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The characterisation of the antimycobacterial activity of a Pseudomonas isolate

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Chapter One:

1. Introduction:

Today we live in a world that has advanced in the areas of science and technology with modern medicine being the forefront of some of the prominent discoveries relevant to drug development against infectious diseases. However, diseases such as, Tuberculosis have used these drug discoveries to progress to a form where the causative bacterium manifests with multiple and extensive drug resistance. Multiple drug resistant (MDR) strains possess resistance to isoniazid and rifampicin whilst extensively drug resistant (XDR) strains are resistant to three of the six classes of second line TB drugs (explained in 1.4). The first case related to these strains; was reported in South Africa on the 1st of September 2006 in an area called Tugela Ferry. Of the 544 patients found to be infected with TB, 221 of them had the MDR TB phenotype and 53 of the latter had the XDR TB phenotype. To date, 39 hospitals have reported cases in the Kwa-Zulu-Natal province with 30 new cases being reported each month (Singh, Upshur and Padayatchi, 2007). The occurrence of drug resistant strains is underestimated since detection of the disease is only tested for after the current treatment regimen fails, hence, the severity of the situation could be grossly underestimated with dire consequences.

Currently there have been several factors that have contributed to the TB pandemic. Some of these factors are related to socio-economic circumstances, the nature of the TB infection cycle and the lack of new drug targets in TB which has lead to a bottleneck in the production of new drugs. These factors will be briefly looked at as well as what routes that are followed today in terms of drug discovery, and finally a route for the search and discovery of novel compounds with new targets against TB will be proposed.

1.1 Infection cycle of Tuberculosis:

Mycobacterium tuberculosis is a bacillus-shaped bacterium, which can exist in several distinct physiological states depending on the status of the infection. This is one of the

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attributes which makes the infection cycle a complicated process. *M. tuberculosis* is transferred into the alveoli of the lung by cough droplets. The pathogen infects the dendritic cells (Saunders and Britton, 2007) thereby eliciting the release of macrophages by CD4 and CD8 cells. The infected cells are then phagocytosed by the macrophage cells (Davis and Ramakrishan, 2008). However, *M. tuberculosis* prevents phago-lysomal fusion, acidification of the phagosomes (Saunders and Britton, 2007) and remains in the recycling endosomal pathway (Zu Bentrup and Russell, 2001) thereby preventing complete eradication of the bacteria. The release of macrophages is further inhibited by the bacteria, by the release of interferon (INF-γ) which is released by TH1- like CD4 cells. This leads to persistence of the bacteria in the lung and results in latent Tuberculosis (TB) infection. The release of cytokines by the T cells leads to differentiation of the macrophage cells into epithelioid cells. These cells fuse to form giant cells (Saunders and Britton, 2007) which contribute to granuloma formation. These cells are joined together by T and B lymphocytes and NK cells (Davis and Ramakrish, 2008). The processes involved in granuloma formation are still not fully understood. It has been hypothesised that the formation of granulomas are due to interaction between the innate immunity response and the pathogen (Davis and Ramakrish, 2007). Studies have shown that INF-γ plays an important role in the maintenance and formation of granulomas (Davis and Ramakrish, 2007).

It has been the long standing belief that the granuloma contains necrotic cells which resemble the porous nature of cheese and low metabolic rate (vegetative state) bacteria which are believed to decrease the exponential growth of the bacterial population (Zu Bentrup and Russell, 2001). Recently the structure of the granuloma has been described as a myeloid cell scaffold with a large effector T cell population surrounding it (Egen *et al*., 2008). Studies have revealed that components of the granuloma may possess the ability to move in out of the structure. These recent discoveries have changed the thinking of how granulomas are seen, that is, they could be the transport medium for the bacteria, rather than a protective barrier (Egen *et al*., 2008). The final stage of the disease occurs with the disruption of the granuloma thereby releasing bacteria into the lung tissue (Co *et al.*, 2004). This occurrence manifests as a persistent cough with sputum which allows for the transmission of the bacteria to new hosts (Zu Bentrup and Russell, 2001, Co *et al.*, 2004).

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Taking into account latent TB infection and resuscitation of the bacteria from a dormant state, it has been assumed that there are three physiological states of the bacterial cells within the granuloma; namely, metabolically active bacteria on the outer wall of the granuloma, semi-dormant bacteria and dormant bacteria within (Zu Bentrup and Russell, 2001, Co *et al.*, 2004; Harries, 1997). This assumption has lead to a combination drug therapy approach.

1.2 Combination drug therapy- TB drug targets and their mode of action:

Taking into consideration the complexity of the disease, it is not surprising that the standard treatment for *M. tuberculosis* consists of six drugs. A combinatorial drug therapy approach is used in order to combat resistant phenotypes (Balganesh *et al.*, 2004) and treatment lasts for either six (short course) or twelve months (long course) under constant monitoring. This is known as the DOTS (directly observed treatment short-course) system (Harries, 1997; Zhang, 2005). The reason for such a long period for the TB treatment is related to the nature of the infection cycle, the physiological variation of the bacteria and the mode of action of the drugs. The drugs that have been approved by the world health organisation (WHO) (Harries, 1997) for use in Africa are namely, isoniazid, rifampicin, pyrazinamide, streptomycin, ethambutol and thiacetazone (Harries, 1997).

Most of these drugs and the recent ones present today have three basic strategies to combat bacterial infection, targeting of protein synthesis, disruption of DNA replication and repair and lastly disruption of bacterial cell wall synthesis (Walsh, 2007). A few of the drugs will be discussed in accordance to their target site and mode of action.

Of the bactericidal drugs isoniazid has the most potent affect on the pathogen possessing a 90% killing rate in the first few days of therapy (Harries, 1997). It is able to prevent the formation of mycolic acid (constituent of the mycobacterial cell wall). The drug is activated via a catalase peroxidase (encoded by KatG) from mycobacteria (Zhang, 2005). The activated molecule attaches to NAD^+ which then inhibits enoyl ACP reductase (Zhang, 2005; Sandy *et al.*, 2007). Other reactive species produced

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during the activation of the molecule are believed to cause damage to DNA, carbohydrates, lipids and NAD metabolism (Zhang, 2005). Another drug that targets the cell wall is ethambutol. It targets the enzyme arabinosyltransferase encoded by the *embB* gene. This enzyme is responsible for the polymerization of arabinan of arabinogalactan (polysaccharide) and lipoarabinomannan which are constituents of the cell wall. Hence, cell wall synthesis is affected and arabinogalactan synthesis is inhibited (Zhang, 2005). Recently another drug thiacetazone was found to affect mycolic acid synthesis by preventing the action of cyclopropane mycolic acid synthases. These synthases are responsible for the conversion of double bonds onto cyclopropane rings. If these enzymes are inhibited the synthesis of the cell wall is thus prevented (Alahari *et al.*, 2007).

Rifampicin and pyrazinamide target specific stages of the life cycle of the pathogen. Rifampicin acts against the semi-dormant bacteria and actively growing bacteria (Zhang, 2005). It covalently binds to the β-subunit of the DNA dependent RNA polymerase (encoded by the *rpoB* gene) which prevents RNA synthesis (Zhang, 2005). Pyrazinamide acts on the bacterium whilst it is in the acidic medium of the macrophages (Harries, 1997). This drug is an analogue of nicotamide. It is converted to its active form, pyrazinoic acid, by nicotinamidase or pyrazinamidase (Zhang *et al.*, 2003; Zhang, 2005). An acidic medium is required for its conversion and its maintenance in an un-protonated form. This allows the molecule to pass through the cell and acidify the bacterial cytoplasm. The proton motive force is thus disrupted resulting in a depletion of energy for the cell and hindering the membrane transport systems (Zhang, 2005).

Another process such as mRNA synthesis is affected by streptomycin (an aminoglycoside). It is believed to be active on the 30S subunit of the ribosomes at the S12 ribosomal protein and 16s rRNA protein (binds to the A site) (Zhang, 2005). Its activity leads to the misreading of the genetic code by affecting translation which results in a disruption of protein synthesis and causes damage to the cell membrane (Zhang, 2005). Other drugs with a similar type of activity are kanamycin, amikacin, viomycin and capreomycin. Thiacetazone is a drug that also affects proteins. Studies done *in vitro* have shown that the drug can be activated by monooxygenase EtaA which also activates thioamide ethionamide (Qian and de Montellano, 2006; Alahari

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et al., 2007). The activation results in the formation of a thiacetazone carbodimide metabolite. This metabolite is believed to bind covalently to nucleophilic residues in proteins which results in a deleterious effect on the function of the cell (Qian and de Montellano, 2006).

The second line drugs that have been introduced into the treatment regimen are the quinolone drugs which have a different target site, that is, they inhibit the bacterial type II and IV topoisomerase and DNA gyrase. DNA gyrase is essential for maintenance of the supercoil structure of DNA (Sriram *et al.*, 2006). The quinolone drugs have a common chemical structure with respect to 4-oxoquinoline-3-carboxylic acid. The cores of these molecules consist of carbon atoms. The variation occurs at positions six and eight of the carbon ring which would contain either nitrogen of hydrogen residues (Fuhr *et al.*, 1992). The antimycobacterial activity of this compound is attributed to the cyclic alkyl groups in its structure (Vangapandu *et al.*, 2004). The drugs mentioned above are different to one another with respect to their targets which can be clearly seen in Table 1 below.

Table 1: Targets and mechanisms of some TB drugs (Zhang, 2005):

Drugs	Targets	Process affected
Isoniazid	Acyl carrier protein reductase	Cell wall synthesis
Rifampicin	B subunit of RNA polymerase	RNA synthesis
Pyrazinamide	Membrane electron metabolism	Transportation via the membrane
Streptomycin	Ribosomal proteins S12 and 16s	Protein synthesis
Ethambutol	Arabinosyltransferase	Cell wall synthesis
Thiacetazone	Not known	Not known
Quinolones	DNA gyrase	DNA synthesis

1.3 Emergence of drug resistance:

There are many problems that are associated with the TB treatment regimen. One of the major problems associated with it is that the period of the treatment is too long.

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Taking into consideration that approximately ninety five percent of the countries that are affected by TB are developing countries, it is not surprising that the necessary health care needed for the implementation and maintenance of the DOTS system is insufficient. The long treatment periods has caused a decrease in patient compliance (Harries, 1997; Zhang, 2005; Bakker-Woudenberg, 2005; Morris *et al.*, 2005) which has lead to the emergence of acquired resistance (Morris *et al*, 2005) resulting in rise of MDR-TB resistant strains of *M. tuberculosis* (resistant to both rifampicin and isoniazid) (Dormann and Chaisson, 2007). Furthermore, the treatment of MDR-TB with second line drugs (newly developed drugs) has also lead to the development XDR-TB.

XDR-TB refers to *M. tuberculosis* that are resistant to rifampicin, isoniazid, quinolone drugs and at least one of the injectable drugs capreomycin, kanamycin and amikacin (Dormann and Chaisson, 2007). Most of the resistance can be attributed to mutations that occur at specific genes (See Table 2) which encompass the following modifications: 1) pumping out of the antibiotic from the cell, 2) hydrolytic deactivation of the compound and lastly 3) mono-methylation or di-methylation of an adenine residue A2058 (in erythromycin) (Walsh, 2000). With respect to XDR-TB some mutations have lead to an up-regulation of the enzyme alkylhydroperoxidase. This enzyme acts as a substitute for catalase peroxidase (produced by mycobacteria) in a metabolic pathway (Caws *et al.*, 2006). These enzymes are responsible for the degradation of toxic H_2O_2 (toxic to microbes) which is produced by macrophages (Bartos, Falkinham and Pavlik, 2004).

According to the World Health Organisation approximately one billion people will be newly infected with TB, 200 million people will be ill from the disease and 35 million people will die from the disease between 2000 and 2020 (Morris *et al.*, 2005). There is an urgent need for the development of TB drugs however no drugs with novel targets have been developed in the past 40 years (Zhang, 2005).

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Table 2: Genes containing mutations that are believed to confer resistance against the respective antimycobacterial drugs (Zhang, 2005; Maus *et al.*, 2005; Banerjee *et al.*, 1996; Caws *et al.*, 2006; Vilcheze and Jacobs, 2007)

Anti-mycobacterial Drug	Mutated genes that confer resistance against the Anti-mycobacterial drug	Gene product or mechanism affected
Isoniazid	*Kat G* , *inhA, ndh*	Catalase perioxidase (drug activator)
Rifampicin	*rpoB*	B- subunit of RNA Polymerase
Pyrazinamide	*pncA*	Nicotinamidase (drug activator)
Streptomycin	*rpsL, rrs*	30S subunit of ribosome, S12 ribosomal protein and 16s RNA protein synthesis
Viomycin	*tlyA, rrs*	16s RNA protein synthesis
Kanamycin	*Rrs*	16s RNA protein synthesis
Capreomycin	*tlyA, rrs*	16s RNA protein synthesis
Quinolones Fluoroquinolones R207910	*gyrA, gyrB* *atpE*	
Ethionamide	*inhA, etaA* or *ethA*	
Ethambutol	*Emb CAB* operon	Arabinosyl transferase from *emb B*
Amikacin	*Rrs*	16s RNA protein synthesis

1.4 TB drug discovery:

There are several approaches to drug discovery which have lead to several molecules being isolated and developed into drugs. The majority of drugs discovered against TB have been either natural products or derived from natural products. Approximately 59% of antibacterial drugs have been from natural products between the period of 1981 to 2006 (Newman and Cragg, 2007). These discoveries have been the result of

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high throughput screening (HTS) of whole cells, target molecules and biosynthetic pathways (Payne *et al.*, 2007). The latter two approaches take into consideration finding a target in the pathogen and recognising a compound which has inhibitory activity towards a microorganism *in vitro* (Balganesh *et al.*, 2004), whilst the former screens for antimicrobial activity from the onset (Walsh, 2007; Payne *et al.*, 2007). Most of the drugs present today have been identified in this manner. Optimisation of these molecules has spurred a pharmacological era where molecules are modified in order to enhance their activity; however, this process is time consuming (Payne *et al.*, 2007; Walsh, 2007). For example, the GlaxoSmithKline group identified 300 target genes of a pathogen but only 160 were found to be essential genes (Payne *et al.*, 2007). From this they did HTS focusing on the three aforementioned approaches (67 targets and 3 whole cell screens) which yielded 5 lead compounds over a 7 year period. Shortcomings were attributed to difficulties in isolation procedures (only a few environmental isolates can be grown in the lab), the lack of genome sequences to search for presence of intrinsic resistance (Morris *et al.*, 2005) or that the compounds of interest were toxic to both the pathogen and human cells (Payne *et al.*, 2007; Balganesh *et al.*, 2004). Hence, many scientists have opted to search for an antimicrobial compound via whole cell screening and find the mode of action thereafter. This method not only screens directly for antimicrobial activity but can also be refined with a secondary screen in order to determine the mode of action. Determination of the mode of action is important in order to optimise production of the compound via fermentation processes (Walsh, 2007; Payne *et al.*, 2007).

The screening of whole cells route in drug discovery for TB (Balganesh *et al.*, 2004) is partially responsible for the discovery of the new TB drug, R207910 (Cole and Alzari, 2005). The Johnson and Johnson group had screened for compounds that showed inhibitory mode of action against mycobacteria (Cole and Alzari, 2005). The compound was then modified with respect to its structure and R207910 was developed. It showed inhibition of the membrane bound enzyme which is responsible for the production of ATP (Cole and Alzari, 2005).

Hence, a combination of the two aforementioned approaches should be investigated. The screening of molecules should be focused to particular bacteria with ‘known’ or ‘potential’ machinery and mechanisms to produce secondary compounds with

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antimicrobial activity (Walsh, 2000). The figure below shows that the class of Gammaproteobacteria is the second highest producer of secondary metabolites amongst the fully sequenced genomes. The family of Pseudomonadaceae is apart of this class, hence it is quite reasonable to infer that this family is capable of producing secondary metabolites (Muller and Bode, 2005).

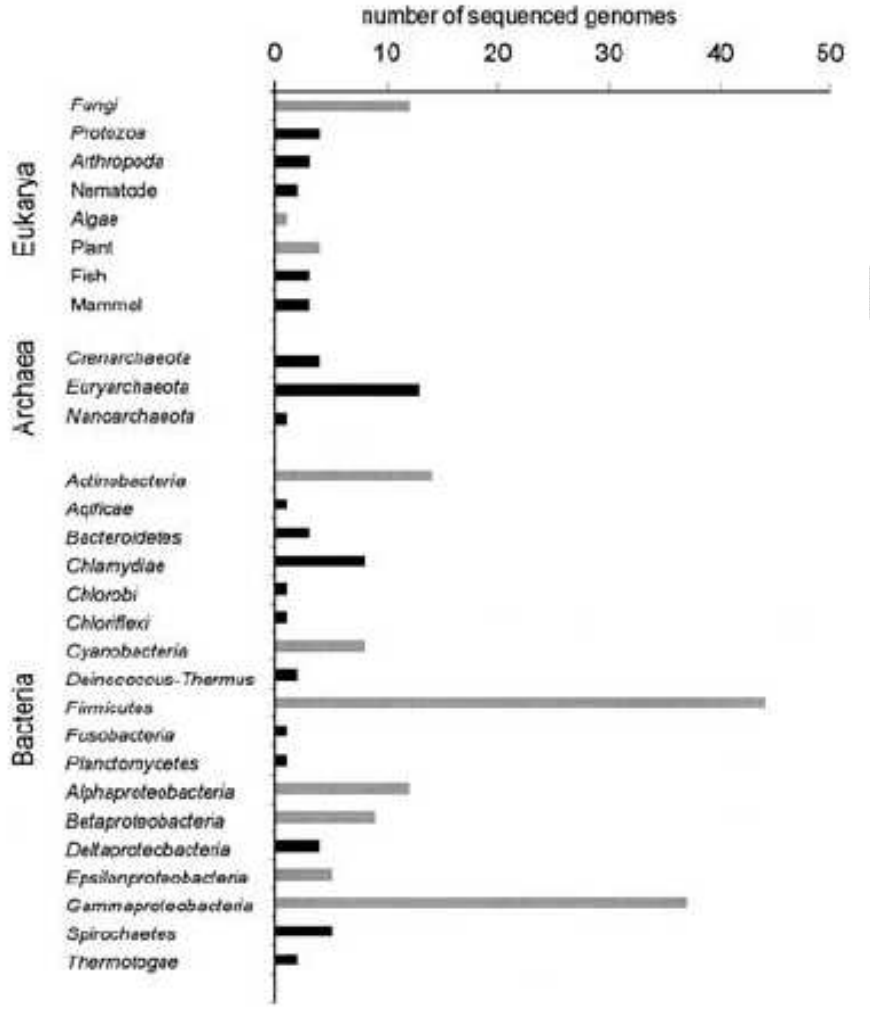


Figure 1: Distribution of the amount of sequenced genomes of organisms that can produce secondary metabolites. The organisms highlighted in grey have been verified via experimental means to produce secondary metabolites (Muller and Bode, 2005).

1.5 Potential for anti-mycobacterial compounds in *Pseudomonas spp*:

The Pseudomonadaceae is a large family of gram negative bacteria and form part of the subclass Proteobacteria (Nelson, 2002). The genera included in this family are *Pseudomonas*, *Xanthomonas*, *Zoogloea* and *Frateuria*. The first genus, *Pseudomonas* contains opportunistic pathogenic bacteria harmful to both animals and humans. Some

members are phytopathogenic (Ochi, 1995). The bacteria are involved in metabolic activities such as element cycling, degradation of biogenic and xenobiotic pollutants due to the production of secondary metabolites (Nelson, 2002). Approximately 47% of sequencing projects of biotechnological relevant bacteria have shown gene clusters that are associated with the production of secondary metabolites (Muller and Bode, 2005). There have been 35 of the fully sequenced genomes from the order of gammaproteobacteria that have had secondary metabolite production certified by experimental means (Muller and Bode, 2005).

It has been hypothesised that one of the reasons this group of bacteria produce secondary molecules is due to their physiological and genetic diversity (Spiers, Buckling and Rainey, 2000). Physiological diversity is attributed to the various niches that this group of bacteria can be found in for example, terrestrial, freshwater and marine environments. The latter is thought to be the result of various factors, such as genome flexibility with respect to genome sizes and exchange of genetic elements through plasmids, transposons and phages. For instance, the bacterium specie *Pseudomonas stutzuri* has a genome size ranging from 3.7 to 4.7 Mbp which implies the gain or loss of genetic sequences (Spiers, Buckling and Rainey, 2000). Due to the size of these sequences one could infer that entire metabolic pathways could be exchanged. However, in order to utilise the various genetic components attained the genome needs to contain regulatory machinery such as activators namely protein kinases. *Pseudomonas aeruginosa* has 9 histidine kinases for every Mbp whilst *E. coli* has only 5 for every Mbp (Spiers, Buckling and Rainey, 2000). The presence of protein kinases allows the bacteria to switch on or off, certain stress activated pathways in different conditions and leads to the production of various compounds. Many of them are produced either by non-ribosomal peptide synthases (NRPs) or by polyketide synthases (PKs) (Walsh, 2007; Spiers, Buckling and Rainey, 2000).

Therefore, it is not surprising that this genus of bacteria are producers of biosurfactants, phytotoxic, antimicrobial lipopeptides, which include and cyclic lipopeptides (CLPs) (particularly in plants) (Bender *et al*., 1995), bacteriocins and polyketides (Parret and De Mot, 2002; Gross *et al.*, 2006). *Pseudomonas* antimicrobial activity can be attributed to the production of various compounds such as bacteriocins, antibiotics, proteases, lysozymes (Das *et al.*, 2006), CLPs with respect

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to phytopathogens (Bender *et al*., 1995; Raaijmaakers, *et al*., 2006), bacteriocins and polyketides (Walsh, 2007; Spiers, Buckling and Rainey, 2000).

1.6 Antimycobacterial activity of *Pseudomonas* compounds:

The probability of *Pseudomonas* producing an anti-mycobacterial compound is high taking into consideration the different antimicrobial compounds that the genus produces. For the purposes of this thesis CLPs, bacteriocins and polyketides will be discussed briefly.

One of the primary drugs developed for TB, erythromycin, was found to be the by-product of a polyketide pathway (Stauton and Weissman, 2001). Polyketides are molecules that are diverse in structure and have antibiotic, chemotherapeutic and anti-parasitic abilities. Synthesis of these compounds is quite similar to that of fatty acid synthesis. It begins with acetyl-coenymeA (two carbon atoms) or propionyl-co-enzymeA (three carbon atoms) or butyryl-co-enzymeA (four carbon atoms) as the primary building blocks. However, unlike fatty acid synthesis, ketoreduction, dehydration and enoyl reduction occur simultaneously or as a combination of just two of the processes. Thereafter the compound can be modified further to form a ring structure (Wawrick *et al.*, 2005). Hence, there is a potential of novel compounds to be produced. Some of these compounds are produced as by-products of the polyketide pathway; for example, mupirocin (pseudomonic acid), pyoluteorin and 2,4 diacetylphloroglucinol a biocontrol agent (Gross *et al.*, 2006) which are produced by the genus *Pseudomonas* (Bender *et al.*, 1999). Even though erythromycin resistant *M. tuberculosis* is prominent, a ketolide telithromycin was active against the resistant phenotype bacteria (Falzari *et al.*, 2005).

Other ribosomally synthesised proteins are bacteriocins (Holtsmark, Eijsink and Brurberg, 2008) which provide an advantage over competition for nutrients in their environmental niches (Parret and De Mot, 2002). There are three classes namely Class I, IIa, IIb, IIc and Class III which are distinguished in terms of structure. With respect to bacteriocins, pyocins have been gaining popularity in terms of searching for new antimicrobial molecules. They are produced by various *Pseudomonas* strains namely, *P. aeruginosa PAO1*, *Pseudomnas putida* (Parret and De Mot, 2002) and

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Pseudomonas syringae phaseolicpola (Guven, 2000). Pyocins are phage tails that have been modified via evolutionary mechanisms into bacteriocins. These molecules generally target intracellular DNA and degrade it (Parret and De Mot, 2002). However, they can also act as pore formers and function as tRNase (Iwalokun *et al.*, 2006).

CLPs from *Pseudomonas* have also been able to show anti-mycobacterial activity. A number of cyclic lipopeptides have been identified within the *Pseudomonas* species (Nielsen *et al.*, 2002). The compounds possess antimicrobial, cytotoxic or surfactant properties and are ribosomally synthesised. Structurally these compounds are made up of short oligopeptides linked to a fatty acid tail and the presence of a lactone ring between the two amino acids (Figure 1). The CLPs are classified on the basis of the composition and length of their fatty acid tail as well as the length and constituents of the peptide moiety (refer to Table 4) (Raaijmaakers *et al*., 2006). It is believed that lipopeptides (Makotvitzki, *et al*, 2006) and CLPs could have a mode of action that is similar to the carpet mechanism. It entails the accumulation of the peptide on the surface of the cell to a particular threshold where the permeation of the membrane follows in a detergent-like manner (Makotvitzki, *et al.*, 2006). Taking into consideration that mycobacterial cell membranes possess high lipid content approximately 50% to 60% (Fitzmaurice and Kolattukudy, 1998), a molecule with biosurfactant like activity could be an effective antimycobacterial agent.

There have been reports of activity against *Mycobacterium smegmatis* from two *P. syringae* pv syringae strains, namely strains 508 and B359 (Grgurina *et al*, 2005; Buber *et al*, 2002). A novel lipodepsipeptide (syringopeptin) was extracted from *P. syringae* pv lachrymans strain 508. (Grgurina *et al*, 2005). Agar diffusion assays performed with *M. smegmatis* showed the formation of clear zones around the wells which were inoculated with the extract. *M. smegmatis* forms part of the family of mycobacteria which include *M. tuberculosis* (Grgurina *et al*, 2005). Thus there is the possibility that *Pseudomonas spp* could produce a product that could be developed into an anti-mycobacterial agent.

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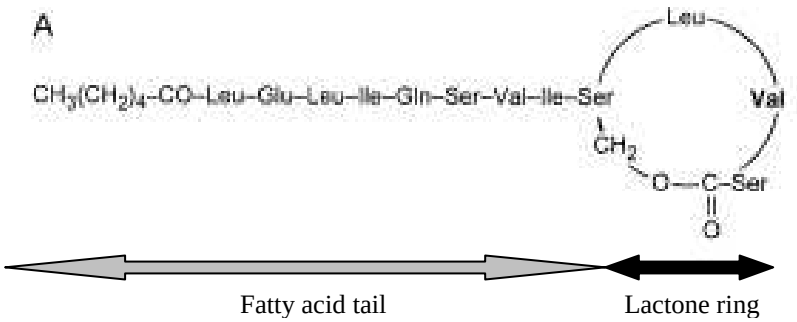


Figure 2: Structure of the cyclic lipopeptide, putisolivin I from *P.putida* PCL1445 (Kuiper *et al*, 2004)

1.7. Motivation:

A *Pseudomonas* isolate with inhibitory activity against *M. smegmatis* was discovered during isolation of mycobacteriophage from soil samples; by a Masters student L.K Lukusa. Growth of the isolate on petri dishes containing *M. smegmatis* in the agar exhibited inhibition of the mycobacteria. 16s RNA sequence analysis showed that the isolate was closely related to several designated *Pseudomonas putida* strains with known secondary metabolite production (see Appendix B, Table 11). As previously mentioned, *Pseudomonas* strains have been shown to produce various compounds which act as antimicrobials. From this it was inferred that there is a possibility that the activity produced was a result of the production of a compound with anti-mycobacterial activity. Taking into consideration that antimicrobial resistant TB strains are present, there is a sense of urgency to find alternative treatments to combat the disease. Since *M. smegmatis* falls within the same family of mycobacteria, this *Pseudomonas* isolate could act on pathogenic strains such as *Mycobacterium bovis*. Should its activity be successful, the possibility that it could be active against TB and the resistant strains is to be investigated. In this study, we proposed to isolate and characterize the antimycobacterial activity of the *Pseudomonas* isolate (*Pseudomonas* αmb-antimycobacterial-).

1.8 Research Question:

Does the unknown *Pseudomonas* isolate possess any specific antimicrobial properties against *M. smegmatis*?

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1.9 Main Objective:

Extraction and characterization of the anti-mycobacterial activity of the *Pseudomonas* αmb isolate.

1.10 Sub-objectives:

a) Extraction of the antimycobacterial compound in the form of a crude extract.

b) Purification of the crude extract and preliminary characterisation of the antimycobacterial compound/s with respect to:

(i) Solubility, polarity and presumptive molecular mass of the antimycobacterial compound
(ii) Assessment of its activity against *M. smegmatis* mc^2155 and other environmental strains

c) Isolation of a *Pseudomonas* αmb isolate lacking antimycobacterial activity made using transposon mutagenesis as a preliminary step to determining the genetic mechanism of the activity.

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