detergents such as polyethylene glycol sorbitan monooleate (Tween 80) and SDS weaken the bacterial cell wall. Tween 80 is a nonionic surfactant as well as an oil-in-water emulsifier. Exposure to Tween 80 has been shown to weaken the cell wall of mycobacteria and increase susceptibility to antimicrobial agents (129). Tween 80 can be substituted by Tyloxapol, which is a non-hydrolyzable detergent (131). Sodium dodecyl sulfate (SDS) is also a cell wall-perturbing agent (131). To determine whether a cell wall permeability defect could increase sensitivity to the *Pseudomonas*-derived extract, indicator plates were supplemented with one of the following detergents - Tween 80 (0.05%), sodium dodecyl sulphate (SDS, 0.01% and 0.001%) or Tyloxapol (0.02%) - and exposed to the extract. Indicator plates supplemented with GS only served as a control, and two independent extracts analyzed. In addition, in order to determine the effect of the various detergents on the growth of MSM in indicator plates in the absence of the inhibitory activity, an additional control was implemented: specifically, indicator plates supplemented differentially with the various detergents were incubated without any exposure to the inhibitory extract and compared to the corresponding indicator plates to which extract was exposed. That is, all plates labelled “A” (in Fig. 3.17) were compared to plates labelled “B”. The data are from replicate experiments performed at least twice.

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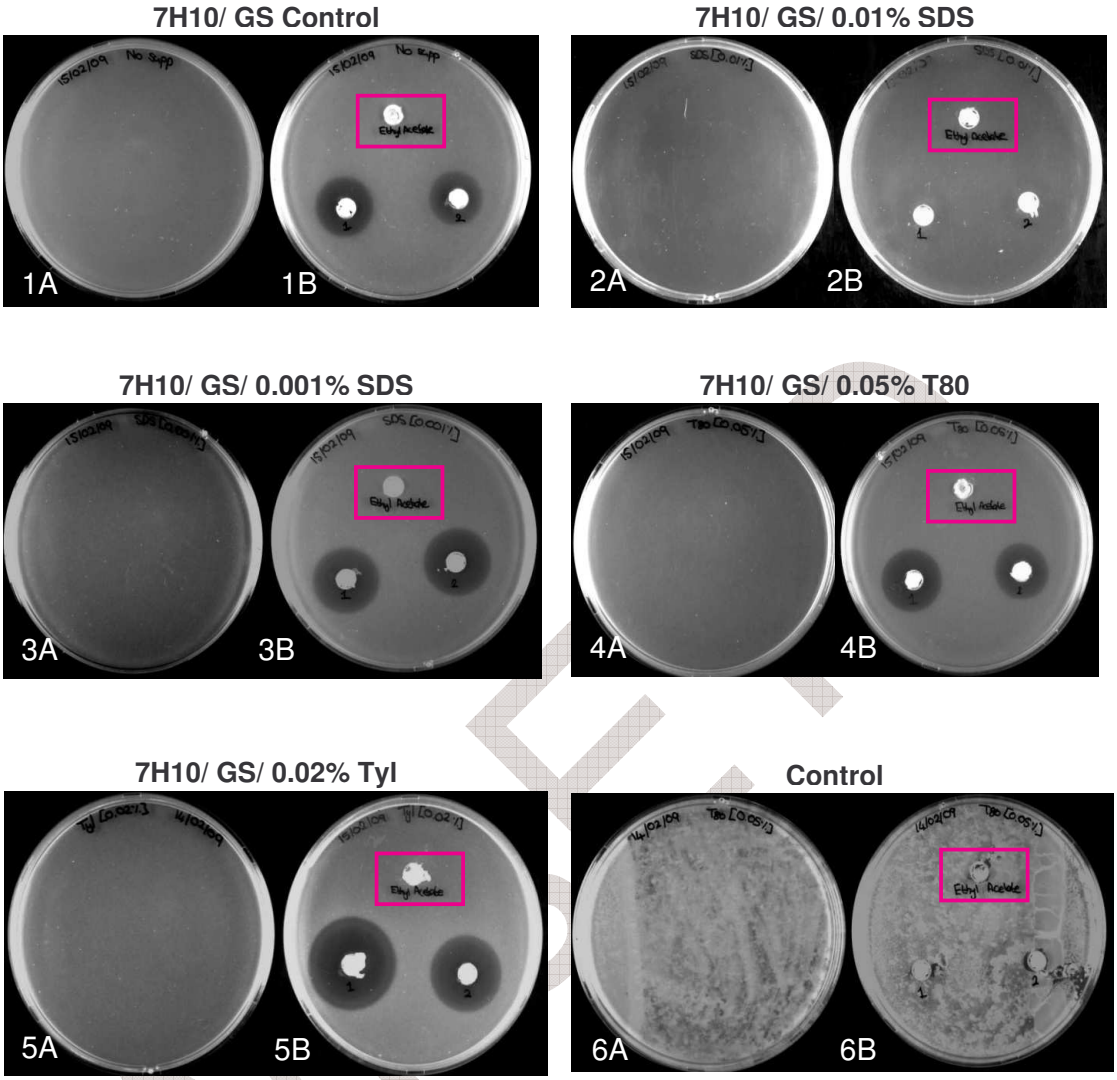


Figure 3.17: MSM indicator plates differentially supplemented with cell wall perturbing agents. Two independent extracts were tested and the ethyl acetate control is highlighted in pink. Indicator plates supplemented with GS (1A-1B) served as a control for the comparison of ZOIs, while 7H10 GS plates with a bacterial lawn (6A-6B), served as a second control to ensure that viable cells were added to the indicator plates. All plates labelled (A) were not exposed to the extract, but incubated simultaneously with those that were exposed (B).

The presence of SDS (0.001%) or Tyloxapol (0.02%) in the indicator plates resulted in the most significant increase in the apparent potency of the inhibitory extract (Figs. 3B and 5B compared to Fig. 1B). In fact, it appeared as if MSM was hypersensitive to the extract in the presence of Tyloxapol, indicated by the large

ZOI in Fig. 5B compared to Fig. 1B. No bacterial growth was observed in Fig. 2A compared to Fig. 1A, indicating that SDS at a concentration of 0.01% was inhibitory to MSM growth in indicator plates. Tween 80 appeared to have no significant effect on sensitivity to the extract, as the ZOIs observed in Fig. 4B are comparable to those in the control, Fig. 1B. The control that was created, in Fig. 6A and 6B, indicated that the cells added to the indicator plates were viable, as a bacterial lawn formed on 7H10/GS plates. This experiment also highlighted the inability of the concentrated extract to inhibit growth of an established culture, as no ZOI was detectable when extract was added to a pre-existing bacterial lawn (Fig. 3.17, 6B). This implies that the inhibitory compound(s) are not able to reach the target(s) when the cell density is too high, a possibility that could be tested by plating a bacterial lawn with a significantly lower cell density followed by exposure to the extract.

3.9 The *Pseudomonas*-derived inhibitory compound(s) may have a Gram-restricted target range

For drug development from natural products, it is important to consider whether the active compound(s) inhibits the target organism specifically, or whether it exerts a general effect, targeting a number of unrelated organisms ("broad spectrum"). Therefore, to determine the target specificity, the *Pseudomonas*-derived extract was tested against selected indicator organisms including Actinobacteria such as *Mycobacterium*, *Rhodococcus* and *Nocardia* spp.; other Gram-positive bacteria including *Bacillus* and *Staphylococcus* spp. and the Gram-negative *E. coli.* For this experiment, indicator plates were prepared using growth media appropriate to the specific organism under investigation (See Table 5.1, Appendix A). Each bacterial strain was exposed to the inhibitory extract dissolved in 100% ethyl acetate or 50% ethanol. Each of the different bacteria assayed possesses a characteristic growth rate. Therefore, plates were incubated at 37˚C for different time periods: 1 day for *Bacillus* spp. and *E. coli*; 2 days for *Mycobacterium* spp. and *Staphylococcus* spp.; and 5 days for the remaining actinobacteria. ZOIs, observed as clearings in the agar, were recorded for comparative analyses (Fig. 3.18).

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Although a slight inhibitory effect was observed against the Gram-negative *E. coli,* the inhibition was insignificant in comparison to the Gram-positive bacteria, which appeared to be significantly more sensitive to the extract (Fig. 3.18). *E. coli* was selected as the representative Gram-negative species, of which only two strains were tested against the crude extract. To further investigate potential Gram-specificity of the *Pseudomonas*-derived extract, a variety of Gram-negative bacteria should be examined. As such, our data do not eliminate the possibility that the crude extract has activity against Gram-negative organisms. The *Nocardia* strains appeared to be hypersensitive to this extract, as large ZOIs were observed in comparison to the ZOIs of the other bacterial species. Notably, an increased inhibitory effect was observed when 100% ethyl acetate was used as a solvent. This was consistent with previous observations (Fig. 3.10), and again suggested the importance of the solvent for activity. The data are from a representative experiment that was performed in duplicate.

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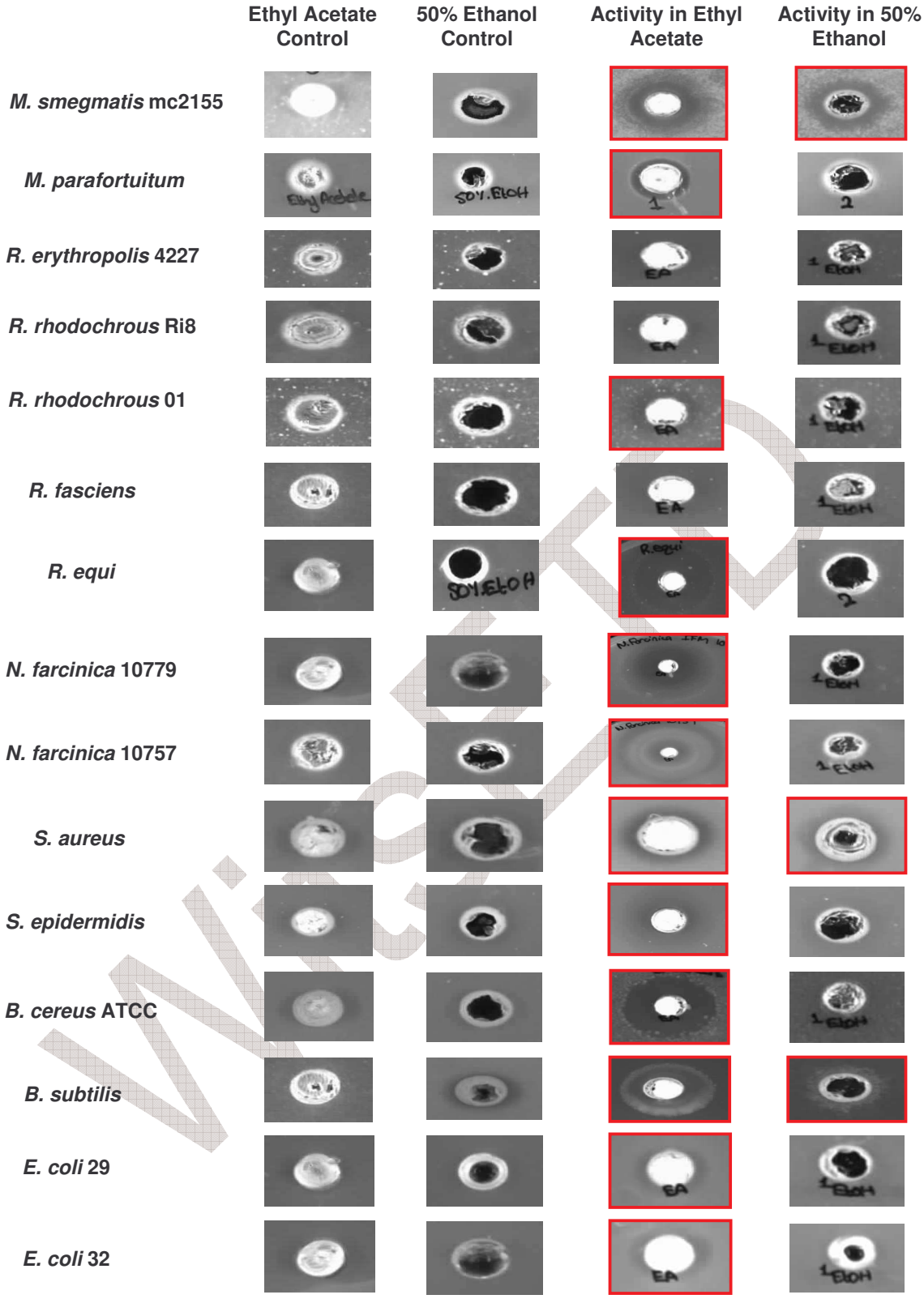


Figure 3.18: **The *Pseudomonas*-derived extract inhibits growth of Gram-positive organisms and its inhibitory effect is solvent dependent.** The Gram specificity of the crude extract was examined by assaying its growth inhibitory effect on a panel of representative Gram-positive organisms,

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actinomycetes, and the Gram-negative *E. coli*. From these data, the extract appears to have maximum effect against Gram-positives, however the panel of representative Gram-negatives needs to be expanded (see text for details). In addition, these data reveal an apparent solvent-dependent effect on inhibitory activity.

3.10 Mutants resistant to the *Pseudomonas*-derived extract cannot be spontaneously isolated

A common strategy to elucidate an unknown MOA is to characterize mutants resistant to the compound(s) of interest. This approach exploits the fact that that mutation(s) in the target or complementary gene(s) are generally associated with drug resistance (50, 85, 93), and is especially true in an organism such as MTB in which resistance is conferred exclusively by chromosomal mutations. Spontaneously-arising resistant mutants can often be isolated by serially plating log-fold dilutions of a drug-susceptible wild-type culture onto medium containing various concentrations of the antibiotic (35, 85). Since we were utilizing a crude extract of the *Pseudomonas*-derived compound(s), it was not possible to use varying concentrations to isolate spontaneous mutants; instead, different dilutions of the extract were applied. Log-fold dilutions of a wild-type MSM culture were serially plated onto 7H10 medium containing the extract at a range of dilutions: specifically, 100 µL, 500 µL and 1 ml of the crude extract per 25 ml agar plate. Extract-free GS plates were used as a control. Although the experiment was repeated in triplicate, the following results were not reproducible, perhaps indicating the complexities associated with applying this method to an extract that likely comprises multiple active compounds. The extent of inhibition of MSM growth was in direct proportion to the volume of the extract applied, with maximum inhibitory effect obtained at the highest volume (1ml/plate) and almost no inhibition at the lowest (100 µl/plate) (Fig. 3.19). The data are from replicate experiments performed at least three times.

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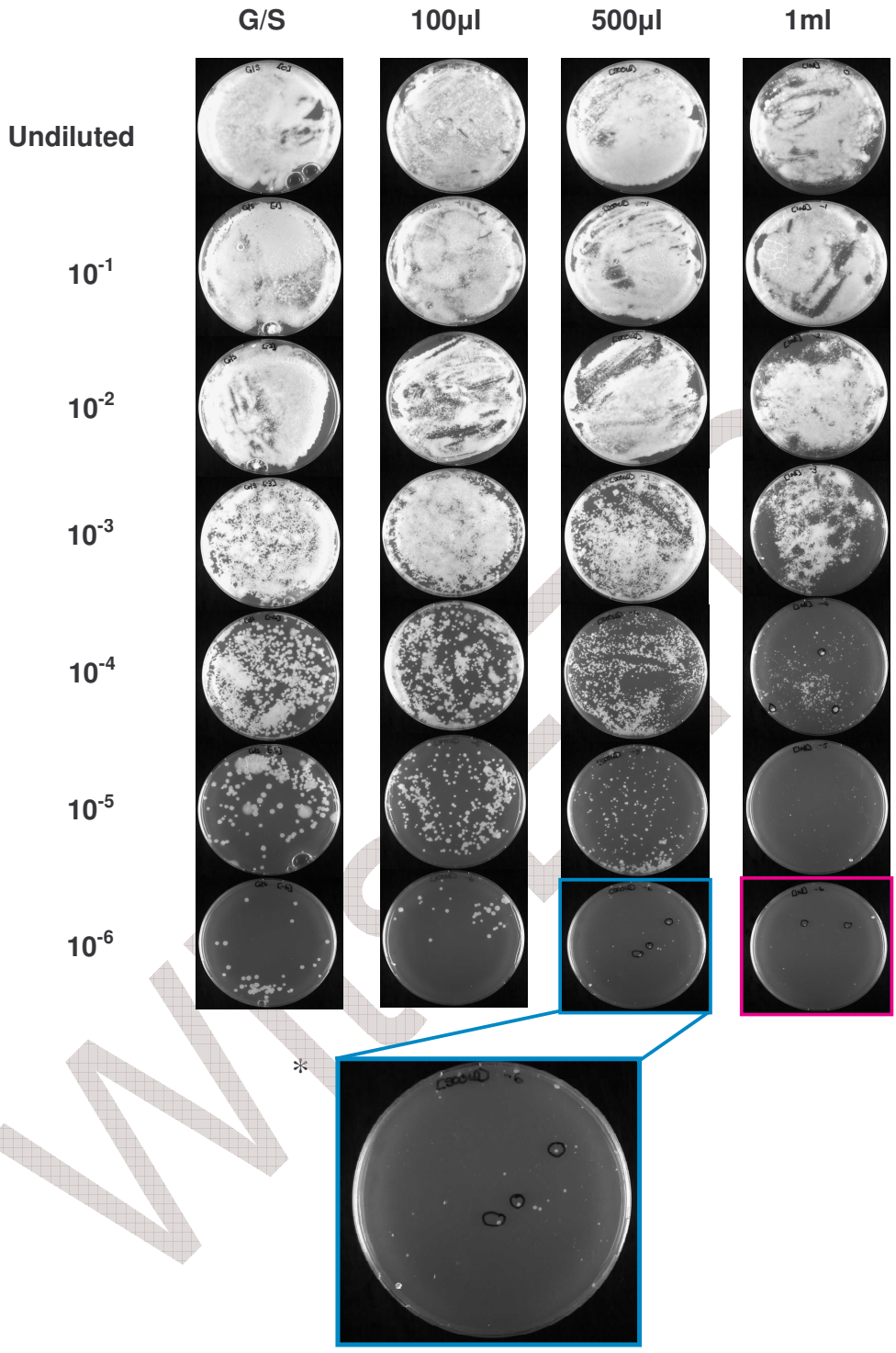


Figure 3.19: Growth inhibition of MSM on solid 7H10 medium supplemented with increasing volumes of the crude extract. Log-fold dilutions of a wild-type MSM culture were plated onto solid 7H10 agar plates containing 100ul, 500ul, and 1ml *Pseudomonas*-derived extract per plate, respectively. Putative resistant mutants were isolated from plates supplemented with 500µl and 1ml of the extract, as indicated by the circled areas (magnified images*).

Several putative mutants were isolated and cultured in liquid 7H9 medium (GS+Tween 80). The mutants exhibited unique growth characteristics: the cells appeared to settle to the bottom of the flask in small clumps, and did not yield an homogenous suspension in the liquid medium. To confirm the heritability of the resistance phenotype (and, therefore, genotype), indicator plates containing each of the selected mutants were prepared and exposed to the extract. However, the "mutants" that had been identified as resistant on the original plates were not resistant to the inhibitory extract in the indicator plate assay (Fig. 3.20). If anything, these isolates appeared somewhat hypersensitive to the extract, as suggested by the larger ZOIs in Fig 3.20A and 3. 20B compared to the ZOI in Fig. 3.20C. However, the basis for this effect is unclear. The data are from a representative experiment that was performed in duplicate.

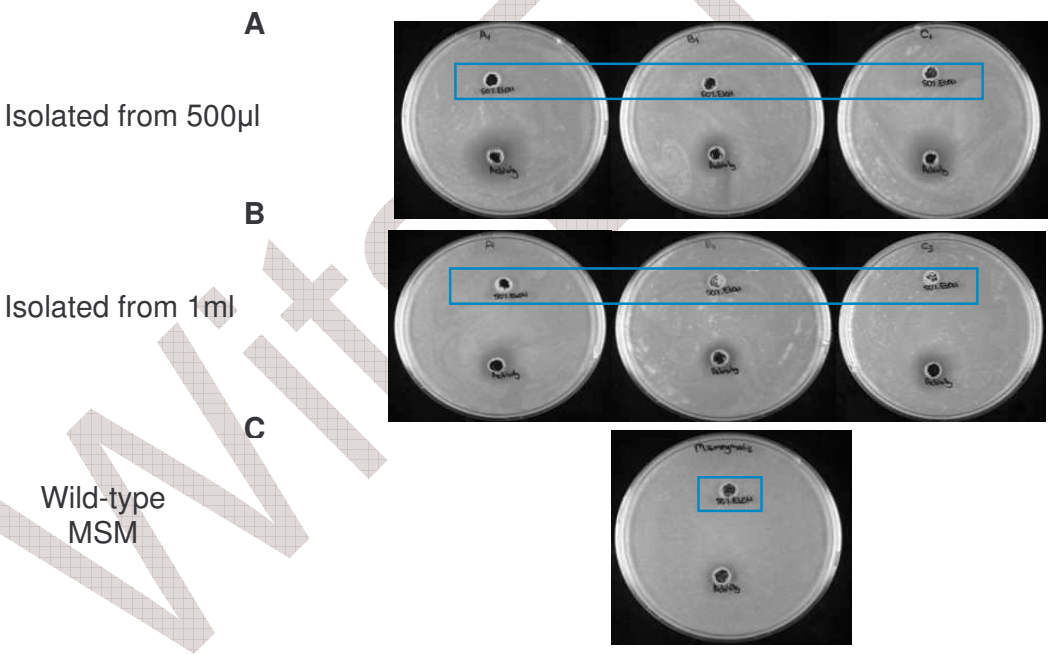


Figure 3.20: Putative resistant "mutants" were not resistant to the inhibitory extract. Indicator plates containing possible "extract-resistant" mutants originally isolated from plates containing 500µl (A) or 1ml (B) of the extract. An indicator plate containing wild-type MSM was used as a control (C). Ethyl acetate control is highlighted in blue.

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3.11 Susceptibility of MSM in liquid culture to the inhibitory compound(s) is increased in the presence of Tyloxapol

We showed previously that MSM was hypersensitive to the inhibitory extract in indicator plates supplemented with Tyloxapol (Fig. 3.17, 5B). In order to determine whether the apparently synergistic effect of Tyloxapol on the extract-mediated growth inhibition applied similarly in liquid medium, a broth microdilution assay was set up as previously described (See Section 3.4) using 7H9 supplemented with either Tween 80 (0.05%) or Tyloxapol (0.02%). In addition, to investigate whether the nature of the carbon source affects susceptibility, the liquid medium was prepared with either GS or Albumin-Dextrose-Saline (ADS) which is often used as a supplement for MTB (57). From these assays, it was established that the nature of the supplement (GS vs. ADS, Tyloxapol vs. Tween 80) impacted the MIC measurement. In general, MSM appeared to grow better in 7H9 supplemented with GS than with ADS: the GS supplemented cells ("7H9 media" control wells; Fig. 3.21) developed the pink colour indicative of bacillary growth 15 hrs after the addition of Alamar blue, whereas the ADS-supplemented cultures ("7H9 media" control wells; Fig. 3.22) remained blue after the same incubation period. Moreover, in GS supplemented medium, Tween 80 and Tyloxapol had a significant effect on the MIC: the addition of Tyloxapol to the growth medium resulted in a significantly lower MIC than that observed with Tween 80 (Fig. 3.21), reinforcing similar observations on solid medium (Fig. 3.17, 5B compared to Fig. 3.17, 4B). The data are from a representative experiment that was performed in duplicate.

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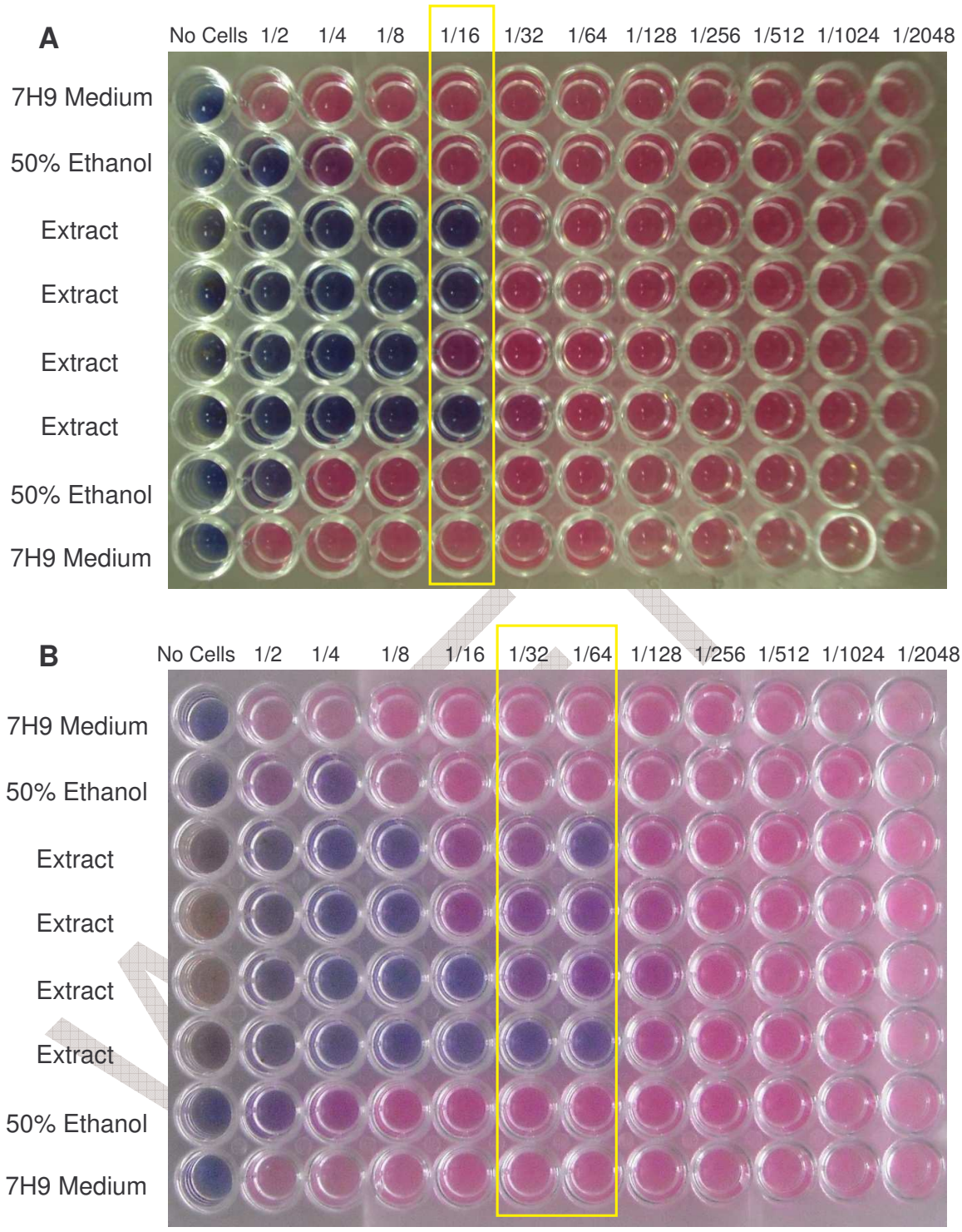


Figure 3.21: The inhibitory effect of the extract on MSM grown in GS-supplemented medium with Tween 80 or Tyloxapol. The effect of the detergent on the inferred MIC of the *Pseudomonas*-derived crude extract was investigated by performing duplicate broth microdilution assays with either Tyloxapol or Tween 80. (A) The MIC occurs at a 1/16 dilution of the neat extract when assayed against MSM grown in GS-supplemented 7H9 medium containing Tween 80 (0.05%),

but lies between 1/32 and 1/64 dilutions when the same medium contains Tyloxapol (0.02%) as detergent. The MIC's are highlighted in yellow.

When ADS was used, supplementation with Tween 80 or Tyloxapol had no significant effect on the MIC: both experiments yielded an MIC at a 1/4 dilution (Fig. 3.22), a value at least two-fold higher than the MIC determined from media supplemented with GS (Fig A and reported previously). This result suggested that the presence of ADS in the liquid medium limited the inhibitory activity of the crude extract. In fact, the lowest MIC was observed when 7H9 was supplemented with GS and Tyloxapol (0.02%). Although these observations suggest that the nature of the C source might affect the activity of the extract, it is equally likely that the presence of albumin in the ADS supplement interferes with the inhibitory compound(s). The molecular basis for this effect will form one aspect of future research into the MOA of the inhibitory compound(s), as indicated below (see Discussion).

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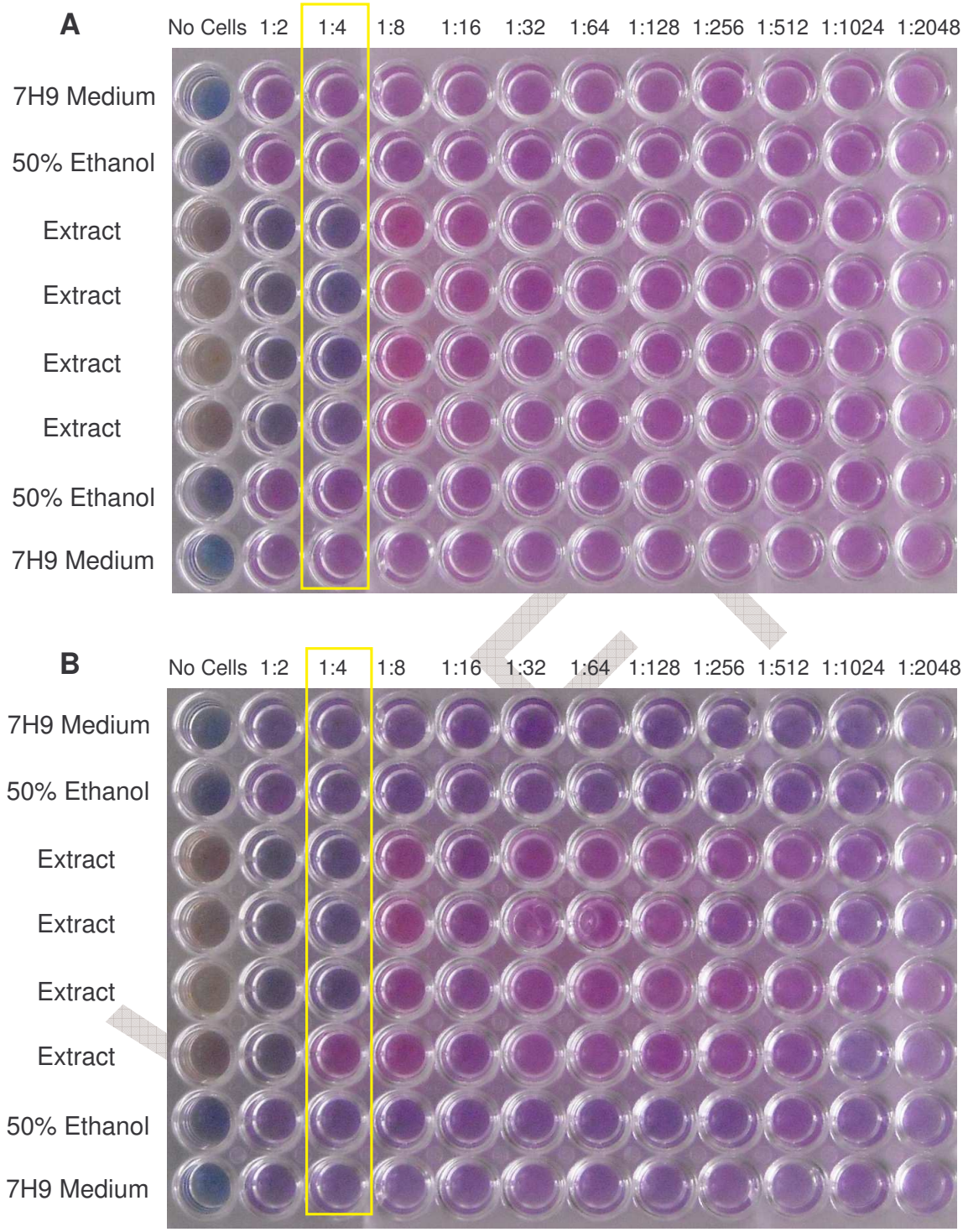


Figure 3.22: The inhibitory effect of the extract on MSM grown in ADS-supplemented medium with Tween 80 or Tyloxapol. (A) The MIC of the inhibitory extract occurs at a 1/4 dilution when 7H9 is supplemented with ADS and Tween 80 (0.05%), and (B) at a 1/4 dilution when 7H9 is supplemented with ADS and Tyloxapol (0.02%). The MIC's are highlighted in yellow.

Nutrient availability not only affects the growth of bacteria, but also the efficacy of antimicrobials. The structure of CLPs can be altered by the nutrient source provided in the bacterial growth medium (142). The addition of bovine serum albumin (BSA) has been previously reported to enhance the growth of tubercle bacilli in liquid medium (42). In contrast to these findings, Sattler and Youmans (1948) reported contradictory results in which BSA did not stimulate growth (113). Both groups did, however, identified the primary role of BSA in binding unesterified fatty acids which are toxic to MTB (42, 113). We observed improved growth of MSM in liquid 7H9 medium supplemented with ADS relative to the same medium supplemented with GS. Although the basis for this observation is unclear, it is possible that albumin (in ADS) might fulfil a similar detoxification role, but this will require further investigation.

3.12 The susceptibility of liquid cultures of MSM to cell wall inhibitors is increased in the presence of Tween 80

The presence of Tyloxapol in the growth medium appeared to increase the sensitivity of MSM to the inhibitory extract. To determine whether this effect was specific to the crude extract, or was a common feature of all antibiotic compounds, we set up broth microdilution assays to examine the sensitivity of MSM grown in the presence of Tween 80 or Tyloxapol to various antibiotics. For these experiments, we selected representative antibiotics: two cell wall biosynthesis inhibitors - vancomycin (VAN) and carbenicillin (CAR), both of which are inhibitors of peptidoglycan (60, 81) - as well as two antibiotics that target processes other than cell wall biosynthesis – KAN, a protein synthesis inhibitor via interference of translation (74), and RIF, which inhibits transcription by binding to the β-subunit of RNA polymerase (24). On the basis of the observations described in the previous section (above), all liquid media used in this experiment were supplemented with GS. The presence of Tween 80 in the liquid medium increased the sensitivity of MSM to the cell wall inhibitors, CAR and VAN (Fig. 2.33). In contrast, the sensitivity of MSM to KAN and RIF remained unchanged regardless of the addition of Tween 80 (Fig. 3.23) or Tyloxapol (Fig. 3.24). This strongly suggests that the cell wall might be a target for our compound(s). It is interesting,

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however, that we did not see enhanced efficacy with Tween 80 although this detergent does improve efficacy of VAN and CAR. The data are from replicate experiments performed at least twice.

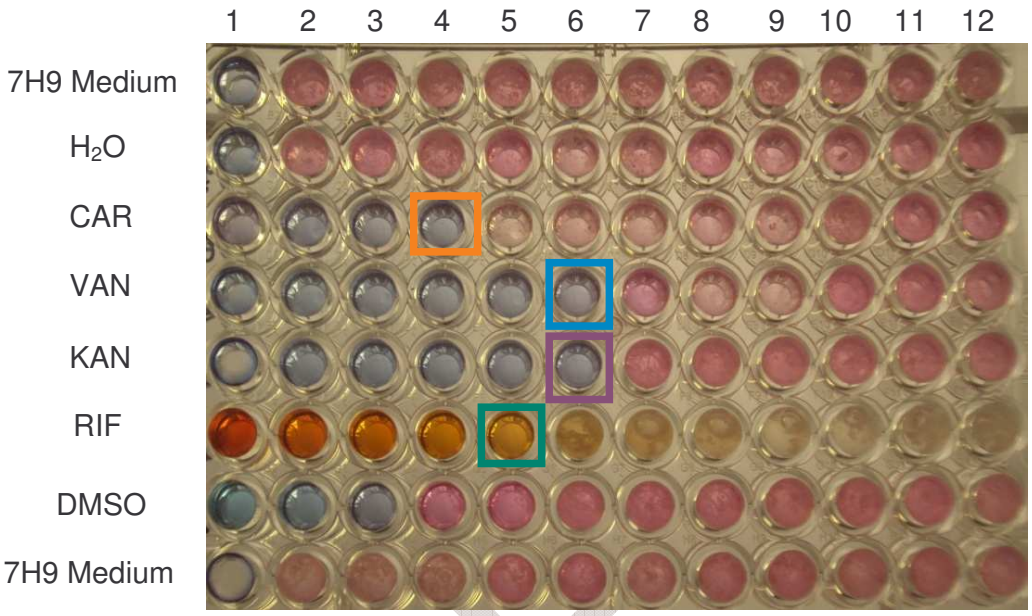


Figure 3.23: Effect of Tween 80 on antibiotic-mediated inhibition of MSM. Starting concentrations in lane 1: CAR, 50mg/ml; VAN, 100 µg/ml; KAN, 80 µg/ml and RIF, 1000 µg/ml. MICs are highlighted: CAR, 3.125 mg/ml (orange), VAN, 1.56 µg/ml (blue); KAN, 1.25 µg/ml (purple) and RIF, 31.25 µg/ml (green).

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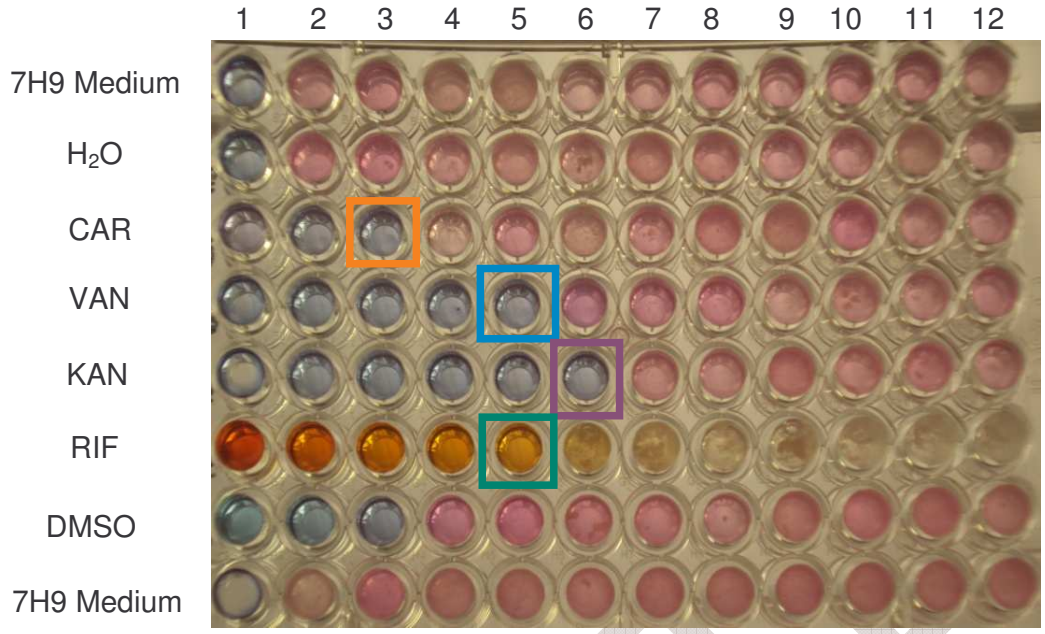


Figure 3.24: Effect of Tyloxapol on antibiotic-mediated inhibition of MSM. Starting concentrations in lane 1: CAR, 50mg/ml; VAN, 100 µg/ml; KAN, 80 µg/ml and RIF, 1000 µg/ml. MICs are highlighted: CAR, 6.25 mg/ml (orange), VAN, 3.125 µg/ml (blue); KAN, 1.25 µg/ml (purple) and RIF, 31.25 µg/ ml (green).

3.13 The extract is active against MSM at concentrations comparable with established antimycobacterial agents

DMSO is the most common solvent in which antibiotics are dissolved (19). Therefore, we assessed its potential as an alternative solvent for the *Pseudomonas*-derived extract. Two independent extracts were dried down to solid pellets, which were weighed (Table 3.2) and then dissolved in 2 ml of DMSO. Prior to resuspension in DMSO, Extract 2 had been stored for 5 weeks at -20℃. Both extracts were incompletely soluble in DMSO, as evidenced by the presence solid particulate matter in the DMSO suspension. In order to ensure a homogenous solution, the insoluble matter was removed and weighed, and this mass deducted from the initial mass to allow the calculation of an approximate final concentration of 16.5 mg/ml for Extract 1 and 7.5 mg/ml for Extract 2 (Table 3.2).

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Table 3.2: Calculation of final concentrations of Extract 1 and Extract 2

	Initial Mass (mg)	Mass of insoluble deposits (mg)	Final Mass (mg)	Estimated Final Concentration (mg/ ml)
Extract 1	68	35	33	**16.5**
Extract 2	75	60	15	**7.5**

Both extracts inhibited growth of MSM in indicator plates (Fig. 3.25). Moreover, despite the fact that the concentration of Extract 1 was more than double that of Extract 2, there appeared to be no significant difference in the inhibitory effect of the two extracts against MSM grown on solid 7H10 medium.



Figure 3.25: Inhibition of MSM by the inhibitory extract when dissolved in DMSO. There was no significant difference in the ZOIs despite differences in concentrations between the extracts. DMSO control (A), Extract 1 at a concentration of 16.5 mg/ml (B) and Extract 2 at a concentration of 7.5 mg/ml.

The broth microdilution method was then used to examine the inhibitory effect of the DMSO extracts against MSM in liquid. In both cases, the extract appeared to be quite potent, as inhibition was observed at concentrations of 8 µg/ml and 1.8 µg/ml for Extract 1 and Extract 2 respectively (Fig. 3.26). The data are from a representative experiment that was performed in triplicate.

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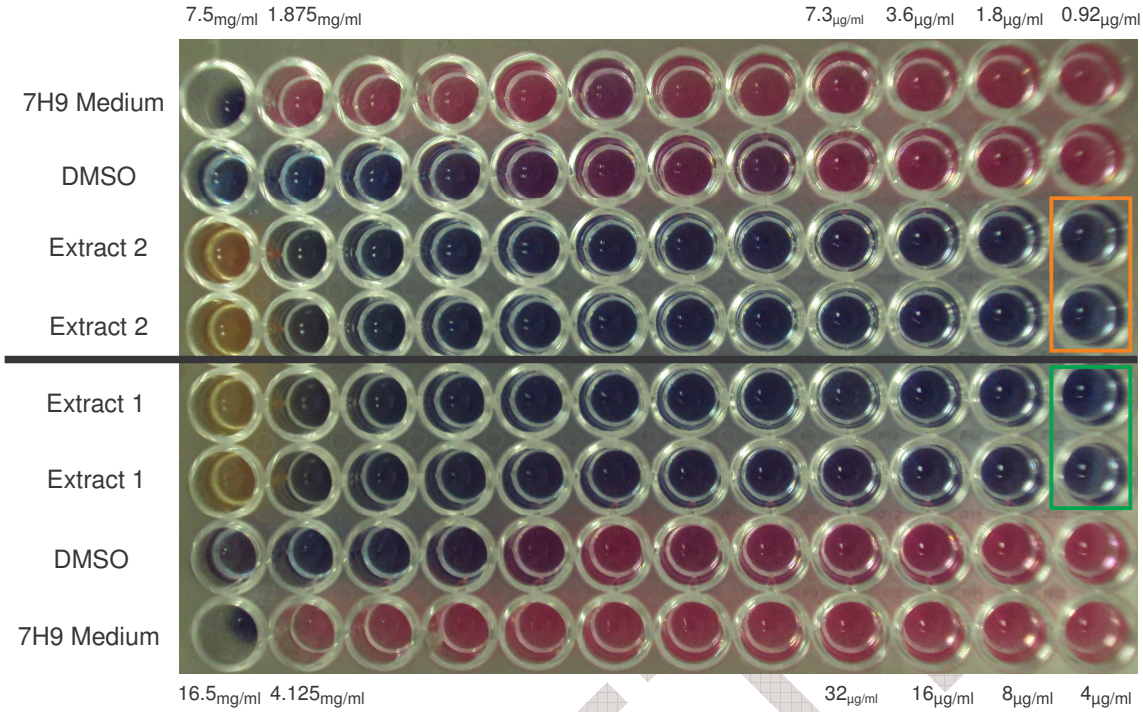


Figure 3.26: Inhibition of MSM, in liquid culture, by Extract 1 and Extract 2. Starting concentrations of Extract 1 and Extract 2, 16.5 mg/ml and 7.5 mg/ml, respectively. Inhibition was observed at concentrations of 4 µg/ml for Extract 1 (green) and 0.9 µg/ml for Extract 2 (orange).

An analysis of the potency of the *Pseudomonas*-derived inhibitory extract in comparison to two established antimycobacterials, KAN and EMB, was then examined. The MIC of Extract 1 ranged between 0.23 µg/ml and 0.55 µg/ml (Fig. 3.27) and between 1.2 µg/ml and 0.63 µg/ml for Extract 2 (Fig. 3.28). That is, the MIC of the *Pseudomonas*-derived inhibitory extract against MSM can reasonably be estimated as falling between 1.2 µg/ml and 0.2 µg/ml. This value compares favourably with the published MSM MICs of EMB (2 µg/ml) and KAN (0.63 µg/ml), especially since these compounds are supplied in purified form whereas the *Pseudomonas*-derived inhibitory compound(s) is present in crude extract. The data are from an experiment that was only performed once, as the MICs of EMB and KAN compared favourably with published MICs.

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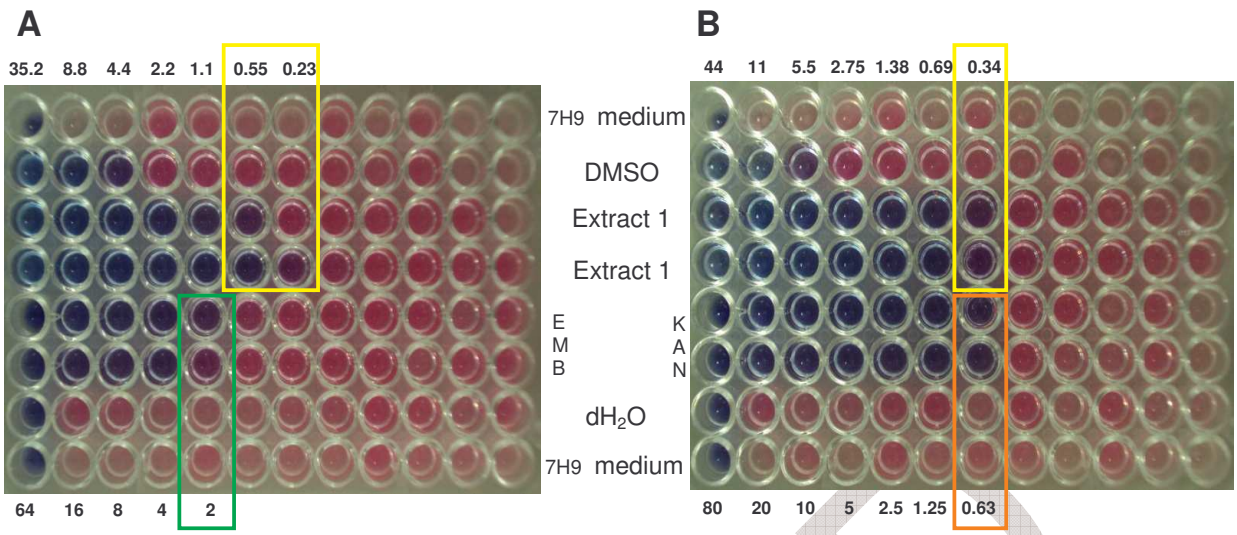


Figure 3.27: Comparison of the growth-inhibitory efficacy of Extract 1 with known antimycobacterials. EMB (A) and KAN (B). All antimicrobials are at concentrations of µg/ml. The MICs of Extract 1 (highlighted in yellow) ranges between 1.1 µg/ml and 0.55 µg/ml. The MIC of EMB is 4 µg/ml (highlighted in green) and the MIC of KAN is 1.25 µg/ml (highlighted in orange).

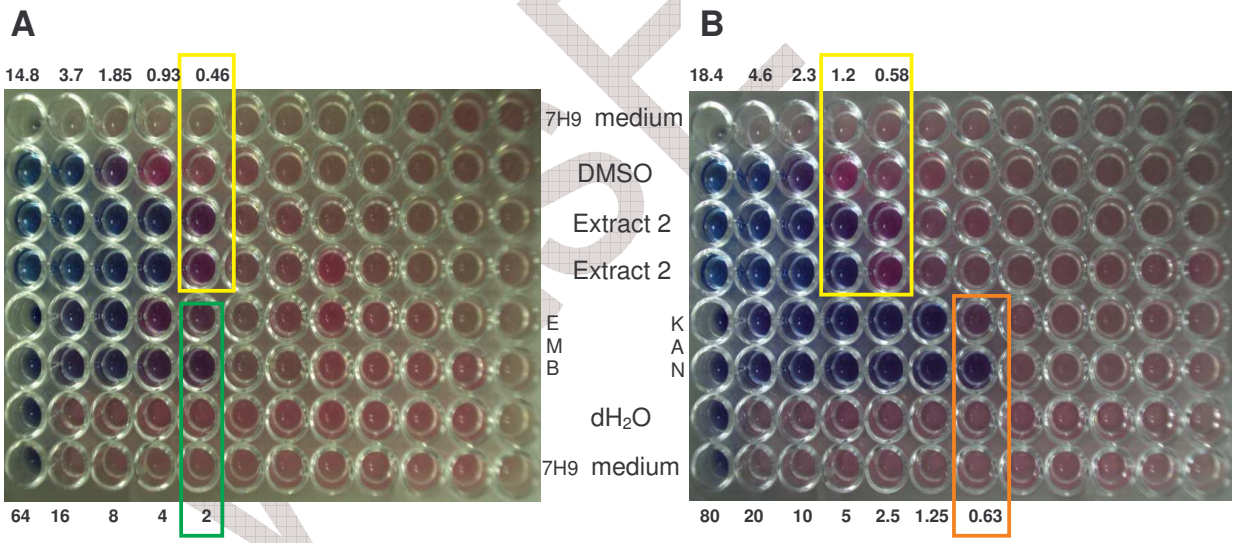


Figure 3.28: Comparison of the growth-inhibitory efficacy of Extract 2 with known antimycobacterials. EMB (A) and KAN (B) All antimicrobials are at concentrations of µg/ml. The MIC of Extract 2 (highlighted in yellow) ranges between 1.2 µg/ml and 0.46 µg/ml. The MIC of EMB is 2 µg/ml (highlighted in green) and the MIC of KAN is 0.63 µg/ml (highlighted in orange).

3.14 The *Pseudomonas*-derived extract inhibits growth of MTB

To determine the effect of the inhibitory extract on MTB, log-fold dilutions of an exponential phase culture of H37Rv were serially plated onto 7H10 medium containing 1ml of the extract. After 3-4 weeks of incubation at 37℃, inhibition of growth was not observed. However, we hypothesized that the prolonged incubation period at 37℃, might have impacted negatively on the activity of the inhibitory compound(s).

Therefore, we switched to the broth microdilution method (32, 41) which allows results to be scored within two weeks of incubation, to test the inhibitory extract, dissolved in DMSO, against MTB. The effect of the extract dissolved in DMSO was compared to that of RIF and KAN. The broth microdilution method was modified when testing against MTB by adding media only to the outer perimeter wells of the 96-well microtitre plate to prevent dehydration in experimental wells during the incubation period. The MIC of Extract 1 was compared to that of RIF (Fig. 3.29). The starting concentration of RIF (25µg/ml) was too high, as killing was observed in all wells even where diluted 1000-fold. The published MIC of RIF for MTB ranges between 0.01- 0.02µg/ml (28, 41). Therefore in order to observe this MIC, the starting concentration of RIF should be much lower, possibly 4µg/ml. Despite this oversight, we estimated the MIC of Extract 1 at 16µg/ml. The data are from a representative experiment that was performed in triplicate.

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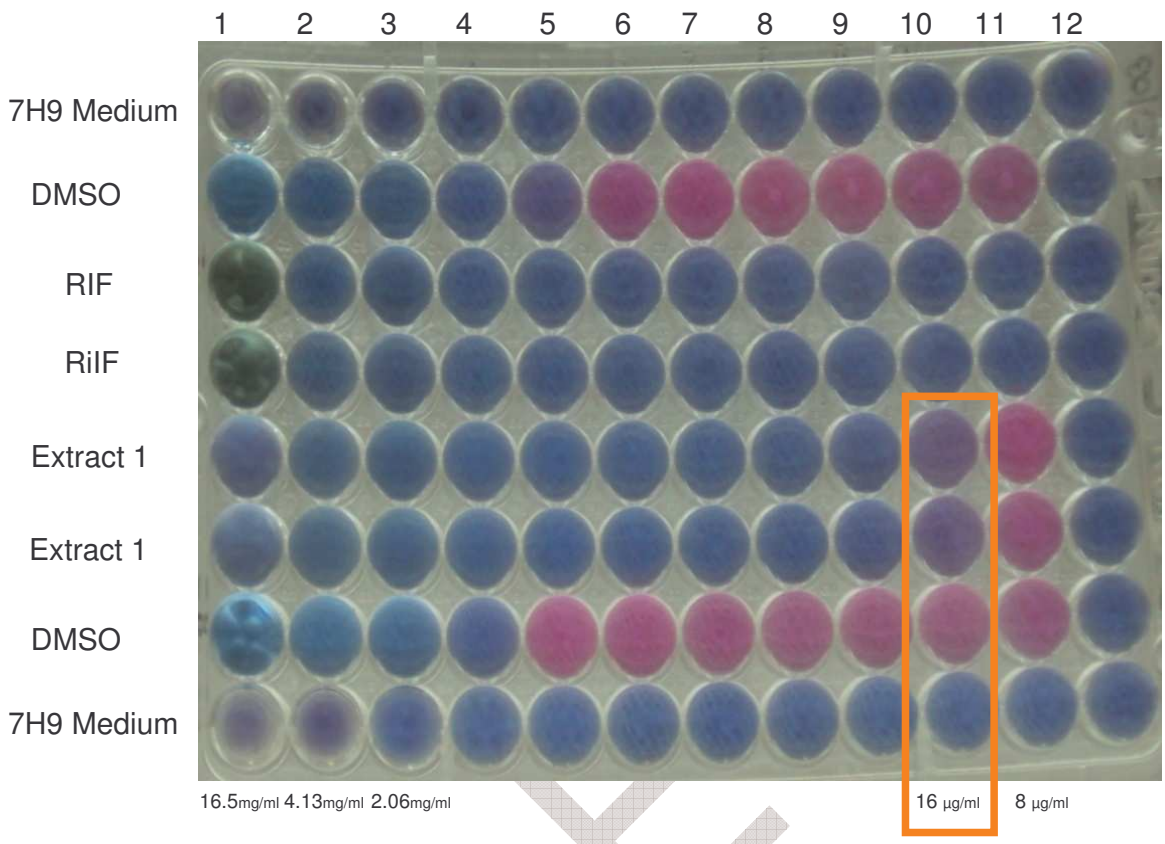


Figure 3.29: Examination of the inhibitory effect of Extract 1 against MTB and comparison to the MIC of RIF. No cells were added to the outer perimeter wells of the micotitre plate. Starting concentrations, in lane 1, for RIF is 25 µg/ml and 16.5 mg/ml for Extract 1. The MIC of the inhibitory extract is 16 µg/ml (highlighted in orange).

Additionally, the MIC of Extract 2 was compared to that of KAN (Fig. 3.30). In this experiment, the MIC of KAN was observed to be 3.125 µg/ml while the MIC of Extract 2 is 14.6 µg/ml. The MIC of KAN in MTB ranges between 1- 8 µg/ml, as reported (2, 41), which is consistent with the findings in this study.

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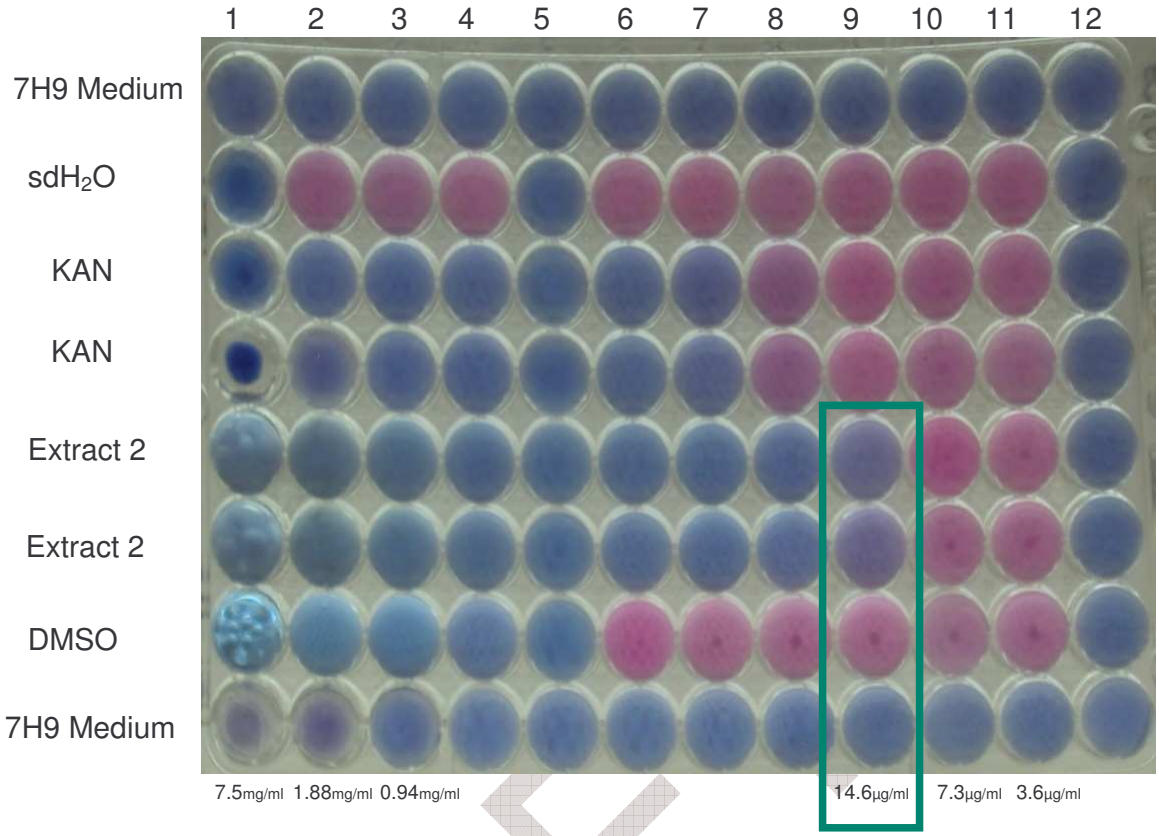


Figure 3.30: Examination of the inhibitory effect of Extract 2 against MTB and comparison to the MIC of KAN. No cells were added to the outer perimeter wells of the micotitre plate. Cells were unintentionally excluded from Lane 5, however this does not interfere with the MIC of KAN or Extract 2. Starting concentrations, in Lane 1, for KAN is 200µg/ml and 7.5mg/ml for Extract 2. The MIC of the inhibitory extract is 14.6 µg/ml (highlighted in green).

Importantly, these data provide good evidence that MTB might be susceptible to the *Pseudomonas*-derived inhibitory extract. Notably, similar results were obtained from duplicate experiments performed with separate extracts. However, these data are preliminary and require further repetition with appropriate controls in order to establish conclusively that MTB is susceptible to the crude extract.

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4.0 DISCUSSION

The rise of resistant pathogens emphasizes the dire need for new therapeutic agents (126). This is especially true for MTB, a major human pathogen that has in recent years been associated with the increasing emergence of multi-drug resistance (116). The majority of effective treatments currently available derives from nature, specifically microorganisms (37). Microbes constitute a rich reservoir of bioactive microbial products, and it is estimated that a mere 1% of the global consortium of microbial producers has been discovered (47). In this study, we identified a bacterial isolate that was shown to be inhibitory to the growth of MSM. Following 16S rRNA analysis, the isolate was designated *Pseudomonas* αMB to reflect its antimycobacterial activity. Subsequently, we demonstrated the stable extraction of the inhibitory compound(s) using ethyl acetate as a solvent, and confirmed that the *Pseudomonas*-derived extract was produced exclusively by our αMB strain. Although the MOA remains unclear, microbiological assays indicated that the inhibitory activity was specific for Gram-positive organisms. Moreover, the crude extract was effective against MTB in broth culture at a concentration that compared favourably to key frontline and secondary anti-TB drugs. As such, the *Pseudomonas*-derived active agent(s) represents a compelling candidate for further investigation as potential lead compound(s) for a novel antimycobacterial agent(s).

The dynamics of secondary metabolite production

Most bioactive compounds are organic molecules of low molecular weight (37). These secondary metabolites are conventionally thought to function as an alternative defence mechanism, conferring a selective advantage on the producer organism (83). However, recent studies have described the function of secondary metabolites in terms of the phenomenon of hormesis which holds that the activity of a compound is concentration-dependent (37): that is, all natural small molecules regulate transcription at lower concentrations (environmental levels) and exhibit antibiosis, or inhibitory activity, at higher (artificial) concentrations (36). Notably, the hormetic properties of small molecules have been confirmed in studies in

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which multiple phenotypes (such as morphology and antibiosis) can be induced by growing environmental bacteria in close proximity on agar plates (128).

Natural environments, such as marine or soil ecosystems, offer a potentially rich source of antimicrobial producers. The bacterial strain characterized in this study was initially isolated from a soil sample on an MSM indicator plate, and was subsequently identified as having 99% sequence similarity to several *Pseudomonas* species (Fig. 3.4). For this reason, we designated the isolate as *Pseudomonas* αMB to denote its demonstrated antimycobacterial effect. Future work, including a combination of molecular genetics and the generation of a complete genome sequence, will be required to situate our isolate accurately within the genus, as well as to confirm the nature of the inhibitory compound(s) and to identify the machinery responsible for its production (discussed below).

Pseudomonas is a genus of γ-proteobacteria, one of four microbial taxa shown to be major producers of currently available antimicrobials (47). Secondary metabolism is a characteristic feature of *Pseudomonas* spp. which produce diverse metabolites including siderophores, CLPs, phytotoxins, and bioactive compounds with antibacterial and antifungal activities (55). The parallel ethyl acetate extractions on *Pseudomonas* αMB and an unrelated *P. putida* strain indicated that the observed antimycobacterial effect was not common to all *Pseudomonas* spp (Fig. 3.12) and, further, eliminated the possibility that the inhibitory effect resulted inadvertently from the ethyl acetate-based extraction protocol. Taking into consideration the phenomenon of hormesis, it is possible that the *Pseudomonas*-derived compound(s) described in this study may have alternative biological functions and that the observed inhibitory effect is a direct consequence of the increased concentration of these molecules on solid agar medium.

A number of factors may trigger secondary metabolite production. These include the depletion of nutrients, the synthesis or addition of an inducer, as well as a decrease in the growth rate of the producer organism (39). Numerous studies have exploited fermentation technology to stimulate the production of bioactive secondary metabolites (98, 99, 142). In the study presented here, a modified version of solid substrate fermentation was used to stimulate the production of the inhibitory activity. This methodology involves fermentation on a solid matrix in the

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absence - or near absence - of free water, though with sufficient moisture to sustain the growth and metabolism of the microorganism (120). We observed maximal production of the inhibitory compound(s) after increasing the incubation time of the 7H10 agar plates containing *Pseudomonas* αMB (Fig. 3.8). Additionally, an insignificant amount of the inhibitory compound(s) was secreted into the agar during the incubation period (Fig. 3.9), indicating that the majority of the inhibitory compound(s) is extracted from the *Pseudomonas* αMB cells. Alternatively, it is possible that the applied protocol is inadequate for the extraction of the inhibitory compound(s) from the agar, and other methods could be examined, such as freeze-drying the agar before extraction (14). Given that the inhibitory effect was significantly increased after a longer fermentation period (Fig. 3.8), it is possible that the depletion of nutrients or the biosynthesis of an inducer may be the trigger(s) for the accumulation of the inhibitory compound(s). The growth medium was unsupplemented 7H10 – that is, standard 7H10 agar without an additional carbon source in the form of glucose or equivalent; therefore, it seems unlikely that an external inducer is operating under these conditions. However, since the growth rate of the *Pseudomonas* was not monitored during the fermentation period, the role of an inducer in stimulating production cannot be definitively excluded. Future studies are required to elucidate the stimuli for production, as well as the specific molecular elements regulating the genetic programme for the biosynthesis of the inhibitory compound(s). To this end, we have obtained a (HiMar-based) transposon which will be used to generate a whole-genome knockout library of *Pseudomonas* αMB (80), and so should facilitate the identification of genes required for the production of the inhibitory compound(s).

The protocol applied in this study (Fig. 3.5) was sufficient for the small scale extraction of the activity. However, as suggested by our preliminary attempts to optimize the extraction, there are many steps within the protocol that might be modified in order to improve the yield of the active compound(s). As mentioned previously, fermentation conditions have considerable influence on the production of secondary metabolites (103). (103). Studies have shown that variation of media composition (79), as well as the life cycle stage of the initial inoculum (that is, isolated from younger versus older cultures) can influence the production of

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different metabolites (103). Therefore, additional modifications of the extraction protocol to improve yield might include the use of controlled fermentation of liquid cultures, which can also have a significant impact on the quantity of inhibitory compound isolated (10, 58). The composition of the growth medium might also be adjusted; for example, the nature of the nitrogen source has been shown to influence secondary metabolite production with not all nitrogen sources supporting the production of certain secondary metabolites (79). In addition, the introduction of oxygen, nitrogen, chlorine, or sulphur can affect the function of the synthesized metabolite(s) (23, 82, 84). For the extraction process, alternative organic solvents such as acetone, chloroform, hexane (10) or methanol (23) may yield more potent activity.

Solubility of the inhibitory compound(s)

We inherited a preliminary extraction protocol based on ethyl acetate (94). Although it proved a useful solvent for the extraction of the inhibitory compound(s), ethyl acetate posed some problems in subsequent experiments, primarily as a result of ethyl acetate-mediated degradation of standard plastic consumables (microtitre plates in particular). Several alternative solvents were evaluated, including 50% ethanol and DMSO. Although 50% ethanol circumvented the problem of degradation, the inhibitory activity of the crude extract seemed to be reduced in 50% ethanol relative to ethyl acetate (Fig. 3.10). This might indicate that inhibition requires the activity of more than one active compound, each having its own biochemical properties including different solubility in different solvents. Notwithstanding the minor reduction in activity, we selected 50% ethanol as an alternative solvent for the bulk of our initial experiments as it was not inhibitory to bacterial growth on its own and, importantly, the ethanol-based extract retained antimycobacterial activity, ensuring that it could be used in broth microdilution system (Fig. 3.11). However, the extract was not completely soluble in 50% ethanol, and moderate quantities of insoluble deposits were observed, again reinforcing the likely presence of more than one active compound. The accumulation of insolubles during extraction procedures appears to be a common occurrence, as others have reported the need to apply subsequent purification

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steps to remove insoluble deposits, such as filtration through a fritted funnel *in vacuo* (23).

Non-polar compounds are predicted to be more soluble in ethyl acetate than 50% ethanol, which is the more polar of the two solvents. The differential (reduced) activity of the crude extract when resuspended in polar (50% ethanol) versus non-polar (ethyl acetate) solvent suggests that the inhibitory compound(s) may be non-polar in nature. In later experiments, DMSO was examined as a possible solvent in order to ascertain the effect of solvent on inhibitory activity (Fig. 3.23 – Fig 3.26). On solid medium, there appeared to be no significant difference for DMSO relative to ethyl acetate (Fig. 3.23). However, when the inhibitory compound(s) was assayed in liquid medium, the potency appeared to be significantly higher when DMSO was used as a solvent compared to 50% ethanol (Fig. 3.24). This might be a direct consequence of the dual hydrophobic and hydrophilic characteristics of DMSO (19), which would ensure the solubility of different compounds within the extract with different solubility characteristics. This possibility is further supported by the moderate solubility of the compound(s) in 50% ethanol which suggests that the compounds are not totally hydrophilic in nature. However, further characterization is required to establish unequivocally that solubility determines the apparent relative potency of the inhibitory agent(s) in different solvents which might be summarized as follows: DMSO > ethyl acetate > 50% ethanol.

Initially, solid media were supplemented with a 1ml volume of the crude extract to test the susceptibility of MTB H37Rv to growth inhibition. At this concentration, no inhibition of MTB was evident on agar plates. MTB has a doubling time of ~24hr when incubated on solid 7H10 agar at 37^{o}C under standard aerobic conditions (77); it is possible, therefore, that the inhibitory compound(s) loses activity during the extended incubation period (three to four weeks) required to enumerate viable CFUs. Also, it is important to note that this experiment utilized 50% ethanol as solvent, which might have influenced the experimental outcome, as discussed. Notably, this result was consistent with our previous observation that the crude extract dissolved in 50% ethanol was not as active against MSM as the equivalent ethyl acetate-dissolved extract (Fig. 3.10).

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The crude extract inhibits growth of Gram-positive organisms

The crude extract was tested against a spectrum of bacteria, including various actinomycetes, Gram-positives and the Gram-negative *E. coli* (Fig. 3.18). Actinomycetes are characterized by highly complex cell walls comprising branched fatty acids called mycolic acids (18). The cell walls of other Gram-positives (for example, *Bacillus subtilis*) are made up of teichoic and lipoteichoic acids, whereas the Gram-negative *E. coli* possesses a cell wall comprising lipopolysaccharides (44). In our assays, Gram-positive bacteria generally appeared to be more sensitive to the growth inhibitory effects of the extract, with the *Nocardia* strains the most sensitive overall (Fig. 3.18). The apparent hypersensitivity of *Nocardia* to the *Pseudomans* αMB-derived compound(s) is of special interest as MTB and *Corynebacterium glutamicum* are most closely related to *N. farcinica* phylogenetically (132) and the closeness of this relationship has suggested Nocardia as a useful model for some aspects of mycobacterial physiology (33, 132).

Although these assays did indicate the ability of the crude extract to inhibit growth of *E. coli*, this effect was minimal and considered insignificant when compared with its strong inhibition of the Gram-positive organisms, including MSM (Fig. 3.18). Nevertheless, we cannot exclude the possibility that there are multiple compounds within this crude extract and, further, that some of these might be active against Gram-negative bacteria. However, as only two strains of the representative Gram-negative *E.coli* species were tested against the crude extract, further examination of the potential Gram-restricted target specificity is required in which more than one Gram-negative species is tested.

This experiment also highlighted the important influence of the solvent on the observed inhibitory activity: for many of the strains tested - for example, *M. parafortuitum* and the *Rhodococcus* spp. - inhibition was only observed when ethyl acetate was used as the solvent, whereas for organisms such as MSM, *Staphylococcus* spp., and *Bacillus* spp., the ethyl acetate-based extraction resulted in increased inhibition. Again, these observations reinforce the possibility that the extract contains multiple compounds with diverse biochemical properties and that the composition of the crude resuspension might be differently affected depending on the nature of the chosen solvent. This effect would be exacerbated

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if the inhibitory activity were a function of multiple active constituents acting synergistically.

The demonstrated activity of the crude extract against Gram-positive bacteria supports the need to investigate the *Pseudomonas* αMB-derived compound(s) further as candidate lead molecules. Gram-positive infections are of particular concern in the hospital environment (109, 141). It is thought that the focus in the 1970s and 1980s on the development of drugs against Gram-negative bacteria might have resulted in the slow evolution and selection of Gram-positive bacteria (7). In turn, this might explain the increasing prevalence of Gram-positive infections. As noted previously, only a few classes of novel antibiotics have been introduced clinically since 1999. These include the streptogramin combination, quinupristin/dalfopristin; the oxazolidinone, linezolid; the lipopeptide, daptomycin; cationic antimicrobial peptides such as ketolides; the glycylcyclines such as tigecycline; the glycopeptides, oritavancin and dalbavancin; and the lipoglycodepsipeptide antibiotic, ramoplanin (61). Further work is required to elucidate the MOA of the *Pseudomonas*-derived extract; however, the fact that the cell wall constitutes the major target of many of the newer Gram-positive antimicrobials (Figure 4.1) suggests that the active compound(s) is likely also to target the cell wall, or cell membrane.

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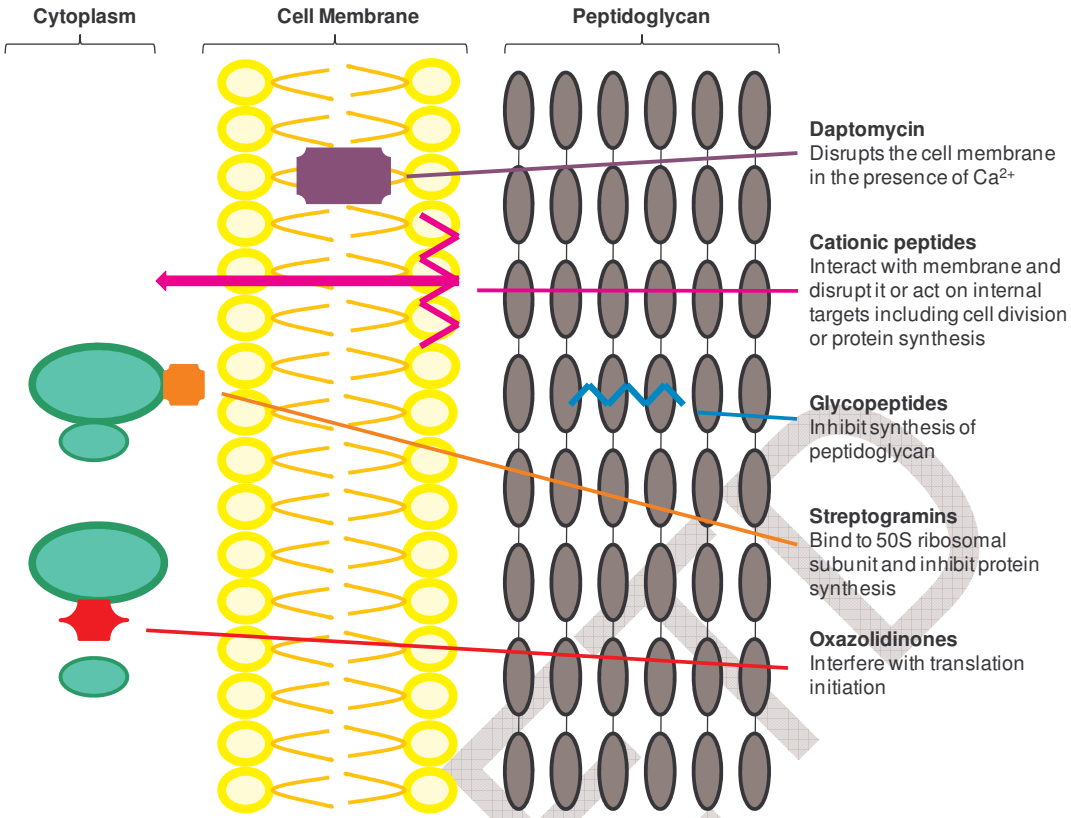


Figure 4.1: The MOA of the newer antimicrobial agents introduced for use against Gram-positive bacteria (Adapted from (62)).

What is the active compound(s) in the *Pseudomonas*-derived extract?

The limited solubility of some antimicrobials affects their drug delivery potential by decreasing the bioavailabilty of the active compounds (86). An example is RIF, which is classified as a class II drug on account of key characteristics: it is highly permeable but has low solubility and high hydrophobicity (86). The relative insolubility of the crude extract in 50% ethanol appeared to correlate with reduced activity against Gram-positive organisms (including MSM) when compared with the same extract dissolved in ethyl acetate. This suggests that the ethyl acetate-soluble compound(s) within the ethanol-insoluble deposits might act synergistically to effect inhibition. A possible candidate active compound is the known antibiotic pyrrolnitrin, or a derivative thereof. Pyrrolnitrins were first isolated from

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Pseudomonas pyrrocinia and, subsequent to their initial discovery, were isolated from a number of other *Pseudomonas* species (130). Of these, pyrrolnitrin 1 has demonstrated antimicrobial activity against fungi, yeast and Gram-positive bacteria, including mycobacteria (130). The possibility of there being more than one active compound acting synergistically is appealing, and is consistent with the relative activity of the extract in different solvents, as well as our inability to isolate a resistant mutant (discussed below). Recently, a U.S. patent was issued to Casida, L.E. for *Burkholderia ambifaria* strain 679-2 based on its broad-spectrum, extracellular antimicrobial activity (23). Following extraction and purification of the inhibitory activity, three distinct compounds were isolated: two of these, pyrrolnitrin and maculosin, had been identified previously while the other was a novel compound named banegasine. Notably, when maculosin and banegasine were tested individually or in combination, antimicrobial activity was not observed. However, increased antimicrobial activity was demonstrated against a spectrum of bacteria using a combination of all three compounds (23). This study highlights the importance of pursuing compound(s) that exhibit a broad spectrum of activity and cautions that measurement of antimicrobial activity of single compounds may exclude promising candidates.

The addition of Tween 80, a non-ionic surfactant, had no significant effect on the growth of MSM or the inhibitory activity of the extract, regardless of the available carbon source (Figs. 3.21 and 3.22). This is equivalent to the data obtained from experiments performed on solid media in the presence of cell wall-perturbing agents (Fig. 3.17). However, the addition of Tyloxapol lowered the MIC of the extract significantly in liquid media containing glucose as the carbon source (Figs. 3.21 and 3.22), reinforcing the observation that Tyloxapol renders MSM, hypersensitive to the inhibitory extract in indicator plates (Fig. 3.17). The addition of Tween 80 has been reported to enhance the growth of MSM, as it is metabolized into oleic acid and converted into triacylglycerols, which can then be used as carbon sources for lipid biosynthesis and other biomass building blocks (125). In contrast, Tyloxapol is a non-hydrolyzable detergent, therefore cells grown in the presence of Tyloxapol do not enjoy the same growth advantage as that conferred by Tween 80 – a factor that might partially account for the apparent hypersensitivity of MSM to the extract in media supplemented with Tyloxapol. The

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effect of SDS on MSM growth in liquid media was not examined. However, supplementation of indicator plates with 0.001% SDS rendered MSM hypersensitive to the *Pseudomonas*-derived extract (Fig. 3.17). Cell wall permeability defects have been shown to enhance susceptibility to lipophilic antibiotics, which include some first line antituberculosis agents such as RIF, EMB and INH (131). Recently, Vandal *et al.* (2009) showed that acid-sensitive MTB mutants with cell wall defects were hypersensitive to SDS and appeared to be more sensitive to lipophilic antibiotics (131). In the study presented here, the hypersensitivity of MSM to SDS suggests the inhibitory compound/s may be lipophilic in structure. However the evidence is only preliminary and further work is required to elucidate the structure of the inhibitory compound(s).

Preliminary elucidation of MOA of the inhibitory compound(s)

As noted previously, natural product extracts have proved a valuable source of antibiotics; however, the attractiveness of natural products is limited to some extent by the high probability of re-isolating known compounds (134). In addition, evidence suggests that in many cases the inhibitory activity is non-specific. In combination, these observations place a premium on the elucidation of the MOA, which is also critical to the potential development of a compound as a drug candidate (101) and impacts both target specificity as well as any subsequent attempts to derivatize the lead molecule for improved pharmacological effect.

One approach to revealing the MOA is through the isolation and characterization of mutant strains resistant to the compound of interest. Our experiments aimed at isolating MSM mutants resistant to the crude extract proved unsuccessful (Fig. 3.19). Moreover, apparent mutants (phenotypically resistant colonies) were associated with resistance phenotypes that were not reproducible in classic heritability experiments (110). In some cases, the colonies isolated from solid media containing inhibitory activity failed to grow in the presence of the extract. It is likely that these conflicting results are attributable to multiple factors, and similar attempts to identify resistant mutants have failed in other screens of natural compounds (102). In addition, as noted above, the presence of more than one

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compound within the *Pseudomonas*-derived inhibitory extract might further confound attempts to produce resistant mutants.

Synergistic activity suggests that the compounds attack different targets (23). Most forms of drug resistance are thought to occur in a single step as a result of a single spontaneous mutation. However, it has been reported that a single mutation may change the susceptibility to more than one type of antimicrobial at a time (111). Therefore the colonies isolated and initially identified as resistant mutants in this study may have mutations that confer resistance to certain compounds within the inhibitory extract but are susceptible to other more potent compounds also present in the extract. Cavalieri *et al.* (1995) reported that the combination of clarithromycin, a drug that showed relatively poor *in vitro* activity against MTB, and various first-line antituberculosis drugs provided a potential solution to multidrug resistance (26). Even though characterization of resistant mutants is a common method of defining the MOA of an antimicrobial compound, it has to be taken into account that mutations conferring resistance are not only located in target genes (50). Again, if there is indeed more than one inhibitory compound within the extract, resistant mutant characterization may not be ideal as target-related resistance mutations may not be identifiable (50).

Whole cell-antibacterial screening

In drug discovery programmes, certain characteristics of potential antimicrobials must be considered before candidates can be developed (102). Aside from the broad experiments highlighted in Fig. 4.2, a number of specific tests (Dr. Helena Boshoff, personal communication) can be performed to further characterize and develop a candidate antimicrobial compound (14). Firstly, determination of target specificity of the candidate compound is required since demonstrated activity against the organism of interest (in this case, MTB) is essential. For this reason, primary screens test the activity of selected compounds against MTB H37Rv, the fully virulent laboratory strain of MTB that has a drug susceptibility profile which is considered representative of drug susceptible clinical isolates (100). In addition, testing for activity against other bacteria (*E. coli*, for example) excludes the possibility that the compound may be a generic cellular toxin, a concern that might

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be further examined in toxicity assays (14, 102). In the study presented here, the two strains of *E. coli*, the selected representative Gram-negative species, both exhibited limited sensitivity to the crude extract, in comparison to the representative Gram-positive species. Further investigation of the Gram-specificity of the *Pseudomonas*-derived extract is required, through the examination of a wide range of Gram-negative organisms. This will form the basis of future work. In addition to Gram-restricted target specificity, preliminary broth microdilution assays demonstrate inhibition of MTB with an apparent MIC of 14-16 µg/ml of crude extract.

In addition to target specificity, it is important to establish that the compound(s) of interest is specifically produced by a particular strain and not a broad range of microorganisms that have been previously exploited. Accordingly, we were able to demonstrate that the inhibitory compound(s) is produced by the *Pseudomonas* αMB strain but not the closely related *P. putida*. A limitation of many natural product studies is the inability to extract the active compound(s) in sufficient quantity. We did not examine production of the *Pseudomonas*-derived compound(s) in liquid medium, as it is not unusual for organisms to produce secondary metabolites on solid medium only. However, liquid medium extractions from different liquid media (nutrient-rich vs. nutrient-limited), may need to be examined in the future, as extraction from liquid might offer an easier purification method (14). We did, however, exclude the possibility that a significant amount of the *Pseudomonas*-derived compound(s) was secreted into the agar. However, the applied protocol might have been inadequate and an alternative would be to first freeze dry the agar before extraction of the compound(s) (14). In terms of the solvents chosen, DMSO is commonly used for *in vitro* assay of many antibiotics; however, it is not ideal for initial assays as the compound(s) cannot be removed once dissolved in this solvent (19). It is for this reason that majority of our experiments were performed with the *Pseudomonas*-derived compound(s) dissolved in 100% ethyl acetate or 50% ethanol, both of which are easy to remove by evaporation.

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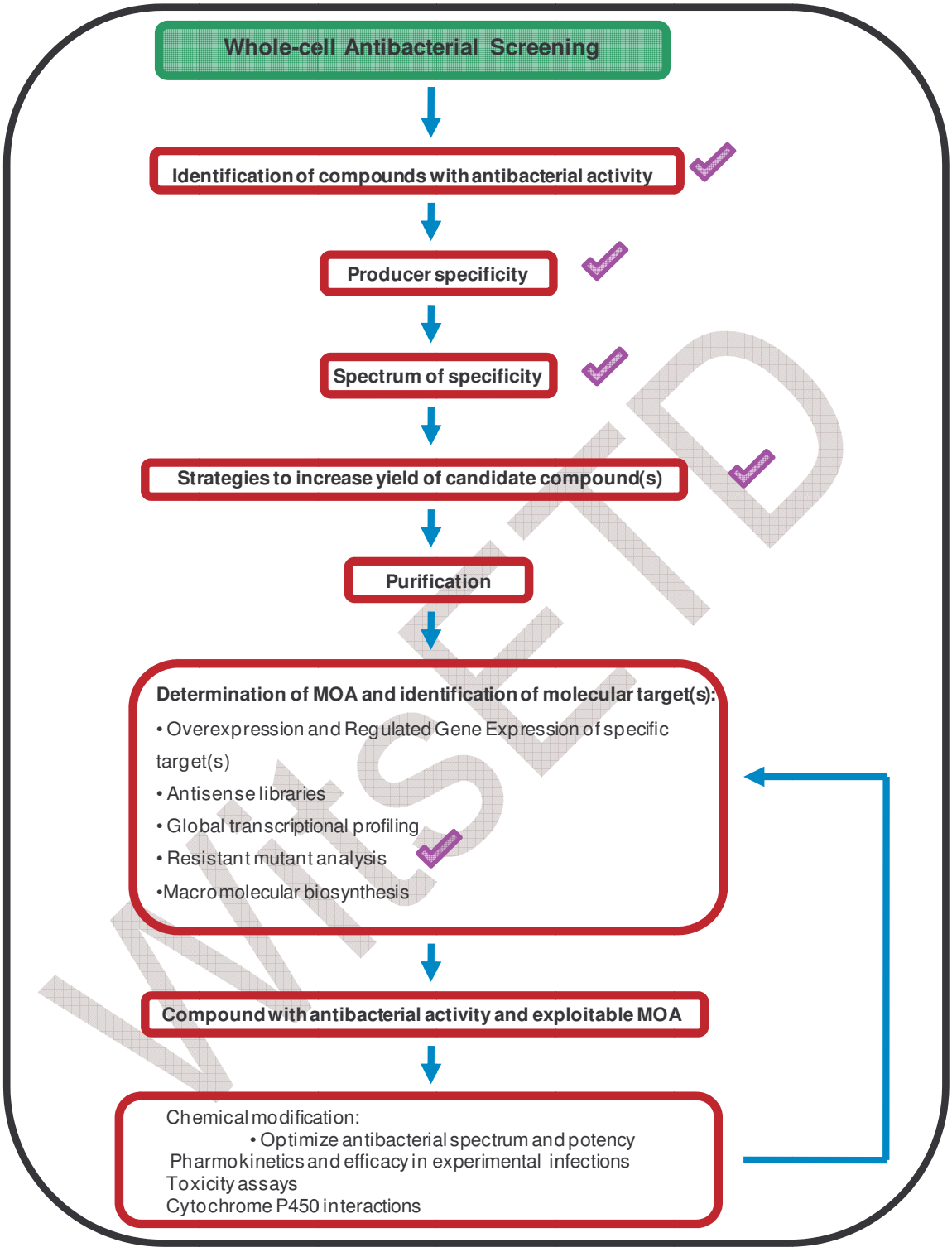


Figure 4.2: Strategies applied to the development of a candidate antibacterial compound identified from whole-cell screening (Adapted from (14, 102)). Strategies attempted in this study (✔).

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Purification of a crude extract is vital to the further development of a candidate compound: it is critical for MOA determination as well as structural and chemical analyses. Therefore, purification of our *Pseudomonas*-derived extract is a future research priority. The MOA of an antimicrobial compound can be determined by means of numerous methods (Fig. 4.2). However microarray technology is the most widely-accepted method, as it allows novel MOAs to be ascertained through comparative analysis with antibiotics of known MOAs. Transcriptional profiling also allows known signatures to be determined (16). For example, the database of transcriptional profiles for a diverse set of drugs and growth-inhibitory conditions not only accurately identifies the MOA of well-established antimicrobials, but also provides fundamental insights into the MOA of unknown compounds, in theory enabling the subsequent identification of novel compounds with novel MOAs (16). Additionally, a signature of general DNA damage response in most cases eliminates a compound as candidate owing to non-specific MOA (for example, DNA intercalating agents).

The determination of bactericidal or bacteriostatic MOAs is imperative to the further development of a candidate compound, which can be determined by the broth microdilution method (56). A bacteriostatic MOA is indicated by the MIC, defined as the lowest antibiotic concentration demonstrating no visible growth, while a bactericidal MOA is defined by the minimum bactericidal concentration (MBC), which can be determined by removing 0.1 ml of the bacterial suspension from subcultures showing no visible growth and inoculating solid media, followed by incubation for a prolonged period (56). Therefore, the MBC is defined as the lowest antibiotic concentration demonstrating 99.9% of killing of the bacterial inoculum. Finally, the isolation of pure compound also enables key primary chemical analyses, such as hydrolysis with protease K for peptide determination, or saponification to determine if the active compound(s) are esters by nature (14).

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CONCLUSION

This study reports the microbiological characterization of a crude extract derived from a putative *Pseudomonas* strain that exerts growth inhibitory effects on Gram-positive organisms including *M. tuberculosis*. The extract exhibited an MIC against MTB in the range 14 – 16 µg/ml in preliminary broth microdilution assays. This appears to be a relatively strong inhibitory effect given that a crude extract was used, and suggests that a potent compound/s within the crude extract may be responsible for the observed inhibition. The inhibition of MTB by a novel *Pseudomonas*-derived extract highlights the importance of exploiting naturally occurring microorganisms as possible sources of antimicrobials. In addition, the extract appears, from preliminary experiments, to have a greater effect on Gram-positive organisms, including MTB, suggesting the potential value of its further characterization, including the purification of the active compound(s) followed by their structural and functional characterization.

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5.0 APPENDICES

A. Bacterial Growth Conditions

Table 5.1: Growth conditions for the various bacterial strains used in this study

Species	Strain	Growth Conditions (solid/liquid media) and (growth temperature)
M. smegmatis	mc^2155	7H10 agar/ 7H9 broth (GS), 37°C
M. tuberculosis	H37Rv	7H10 agar/ 7H9 broth (OADC), 37°C
M. parafortuitum	IFM 0490	7H10 agar/ 7H9 broth (GS), 37°C
R. erythropolis	ATCC 4277	Nutrient agar/ Nutrient broth, 37°C
R. equi		Nutrient agar/ Nutrient broth, 37°C
R. fasciens		Nutrient agar/ Nutrient broth, 37°C
R. rhodochrous	01	Nutrient agar/ Nutrient broth, 37°C
R. rhodochrous	Ri8	Nutrient agar/ Nutrient broth, 37°C
N. farcinica	IFM 10757	Nutrient agar/ Nutrient broth, 37°C
N. farcinica	IFM 10779	Nutrient agar/ Nutrient broth, 37°C
B. cereus	ATCC	Luria Bertani agar/ Luria Bertani broth, 37°C
B. subtilis		Luria Bertani agar/ Luria Bertani broth, 37°C
S. aureus		Luria Bertani agar/ Luria Bertani broth, 37°C
S. epidermidis		Luria Bertani agar/ Luria Bertani broth, 37°C
E. coli	29	Luria Bertani agar/ Luria Bertani broth, 37°C
E. coli	32	Luria Bertani agar/ Luria Bertani broth, 37°C
P. putida	317	Luria Bertani agar/ Luria Bertani broth, 37°C
Pseudomonas	αMB	Luria Bertani agar/ Luria Bertani broth, 37°C

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B. Media, Reagents and Solutions

All media were made to a final volume of 1L with distilled water. Media were sterilized by autoclaving at 121˚C for 15-20 min.

1. Luria-Bertani Agar

10 g Tryptone
10 g NaCl
5 g Yeast Extract
15 g Bacteriological agar
1L distilled water

2. Luria-Bertani Broth

10 g Tryptone broth
10 g NaCl
5 g Yeast Extract
1L distilled water

3. Middlebook 7H10 Agar

19 g 7H10 agar powder (Difco™, USA)
5 ml glycerol (Merck, Germany)
990 ml distilled water
Following sterilization, the solid medium was supplemented with 10 ml Glucose/salt (from a 100x stock solution). For 7H10/ OADC, 900 ml distilled water was added and, following sterilization, enriched with 100 ml OADC (BD Microbiology Systems, USA).

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4. **Middlebrook 7H9 Liquid Medium**

   4.7 g 7H9 broth powder (Difco™, USA)

   2 ml glycerol (Merck, Germany)

   990 ml distilled water

   Following sterilization, media was supplemented with 10 ml Glucose/salt (from a 100x stock solution) and 2 ml Tween 80 (from a 25% v/v stock solution). For 7H9/ OADC, 900 ml distilled water was added and, following sterilization, enriched with 100 ml OADC (BD Microbiology Systems, USA) and 2 ml Tween 80 (from a 25% v/v stock solution).

5. **Nutrient Broth Agar**

   8 g Nutrient broth powder (Difco™, USA)

   15g Bacteriological agar

   1L distilled water

6. **Nutrient Broth**

   8g Nutrient broth powder (Difco™, USA)

   1L distilled water

7. **Glucose/ Salt (100x)**

   20 g glucose

   8.5 g sodium chloride

   100 ml distilled water

   Sterilized by filtration through a 0.22-µm membrane and stored at 4℃.

8. **Tween 80 (25% v/v)**

   25 ml polyoxyethylenesorbitan monooleate (Tween 80) (Sigma-Aldrich, USA)

   75 ml distilled water

   The distilled water was heated to aid dissolving the Tween 80, sterilized by filtration through a 0.22-µm membrane and stored at 4℃.

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9. **Tyloxapol (20% v/v)**

   20 ml Tyloxapol (Sigma-Aldrich, USA)

   80 ml distilled water

   Sterilized by filtration through a 0.22-µm membrane and stored up to 1 year at 4℃

10. **Ethambutol stock solution [1mg/ml]**

    100 mg ethambutol dihydrochloride powder (Sigma-Aldrich, USA)

    100 ml distilled water

    Sterilized by filtration through a 0.22-µm membrane and stored up to 1 month at 4℃.

11. **Albumin-dextrose-saline (ADS) (1L)**

    8.5 g NaCl

    50 g BSA fraction V (Sigma-Aldrich, USA)

    20 g D-dextrose

    950 ml distilled water

    Dissolved NaCl and BSA fraction V first before adding D- dextrose. Adjusted to final volume of 1L. Sterilized clarified solution by filtration through a 0.22-µm membrane. Incubated bottles at 37℃ overnight to detect possible contamination and stored up to 6 months at 4℃.

12. **6 × DNA loading buffer (250 ml)**

    0.3 g Bromophenol blue

    0.3 g Xylenol

    93.6 ml 80% glycerol

    3 ml 0.5 M EDTA

    100 ml distilled water

    Adjusted to final volume of 250 mL with distilled water.

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C. Other Appendices

1. DNA Molecular Weight Markers

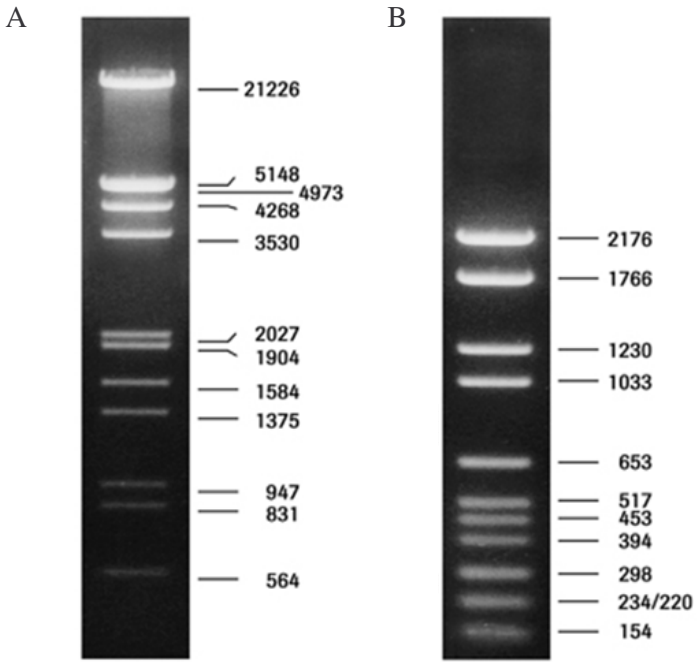


Figure 5.1: DNA Molecular Weight Markers. Marker III (A) and Marker VI (B), from Roche.

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2. Sequence Alignments

```
 Score = 1338 bits (724),  Expect = 0.0
 Identities = 728/730 (99%), Gaps = 0/730 (0%)
 Strand=Plus/Minus

Query  1       ACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCAGAGTTCCCGAAGGCACCAATC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190098  ACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCAGAGTTCCCGAAGGCACCAATC  190039

Query  61      CATCTCTGGAAAGTTCTCTGCATGTCAAGGCCTGGTAAGGTTCTTCGCGTTGCTTCGAAT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190038  CATCTCTGGAAAGTTCTCTGCATGTCAAGGCCTGGTAAGGTTCTTCGCGTTGCTTCGAAT  189979

Query  121     TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189978  TAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTG  189919

Query  181     CGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAAATCTCAAGGA  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189918  CGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAAATCTCAAGGA  189859

Query  241     TTCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTG  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189858  TTCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTG  189799

Query  301     CTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGG  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189798  CTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGG  189739

Query  361     TGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCG  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189738  TGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCG  189679

Query  421     TACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACATCCA  480
               |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189678  TACTCTAGCTTGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACATCCA  189619

Query  481     ACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCT  540
               ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189618  ACTTAACAAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCT  189559

Query  541     CTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAA  600
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189558  CTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAA  189499

Query  601     AACAGCAAGGTATTAGCTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAG  660
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189498  AACAGCAAGGTATTAGCTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAG  189439

Query  661     ACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCC  720
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Sbjct  189438  ACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCC  189379

Query  721     ACTGCTGCCT  730
               ||||||||||
Sbjct  189378  ACTGCTGCCT  189369
```

Figure 5.2: 16S rRNA sequence alignment of the unknown isolate with Proteobacteria. A 730bp portion of the sequenced 16S PCR product from the unknown isolate compared to Proteobacteria 16S sequences within the Genbank database. The alignment indicates 99% sequence similarity to the γ-proteobacteria class (highlighted in pink).

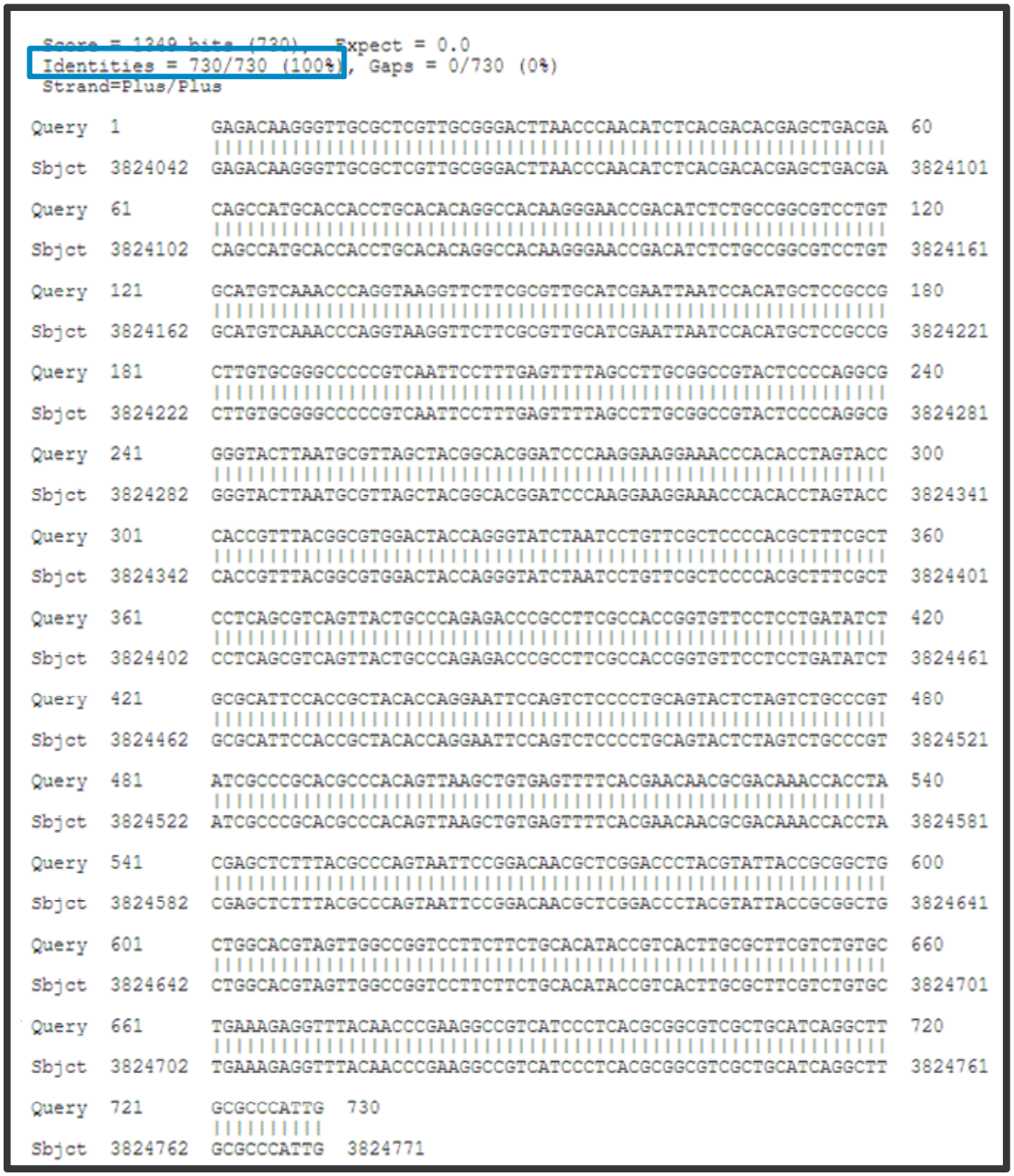


Figure 5.3: 16S rRNA sequence alignment of MSM mc^{2}155 with Actinobacteria. A 730bp portion of the sequenced 16S PCR product from MSM mc^{2}155 compared to Actinobacteria 16S sequences within the Genbank database. The alignment indicates 100% sequence similarity to MSM mc^{2}155 (highlighted in blue).

6.0 REFERENCES

1. 2007. A call to arms. Nat Rev Drug Discov **6:**8-12.
2. **Alangaden, G. J., B. N. Kreiswirth, A. Aouad, M. Khetarpal, F. R. Igno, S. L. Moghazeh, E. K. Manavathu, and S. A. Lerner.** 1998. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **42:**1295-7.
3. **Alcaide, F., G. E. Pfyffer, and A. Telenti.** 1997. Role of *embB* in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob Agents Chemother **41:**2270-3.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-10.
5. **Balganesh, T. S., P. M. Alzari, and S. T. Cole.** 2008. Rising standards for tuberculosis drug development. Trends Pharmacol Sci **29:**576-81.
6. **Baltz, R. H., V. Miao, and S. K. Wrigley.** 2005. Natural products to drugs: daptomycin and related lipopeptide antibiotics. Nat Prod Rep **22:**717-41.
7. **Baquero, F.** 1997. Gram-positive resistance: challenge for the development of new antibiotics. J Antimicrob Chemother **39 Suppl A:**1-6.
8. **Barry, C. E., 3rd, H. I. Boshoff, V. Dartois, T. Dick, S. Ehrt, J. Flynn, D. Schnappinger, R. J. Wilkinson, and D. Young.** 2009. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Microbiol **7:**845-55.
9. **Bascaran, V., L. Sanchez, C. Hardisson, and A. F. Brana.** 1991. Stringent response and initiation of secondary metabolism in *Streptomyces clavuligerus*. J Gen Microbiol **137:**1625-34.
10. **Battu, P. R., and M. S. Reddy.** 2009. Isolation of secondary metabolites from *Pseudomonas fluorescens* and its Characterization. Asian J. Research Chem. **2:**26-29.
11. **Belanger, A. E., G. S. Besra, M. E. Ford, K. Mikusova, J. T. Belisle, P. J. Brennan, and J. M. Inamine.** 1996. The embAB genes of Mycobacterium avium encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci U S A **93:**11919-24.
12. **Bensaci, M. F., and J. Y. Takemoto.** 2007. Syringopeptin SP25A-mediated killing of gram-positive bacteria and the role of teichoic acid d-alanylation. FEMS Microbiol Lett **268:**106-11.
13. **Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood.** 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417:**141-7.
14. **Boshoff, H.** 2010. Personal communication to Dr. D.F Warner.
15. **Boshoff, H. I., S. I. Durbach, and V. Mizrahi.** 2001. DNA metabolism in *Mycobacterium tuberculosis*: implications for drug resistance and strain variability. Scand J Infect Dis **33:**101-5.

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16. **Boshoff, H. I., T. G. Myers, B. R. Copp, M. R. McNeil, M. A. Wilson, and C. E. Barry, 3rd.** 2004. The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism: novel insights into drug mechanisms of action. J Biol Chem **279:**40174-84.
17. **Boshoff, H. I., M. B. Reed, C. E. Barry, 3rd, and V. Mizrahi.** 2003. DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. Cell **113:**183-93.
18. **Boshoff, H. I. M., and C. E. and Barry.** 2006. Is the mycobacterial cell wall a useless target for latent tuberculosis? Drug Discov. Today **3:**237-245.
19. **Bowker, M. J., and P. H. Stahl.** 2008. Drug solubilization with organic solvents, or using micellar solutions or other colloidal dispersed systems, p. 786-812, The practice of medicinal chemistry (Third edition). Wermuth, Camille Georges.
20. **Brennan, P. J., and D. C. Crick.** 2007. The cell-wall core of *Mycobacterium tuberculosis* in the context of drug discovery. Curr Top Med Chem **7:**475-88.
21. **Buber, E., A. Stindl, N. L. Acan, T. Kocagoz, and R. Zocher.** 2002. Antimycobacterial activity of lipodepsipeptides produced by *Pseudomonas syringae* pv syringae B359. Nat Prod Lett **16:**419-23.
22. **Butler, M. S., and A. D. Buss.** 2006. Natural products--the future scaffolds for novel antibiotics? Biochem Pharmacol **71:**919-29.
23. **Cain, C. C., D. Lee, R. H. Waldo, 3rd, A. T. Henry, E. J. Casida, Jr., M. C. Wani, M. E. Wall, N. H. Oberlies, and J. O. Falkinham, 3rd.** 2003. Synergistic antimicrobial activity of metabolites produced by a nonobligate bacterial predator. Antimicrob Agents Chemother **47:**2113-7.
24. **Campbell, E. A., N. Korzheva, A. Mustaev, K. Murakami, S. Nair, A. Goldfarb, and S. A. Darst.** 2001. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. Cell **104:**901-12.
25. **Carraturo, A., K. Raieta, D. Ottaviani, and G. L. Russo.** 2006. Inhibition of *Vibrio parahaemolyticus* by a bacteriocin-like inhibitory substance (BLIS) produced by *Vibrio mediterranei* 1. J Appl Microbiol **101:**234-41.
26. **Cavalieri, S. J., J. R. Biehle, and W. E. Sanders, Jr.** 1995. Synergistic activities of clarithromycin and antituberculous drugs against multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **39:**1542-5.
27. **Chan, E. D., and M. D. Iseman.** 2002. Current medical treatment for tuberculosis. BMJ **325:**1282-6.
28. **Changsen, C., S. G. Franzblau, and P. Palittapongarnpim.** 2003. Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. Antimicrob Agents Chemother **47:**3682-7.
29. **Chatterjee, D.** 1997. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Curr Opin Chem Biol **1:**579-88.
30. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M. A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.

WJSTD

31. **Collins, L., and S. G. Franzblau.** 1997. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. Antimicrob Agents Chemother **41:**1004-9.
32. **Collins, L. A., M. N. Torrero, and S. G. Franzblau.** 1998. Green fluorescent protein reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **42:**344-7.
33. **Coulombe, F., M. Divangahi, F. Veyrier, L. de Leseleuc, J. L. Gleason, Y. Yang, M. A. Kelliher, A. K. Pandey, C. M. Sassetti, M. B. Reed, and M. A. Behr.** 2009. Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide. J Exp Med **206:**1709-16.
34. **Daffe, M., and G. Etienne.** 1999. The capsule of *Mycobacterium tuberculosis* and its implications for pathogenicity. Tuber Lung Dis **79:**153-69.
35. **Daly, J. S., G. M. Eliopoulos, S. Willey, and R. C. Moellering, Jr.** 1988. Mechanism of action and in vitro and in vivo activities of S-6123, a new oxazolidinone compound. Antimicrob Agents Chemother **32:**1341-6.
36. **Davies, J.** 2006. Are antibiotics naturally antibiotics? J Ind Microbiol Biotechnol **33:**496-9.
37. **Davies, J.** 2006. Where have All the Antibiotics Gone? Can J Infect Dis Med Microbiol **17:**287-90.
38. **de Bruijn, I., M. J. de Kock, M. Yang, P. de Waard, T. A. van Beek, and J. M. Raaijmakers.** 2007. Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in Pseudomonas species. Mol Microbiol **63:**417-28.
39. **Demain, A. L.** 1998. Induction of microbial secondary metabolism. Int Microbiol **1:**259-64.
40. **Deng, L., K. Mikusova, K. G. Robuck, M. Scherman, P. J. Brennan, and M. R. McNeil.** 1995. Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. Antimicrob Agents Chemother **39:**694-701.
41. **Domenech, P., M. B. Reed, and C. E. Barry, 3rd.** 2005. Contribution of the *Mycobacterium tuberculosis* MmpL protein family to virulence and drug resistance. Infect Immun **73:**3492-501.
42. **Dubos, R. J., and B. D. Davis.** 1946. Factors Affecting the Growth of Tubercle Bacilli in Liquid Media. J Exp Med **83:**409-423.
43. **Dye, C., and B. G. Williams.** 2000. Criteria for the control of drug-resistant tuberculosis. Proc Natl Acad Sci U S A **97:**8180-5.
44. **Falconer, S. B., and E. D. Brown.** 2009. New screens and targets in antibacterial drug discovery. Curr Opin Microbiol **12:**497-504.
45. **Fange, D., K. Nilsson, T. Tenson, and M. Ehrenberg.** 2009. Drug efflux pump deficiency and drug target resistance masking in growing bacteria. Proc Natl Acad Sci U S A **106:**8215-20.
46. **Fernandes, N.** 2010. MSc Dissertation: Characterization of the activity of biosurfactants produced by *Pseudomonas* species isolated from foods. University of the Witwatersrand, Johannesburg.
47. **Fischbach, M. A.** 2009. Antibiotics from microbes: converging to kill. Curr Opin Microbiol **12:**520-7.
48. **Fischbach, M. A., and C. T. Walsh.** 2009. Antibiotics for emerging pathogens. Science **325:**1089-93.
49. **Franzblau, S. G., R. S. Witzig, J. C. McLaughlin, P. Torres, G. Madico, A. Hernandez, M. T. Degnan, M. B. Cook, V. K. Quenzer, R. M. Ferguson, and**

WisETD

R. H. Gilman. 1998. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. J Clin Microbiol **36:**362-6.

50. **Freiberg, C., and H. Brotz-Oesterhelt.** 2005. Functional genomics in antibacterial drug discovery. Drug Discov Today **10:**927-35.
51. **Gandhi, N. R., A. Moll, A. W. Sturm, R. Pawinski, T. Govender, U. Lalloo, K. Zeller, J. Andrews, and G. Friedland.** 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet **368:**1575-80.
52. **Gerard, J., R. Lloyd, T. Barsby, P. Haden, M. T. Kelly, and R. J. Andersen.** 1997. Massetolides A-H, antimycobacterial cyclic depsipeptides produced by two pseudomonads isolated from marine habitats. J Nat Prod **60:**223-9.
53. **Gillespie, S. H., S. Basu, A. L. Dickens, D. M. O'Sullivan, and T. D. McHugh.** 2005. Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates. J Antimicrob Chemother **56:**344-8.
54. **Givskov, M., L. Eberl, S. Moller, L. K. Poulsen, and S. Molin.** 1994. Responses to nutrient starvation in *Pseudomonas putida* KT2442: analysis of general cross-protection, cell shape, and macromolecular content. J Bacteriol **176:**7-14.
55. **Goldberg, J. B., R. E. Hancock, R. E. Parales, J. Loper, and P. Cornelis.** 2008. Pseudomonas 2007. J Bacteriol **190:**2649-62.
56. **Gomez-Lus, R., F. Adrian, R. del Campo, P. Gomez-Lus, S. Sanchez, C. Garcia, and M. C. Rubio.** 2001. Comparative in vitro bacteriostatic and bactericidal activity of trovafloxacin, levofloxacin and moxifloxacin against clinical and environmental isolates of Legionella spp. Int J Antimicrob Agents **18:**49-54.
57. **Gomez, M., and I. Smith.** 2000. Determinants of mycobacterial gene expression. *In* G. F. Hatfull and W. R. Jacobs (ed.), Molecular Genetics of Mycobacteria. American Society for Microbiolgy Press, Washington, DC.
58. **Gordien, A. Y., A. I. Gray, K. Ingleby, S. G. Franzblau, and V. Seidel.** 2009. Activity of Scottish Plant, Lichen and Fungal Endophyte Extracts against *Mycobacterium aurum* and *Mycobacterium tuberculosis*. Phytother Res.
59. **Grgurina, I., M. Bensaci, G. Pocsfalvi, L. Mannina, O. Cruciani, A. Fiore, V. Fogliano, K. N. Sorensen, and J. Y. Takemoto.** 2005. Novel cyclic lipodepsipeptide from *Pseudomonas syringae* pv. lachrymans strain 508 and syringopeptin antimicrobial activities. Antimicrob Agents Chemother **49:**5037-45.
60. **Hammes, W. P., and F. C. Neuhaus.** 1974. On the mechanism of action of vancomycin: inhibition of peptidoglycan synthesis in Gaffkya homari. Antimicrob Agents Chemother **6:**722-8.
61. **Hancock, R. E.** 2005. Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect Dis **5:**209-18.
62. **Hancock, R. E., and D. S. Chapple.** 1999. Peptide antibiotics. Antimicrob Agents Chemother **43:**1317-23.
63. **Hegan, P. S., V. Mermall, L. G. Tilney, and M. S. Mooseker.** 2007. Roles for *Drosophila melanogaster* myosin IB in maintenance of enterocyte brush-border structure and resistance to the bacterial pathogen *Pseudomonas entomophila*. Mol Biol Cell **18:**4625-36.
64. **Hopwood, D. A., and M. J. Merrick.** 1977. Genetics of antibiotic production. Bacteriol Rev **41:**595-635.
65. **Hutchison, M. L., and D. C. Gross.** 1997. Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. syringae: comparison of the biosurfactant and ion

WISETD

channel-forming activities of syringopeptin and syringomycin. Mol Plant Microbe Interact **10:**347-54.

66. **James, P. D., C. Edwards, and M. Dawson.** 1991. The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat. J Gen Microbiol **137:**1715-20.
67. **Janin, Y. L.** 2007. Antituberculosis drugs: ten years of research. Bioorg Med Chem **15:**2479-513.
68. **Jassal, M., and W. R. Bishai.** 2009. Extensively drug-resistant tuberculosis. Lancet Infect Dis **9:**19-30.
69. **Jawahar, M. S.** 2004. Current trends in chemotherapy of tuberculosis. Indian J Med Res **120:**398-417.
70. **Johnsson, K., King, D.S. and Schultz, P.G.** 1995. Studies on the mechanism of action of isoniazid and ethionamide in the chemotherapy of tuberculosis. J. Am. Chem. Soc **117:**5009-5010.
71. **Karakousis, P. C., W. R. Bishai, and S. E. Dorman.** 2004. *Mycobacterium tuberculosis* cell envelope lipids and the host immune response. Cell Microbiol **6:**105-16.
72. **Khoo, K. H., E. Douglas, P. Azadi, J. M. Inamine, G. S. Besra, K. Mikusova, P. J. Brennan, and D. Chatterjee.** 1996. Truncated structural variants of lipoarabinomannan in ethambutol drug-resistant strains of *Mycobacterium smegmatis*. Inhibition of arabinan biosynthesis by ethambutol. J Biol Chem **271:**28682-90.
73. **Kohanski, M. A., M. A. Depristo, and J. J. Collins.** 2010. Sublethal Antibiotic Treatment Leads to Multidrug Resistance via Radical-Induced Mutagenesis. Mol Cell **37:**311-320.
74. **Kotra, L. P., J. Haddad, and S. Mobashery.** 2000. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother **44:**3249-56.
75. **Kuiper, I., E. L. Lagendijk, R. Pickford, J. P. Derrick, G. E. Lamers, J. E. Thomas-Oates, B. J. Lugtenberg, and G. V. Bloemberg.** 2004. Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Mol Microbiol **51:**97-113.
76. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci U S A **82:**6955-9.
77. **Larsen, M. H., K. Biermann, and W. R. Jacobs, Jr.** 2007. Laboratory maintenance of Mycobacterium tuberculosis. Curr Protoc Microbiol **Chapter 10:**Unit 10A 1.
78. **Lety, M. A., S. Nair, P. Berche, and V. Escuyer.** 1997. A single point mutation in the *embB* gene is responsible for resistance to ethambutol in *Mycobacterium smegmatis*. Antimicrob Agents Chemother **41:**2629-33.
79. **Liangzhi, L. I., Q. Bin, and Y. Yingjin.** 2007. Nitrogen Sources Affect Streptolydigin Production and Related Secondary Metabolites Distribution of *Streptomyces lydicus* AS 4.2501. Chin. J. Ckem. Ettg. **15:**403-410.
80. **Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, J. Villanueva, T. Wei, and F. M. Ausubel.** 2006. An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A **103:**2833-8.

WISE

81. **Livermore, D. M.** 1984. Penicillin-binding proteins, porins and outer-membrane permeability of carbenicillin-resistant and -susceptible strains of Pseudomonas aeruginosa. J Med Microbiol **18:**261-70.
82. **Malik, V. S.** 1980. Microbial Secondary Metabolism. Trends in Biochemical Sciences **5:**68-72.
83. **Maplestone, R. A., M. J. Stone, and D. H. Williams.** 1992. The evolutionary role of secondary metabolites--a review. Gene **115:**151-7.
84. **Martin, J. F., and A. L. Demain.** 1980. Control of antibiotic biosynthesis. Microbiol Rev **44:**230-51.
85. **Matsuhashi, S., T. Kamiryo, P. M. Blumberg, P. Linnett, E. Willoughby, and J. L. Strominger.** 1974. Mechanism of action and development of resistance to a new amidino penicillin. J Bacteriol **117:**578-87.
86. **Mehta, S. K., G. Kaur, and K. K. Bhasin.** 2007. Analysis of Tween based microemulsion in the presence of TB drug rifampicin. Colloids Surf B Biointerfaces **60:**95-104.
87. **Middlebrook, G., and M. L. Cohn.** 1958. Bacteriology of tuberculosis: laboratory methods. Am J Public Health Nations Health **48:**844-53.
88. **Middlebrook, G., M. L. Cohn, W. E. Dye, W. F. Russel, and D. Levy.** 1960. Microbiologic procedures of value in tuberculosis. Acta. Tuberc. Scand. **38**.
89. **Mikusova, K., R. A. Slayden, G. S. Besra, and P. J. Brennan.** 1995. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob Agents Chemother **39:**2484-9.
90. **Mizrahi, V., and S. J. Andersen.** 1998. DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? Mol Microbiol **29:**1331-9.
91. **Mokrousov, I., T. Otten, B. Vyshnevskiy, and O. Narvskaya.** 2002. Detection of embB306 mutations in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. J Clin Microbiol **40:**3810-3.
92. **Motiwala, A. S., Y. Dai, E. C. Jones-Lopez, S. H. Hwang, J. S. Lee, S. N. Cho, L. E. Via, C. E. Barry, and D. Alland.** 2010. Mutations in extensively drug-resistant *Mycobacterium tuberculosis* that do not code for known drug-resistance mechanisms. J Infect Dis **201:**881-888.
93. **Murray, R. W., R. D. Schaadt, G. E. Zurenko, and K. R. Marotti.** 1998. Ribosomes from an oxazolidinone-resistant mutant confer resistance to eperezolid in a Staphylococcus aureus cell-free transcription-translation assay. Antimicrob Agents Chemother **42:**947-50.
94. **Naicker, K.** 2010. The characterization of the antimycobacterial activity of a *Pseudomonas* isolate. University of the Witwatersrand, Johannesburg.
95. **Nash, K. A., Y. Zhang, B. A. Brown-Elliott, and R. J. Wallace, Jr.** 2005. Molecular basis of intrinsic macrolide resistance in clinical isolates of *Mycobacterium fortuitum*. J Antimicrob Chemother **55:**170-7.
96. **Newman, D. J., and G. M. Cragg.** 2007. Natural products as sources of new drugs over the last 25 years. J Nat Prod **70:**461-77.
97. **Nielsen, T. H., D. Sorensen, C. Tobiasen, J. B. Andersen, C. Christophersen, M. Givskov, and J. Sorensen.** 2002. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent Pseudomonas spp. from the sugar beet rhizosphere. Appl Environ Microbiol **68:**3416-23.

WISER

98. **Ohno, A., T. Ano, and M. Shoda.** 1995. Production of a lipopeptide antibiotic, surfactin, by recombinant Bacillus subtilis in solid state fermentation. Biotechnol Bioeng **47:**209-14.
99. **Pandey, A., C. R. Soccol, and D. Mitchell.** 2000. New developments in solid-state fermentation: I- bioprocesses and products. Process Biochemistry **35:**1153 - 1169.
100. **Pauli, G. F., R. J. Case, T. Inui, Y. Wang, S. Cho, N. H. Fischer, and S. G. Franzblau.** 2005. New perspectives on natural products in TB drug research. Life Sci **78:**485-94.
101. **Payne, D. J., M. N. Gwynn, D. J. Holmes, and D. L. Pompliano.** 2007. Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov **6:**29-40.
102. **Payne, D. J., M. N. Gwynn, D. J. Holmes, and M. Rosenberg.** 2004. Genomic approaches to antibacterial discovery. Methods Mol Biol **266:**231-59.
103. **Pfefferle, C., U. Theobald, H. Gurtler, and H. Fiedler.** 2000. Improved secondary metabolite production in the genus *Streptosporangium* by optimization of the fermentation conditions. J Biotechnol **80:**135-42.
104. **Protopopova, M., E. Bogatcheva, B. Nikonenko, S. Hundert, L. Einck, and C. A. Nacy.** 2007. In search of new cures for tuberculosis. Med Chem **3:**301-16.
105. **Raaijmakers, J. M., I. de Bruijn, and M. J. de Kock.** 2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. Mol Plant Microbe Interact **19:**699-710.
106. **Raaijmakers, J. M., D. M. Weller, and L. S. Thomashow.** 1997. Frequency of Antibiotic-Producing Pseudomonas spp. in Natural Environments. Appl Environ Microbiol **63:**881-887.
107. **Ramaswamy, S., and J. M. Musser.** 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis **79:**3-29.
108. **Ramaswamy, S. V., A. G. Amin, S. Goksel, C. E. Stager, S. J. Dou, H. El Sahly, S. L. Moghazeh, B. N. Kreiswirth, and J. M. Musser.** 2000. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. Antimicrob Agents Chemother **44:**326-36.
109. **Rice, L. B.** 2006. Antimicrobial resistance in gram-positive bacteria. Am J Infect Control **34:**S11-9; discussion S64-73.
110. **Rosenberg, S. M.** 2001. Evolving responsively: adaptive mutation. Nat Rev Genet **2:**504-15.
111. **Safi, H., B. Sayers, M. H. Hazbon, and D. Alland.** 2008. Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob Agents Chemother **52:**2027-34.
112. **Sandgren, A., M. Strong, P. Muthukrishnan, B. K. Weiner, G. M. Church, and M. B. Murray.** 2009. Tuberculosis drug resistance mutation database. PLoS Med **6:**e2.
113. **Sattler, T. H., and G. P. Youmans.** 1948. The effect of tween 80, bovine albumin, glycerol, and glucose on the growth o*f Mycobacterium tuberculosis* var. hominis (H37Rv). J Bacteriol **56:**235-43.
114. **Schluger, N. W.** 2000. The impact of drug resistance on the global tuberculosis epidemic. Int J Tuberc Lung Dis **4:**S71-5.
115. **Scorpio, A., P. Lindholm-Levy, L. Heifets, R. Gilman, S. Siddiqi, M. Cynamon, and Y. Zhang.** 1997. Characterization of *pncA* mutations in

WitsETD

pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **41:**540-3.
116. **Shenoi, S., and G. Friedland.** 2009. Extensively drug-resistant tuberculosis: a new face to an old pathogen. Annu Rev Med **60:**307-20.
117. **Showalter, H. D., and W. A. Denny.** 2008. A roadmap for drug discovery and its translation to small molecule agents in clinical development for tuberculosis treatment. Tuberculosis (Edinb) **88 Suppl 1:**S3-17.
118. **Singh, J. A., R. Upshur, and N. Padayatchi.** 2007. XDR-TB in South Africa: no time for denial or complacency. PLoS Med **4:**e50.
119. **Singh, S. B., and J. F. Barrett.** 2006. Empirical antibacterial drug discovery--foundation in natural products. Biochem Pharmacol **71:**1006-15.
120. **Singhania, R. R., A. K. Patel, C. R. Soccol, and A. Pandey.** 2009. Recent advances in solid-state fermentation. Biochemical Engineering Journal **44:**13-18.
121. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. Mol Microbiol **4:**1911-9.
122. **Starks, A. M., A. Gumusboga, B. B. Plikaytis, T. M. Shinnick, and J. E. Posey.** 2009. Mutations at *embB* codon 306 are an important molecular indicator of ethambutol resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **53:**1061-6.
123. **Sutcliffe, I. C., and N. Shaw.** 1991. Atypical lipoteichoic acids of gram-positive bacteria. J Bacteriol **173:**7065-9.
124. **Takayama, K., and J. O. Kilburn.** 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob Agents Chemother **33:**1493-9.
125. **Tang, Y. J., W. Shui, S. Myers, X. Feng, C. Bertozzi, and J. D. Keasling.** 2009. Central metabolism in *Mycobacterium smegmatis* during the transition from O2-rich to O2-poor conditions as studied by isotopomer-assisted metabolite analysis. Biotechnol Lett **31:**1233-40.
126. **Taubes, G.** 2008. The bacteria fight back. Science **321:**356-61.
127. **Telenti, A., W. J. Philipp, S. Sreevatsan, C. Bernasconi, K. E. Stockbauer, B. Wieles, J. M. Musser, and W. R. Jacobs, Jr.** 1997. The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nat Med **3:**567-70.
128. **Ueda, K., S. Kawai, H. Ogawa, A. Kiyama, T. Kubota, H. Kawanobe, and T. Beppu.** 2000. Wide distribution of interspecific stimulatory events on antibiotic production and sporulation among Streptomyces species. J Antibiot (Tokyo) **53:**979-82.
129. **Van Boxtel, R. M., R. S. Lambrecht, and M. T. Collins.** 1990. Effects of colonial morphology and tween 80 on antimicrobial susceptibility of *Mycobacterium paratuberculosis*. Antimicrob Agents Chemother **34:**2300-3.
130. **van Pee, K. H., and J. M. Ligon.** 2000. Biosynthesis of pyrrolnitrin and other phenylpyrrole derivatives by bacteria. Nat Prod Rep **17:**157-64.
131. **Vandal, O. H., J. A. Roberts, T. Odaira, D. Schnappinger, C. F. Nathan, and S. Ehrt.** 2009. Acid-susceptible mutants of *Mycobacterium tuberculosi*s share hypersusceptibility to cell wall and oxidative stress and to the host environment. J Bacteriol **191:**625-31.
132. **Ventura, M., C. Canchaya, A. Tauch, G. Chandra, G. F. Fitzgerald, K. F. Chater, and D. van Sinderen.** 2007. Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. Microbiol Mol Biol Rev **71:**495-548.

WISE TD

133. **Vilcheze, C., and W. R. Jacobs, Jr.** 2007. The mechanism of isoniazid killing: clarity through the scope of genetics. Annu Rev Microbiol **61:**35-50.
134. **von Nussbaum, F., M. Brands, B. Hinzen, S. Weigand, and D. Habich.** 2006. Antibacterial natural products in medicinal chemistry--exodus or revival? Angew Chem Int Ed Engl **45:**5072-129.
135. **Wagner, D., and L. S. Young.** 2004. Nontuberculous mycobacterial infections: a clinical review. Infection **32:**257-70.
136. **Walsh, C.** 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature **406:**775-81.
137. **Walsh, C.** 2003. Where will new antibiotics come from? Nat Rev Microbiol **1:**65-70.
138. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol **173:**697-703.
139. **WHO.** 2009. Global tuberculosis control - epidemiology, strategy, financing: WHO Report, WHO/HTM/TB/2009.411., Geneva: World Health Organization.
140. **WHO.** 2009. Treatment of tuberculosis: guidelines. Fourth Edition. WHO/HTM/TB/2009.420. Geneva: World Health Organization.
141. **Woodford, N., and D. M. Livermore.** 2009. Infections caused by Gram-positive bacteria: a review of the global challenge. J Infect **59 Suppl 1:**S4-16.
142. **Yang, S. Z., D. Z. Wei, and B. Z. Mu.** 2007. Determination of the structure of the fatty acid chain in a cyclic lipopeptide using GC-MS. J Biochem Biophys Methods **70:**519-23.
143. **Yoneyama, H., and R. Katsumata.** 2006. Antibiotic resistance in bacteria and its future for novel antibiotic development. Biosci Biotechnol Biochem **70:**1060-75.
144. **Young, D. B., H. P. Gideon, and R. J. Wilkinson.** 2009. Eliminating latent tuberculosis. Trends Microbiol **17:**183-8.
145. **Zhang, Y.** 2005. The magic bullets and tuberculosis drug targets. Annu Rev Pharmacol Toxicol **45:**529-64.

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