STUDIES IN MYCOBACTIN BIOSYNTHESIS

by

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Abstract

Tuberculosis (TB) is the leading cause of infectious disease mortality in the world by a single bacterial pathogen, *Mycobacterium tuberculosis*. Current TB chemotherapy remains useful in treating susceptible *M. tuberculosis* strains, however, the emergence of MDR-TB and XDR-TB demand the development of new drugs. Enzymes involved in mycobactin biosynthesis, low molecular weight iron chelators, do not have mammalian homologues; therefore they are considered potential targets for the development of new anti-TB drugs. The aims of this study were to identify potential inhibitors and to investigate the function of the *mbtG* and *AmbtE* and *AMbtF* genes during mycobactin biosynthesis. The full length of *mbtB* and the ArCP domain were successfully cloned and post-translationally modified by MtaA, a broad phosphopantetheinyl transferase from *Stigmatella aurantiaca*, using *Escherichia coli*. Inhibitors identified by virtual screening as well as 13 chemically synthesised PAS analogues were initially investigated in whole-cell assay against *Mycobacterium bovis* BCG Pasteur. Seven of these compounds had interesting growth inhibition under iron-sufficient conditions. The *mbtA* gene was cloned and expressed as soluble protein using *Mycobacterium smegmatis* mc^2155. Preliminary *in vitro* MbtA assays provided hints of its activity, although, the K_M for SAL and ATP have not been determined yet. The *mbtG* and *ambtE* genes have been cloned and expressed in *E. coli* to further investigate their biochemical function in mycobactin biosynthesis.

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Undoubtedly, my parents and brothers have played a fundamental role during my life; they have always been with me both in the pitfalls and success. In fact, the first scholarship that I received was from my parents who supported me during all my studies until I completed a university degree. Without them I would never achieve what I have now. Their love and support have encouraged me to keep working hard.

It is always difficult to choose what to do next after the undergraduate studies. However, my experience as a co-worker in the region hospital of my hometown, where I used to collaborate in the diagnosis of tuberculosis, determined my decision to do research and pursue a post-graduate degree in this area. My conviction that Science is a tool to unmask and understand nature for the benefit of human being as well as for the conservation of nature itself was another reason.

Selecting a place to achieve these aims is not easy either. However, I started another fascinating path in my life here in Birmingham in Prof. Gurdyal S. Besra's laboratory. I would like to express my gratitude to him for giving me an opportunity to do tuberculosis research in his laboratory. Also, I am very grateful to the Ford Foundation International Fellowship Program and the Mexican National Council for Research (CONACYT) for granting me a funded Ph D.

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List of abbreviations.

ACN	Acetonitrile
ACP	Acyl carrier protein
AG	Arabinogalactan
AIDS	Acquired immuno-deficiency syndrome
AMI	Amikacin
Araf	Arabinofuranosyl
ArCP	Aryl carrier protein
ATP	Adenosine-5'-triphosphate
BC	Before Christ
BCG	Bacillus Calmette-Guérin
bp	Base pair
BVMOs	Baeyer-Villiger monooxygenases
CAP	Capreomycin
CCPs	Clathrin-coated pits
CD1	Cluster of differentiation1
CM	Cytoplasmic membrane
CMN	*Corynebacterium*, *Mycobacterium* and *Nocardia* group
CoA	Coenzyme A
CP	Carrier protein
CPM	Counts *per* minute
DAT	Diacyl trehalose
Ddl	D-Cycloserine-D-alanine synthetase
DDM	Didehydroxymycobactin
DHB	2,3-Dihydrobenzoate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOTS	Directed observed treatment short course program
DtrR	Diphtheria toxin repressor protein
E°	Standard reduction potential
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol

EPS	Epidemic-pathogenic selection
ER	Endoplasmic reticulum
ESAT-6	Early secreted antigen-6 kDa
ES-MS	Electrospray-mass spectrometry
EtBr	Ethidium bromide
ETH	Ethionamide
FAAL	Fatty acyl-AMP ligase
FAD^+	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FAS	Fatty acid synthase
FDMOs	Flavin-dependent monooxygenases
Fe	Iron (Fe^{3+}= ferric iron; Fe^{2+}= ferrous iron)
FMN^+	Flavin mononucleotide
$FMNH_2$	Reduced flavin mononucleotide
FQs	Fluoroquinolones
FT-ICR-MS	Fourier transformation-ion cyclotron resonance-mass spectrometry
Fur	Ferric uptake protein
g	Grams
GC	Guanine and cytosine genome content
HBC	High burden countries
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid)
HH	Haemochromatosis
HIV	Human immuno-deficiency virus
HPLC	High-performance liquid chromatography
HWMP	High molecular weight protein
IdeR	Iron dependent regulator protein
INH	Isoniazid
IPTG	Isopropyl-thio-β-D-galactopyranoside
ISO	Isoaxyl
KAN	Kanamycin
kbp	Kilobase pair
L	Litre

LAM	Lipoarabinomannan
LB	Luria-Bertani
Lf	Lactoferritin
M	Molar
mAGP	Mycolyl-arabinogalactan-peptidoglycan
MALDI-MS	Matrix-assisted laser desorption ionization-mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
Mb	Megabase pair ($1x10^6$ bp)
MBs	Mycobactins
MDR-TB	Multi-drug Resistant-tuberculosis
meso-DAP	*meso*-Diaminopimelic acid
mg	Milligram
MIC	Minimum inhibitory concentrations
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MurNGLy	Muramic acid N-glycolylated
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMOs	Microbial *N*-hydroxylating monooxygenases
Nramp	Natural resistant associated macrophage protein
NRPSs	Nonribosomal peptide synthetases
OD	Optical density
OM	Outer membrane
PAEs	Peptide arylation enzymes
PAGE	Polyacrylamide gel electrophoresis
PAS	*para*-Amino salicylic acid
PAT	Pentaacyl trehalose
PB	Proskauer and Beck
PBT	Periplasmic binding protein-dependent transport

PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimycocerosate
PE	Proline-Glutamic acid motif sequence
PG	Peptidoglycan
PGL	Phenolic glycolipid
PGRS	Polymorphic GC-rich sequence class
PIM_6	Phosphatidylinositol hexamannoside
PKSs	Polyketide synthases
PMSF	Phenylmethanesulfonylfluoride
P-pant	Phosphopantetheine group
PPE	Proline-Proline-Glutamic acid motif sequence
PPTase	Phosphopantetheinyl transferase
PVM	Parasitophorous vacuole membrane
PZA	Pyrazinamide
RIF	Rifampicin
RNA	Ribonucleic acid
ROIs	Reactive oxygen intermediates
rpm	Revolution *per* minute
rRNA	Ribosomal RNA
SAL	Salicylic acid
SBP	Substrate binding protein
SDS	Sodium dodecyl sulfate
SL	Sulphated tetra-acyl trehalose
SNPs	Single nucleotide polymorphisms
TB	Tuberculosis
TE	Thioesterase domain
Tf	Transferrin
TFA	Trifluoroacetic acid
TMM	Trehalose dimycolate
TZA	Thiacetazone
μCi	Microcurie

μg	Microgram
μL	Microlitre
μM	Micromolar
V	Volts
v/v	Volume/volume
VIO	Viomycin
w/v	Weight/volume
WHO	World Health Organization
XDR-TB	Extensively Drug Resistant-Tuberculosis

1. General Introduction

1.1 Tuberculosis, an ancient human disease.

Tuberculosis (TB), one of the oldest recognised human diseases, is still one of the biggest killers among infectious diseases, despite the worldwide use of the BCG vaccine and several antibiotics to combat it. TB is caused by the successful intracellular pathogen, *Mycobacterium tuberculosis*, a Gram-positive bacterium. Although, *M. tuberculosis* is the main pathogen causing TB in humans, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canettii* and *Mycobacterium microti* may also cause TB (Brosch *et al*., 2001) and are collectively called the *M. tuberculosis* complex. Also, within the genus there are other members of medical, veterinary and environmental importance (Ratledge & Stanford, 1982). Interestingly, many mycobacterial species can become human opportunistic pathogens in immunodepressed individuals, signifying a high adaptation of the genus to different environments (Katoch, 2004).

TB has been recognised in human history, from the dawn of civilisation to the modern era, reflecting a constant co-evolution between host and pathogen. Molecular evidence gained from analysis of ancient osteological samples, derived from amplification of specific regions of *M. tuberculosis* DNA, supports the existence of TB through ancient human populations (Donoghue *et al*., 2004; Zink *et al*., 2007). The disease may have also been present in human populations dated as far back as 9250-BC as observed in skeleton remains found in Ain Ghazal Jordan (Crubézy *et al*., 2006; El-Najjar *et al*., 1996). DNA from members of the *M. tuberculosis* complex has been detected in several ancient Egyptian mummies dated at more than 5,000 years ago (Crubézy *et al*., 1998; Nerlich *et al*., 1997; Zink *et al*., 2003a; Zink *et al*., 2003b), suggesting that the disease was common among that population (Zink *et al*., 2001). In Europe, there is also evidence that TB was present in the Iron Age (4000-2000 BC) (Formicola *et al.*, 1987; Mays & Taylor, 2003; Taylor *et al*., 2005). In South America, TB has been found in pre-Columbian mummies suggesting that the tubercle bacillus was present before contact with European populations (Arriaza *et al*., 1995; Konomi *et al*., 2002; Salo *et al*., 1994). In Asia, *M. tuberculosis* DNA has been found in 2000-year-old human skeletons (Fusegawa *et al*., 2003), as well as *Mycobacterium leprae* DNA dating from 555 BC (Tayles & Buckley, 2004).

Despite this compelling evidence, the origin and the evolutionary time-frame of *M. tuberculosis* remains controversial. Most of the *Mycobacterium* members are natural inhabitants of soil or water inhabitants and it is logical to assume that the genus had its origin in such environments (Falkinham, 2002). How they became pathogenic is still an unsolved question, but probably some members adapted to new niches, under certain circumstances, leading to the emergence of a new ecotype (Cohan, 2002).

It has been speculated that *M. tuberculosis* has evolved from *M. bovis*, by specific adaptation of an animal pathogen to the human host (Stead *et al*., 1995). However, genome comparison of members of the *M. tuberculosis* complex has shown other scenarios; either both bovine and human tuberculosis evolved almost at the same time which suggests a different common ancestor for both bacilli, the theoretical *Mycobacterium prototuberculosis*, or humans infected cattle resulting in the divergence of *M. bovis* (Gutierrez *et al*., 2005; Sreevatsan *et al*., 1997a). Most of the data analysed point to *M. tuberculosis* as the main causative agent of human TB, however, recent data from a study of four archaeological samples from a single site in South Siberia indicate that skeletal lesions were attributable to infection with *M. bovis* (Taylor *et al*., 2007). In addition, genotyping analysis on the *oxyR*285 and *pncA*169 loci, which can be used to distinguish *M. tuberculosis* from *M. bovis*, found single nucleotide polymorphisms (SNPs) that are typical in *M. bovis* (Taylor *et al*., 2007). The apparent absence of *M. bovis* in ancient skeletal and mummified humans (Donoghue *et al*., 2004) and the discovery of DNA of tubercle bacillus amplified from a North American Pleistocene extinct bison, showed a genetic profile resembling either *M. africanum* or *M. tuberculosis* more closely than it does *M. bovis* (Rothschild *et al*., 2001), supporting the new hypothesis.

Analysis of diverse housekeeping genes among modern members of the *M. tuberculosis* complex has suggested a recent evolutionary bottleneck that may have occurred 20,000 to 35,000 years ago (Brosch *et al*., 2002; Sreevatsan *et al*., 1997a). Nevertheless, recent findings and characterisation of East African *M. tuberculosis* strains suggested an age of 2.6-2.8 million years (Gutierrez *et al*., 2005). The same study highlights two interesting

scenarios in the co-evolution of the tubercle bacillus and humans. Firstly, the origin of tuberculosis could be much older than the plague, typhoid fever, or malaria, and may have affected early hominids. Secondly, the fact that the analysed strains came from East Africa, a region where early hominids were present 3 million years ago, suggests that tubercle bacillus emerged in Africa and then evolved following the expansion of a successful clone to the rest of the world, probably parallel to the human migration out of Africa (Gutierrez *et al*., 2005).

The long lasting association of the tubercle bacillus with the early hominids is likely to have directed the intrinsic relation between the host and the pathogen, leading to the outcome of a very successful pathogen. Thus, early humans provided selection pressure for different phenotypes: strains causing the active disease and the ones which become latent, and probably the latter survived as an effective evolutive mechanism. Moreover, *M. tuberculosis* is still evolving and this is demonstrated with the appearance of new strains resistance to drugs (Ernst *et al*., 2007).

1.2 Epidemiology of TB: a persistant human worldwide disease.

The prevalence of TB has been documented through human history. The molecular analyses mentioned earlier suggest that ancient Egyptians suffered TB 5000-BC. Chest pain, coughing and blood in the sputum, the common symptoms of pulmonary TB, were described by Hippocrates (~ 500 B.C.) from patients who may have been infected with the bacillus. He termed the disease phthisis and the frequency of his descriptions of patients with TB-like symptoms indicates that the disease was prevalent (Daniel *et al*., 1994).

During the 16th and 17th centuries Europe had high death rates associated with TB, reaching maximum levels in the first half of the 19th century in which was estimated that one-quarter of all Europeans died of TB. European immigrants brought the disease to the New World, but the levels of mortality never reached the levels found in Europe (Daniel *et al*., 1994). These high mortality rates were due to the appalling socioeconomic conditions,

overcrowding, poor nutrition, and lack of hygiene and sanitation, which prevailed during the early years of the industrial revolution (Murray, 1989).

Although, the "Consumption, white plague or King's Evil disease", as TB was named, was recognised as a very contagious and frequent disease during this time, the etiological agent was not identified. In 1882, a breakthrough in medical history was made by Robert Koch who discovered and described the agent of TB (Sakula, 1982).

In the last half of the 19th century, mortality due to TB decreased, in part due to the following factors: improved socioeconomic conditions, application of sanitation measures, and natural selection of humans resistant to TB may have played a major role (Murray, 1989). However, the decline was too rapid to be explained by these changes since the relationship between socio-economic factors and TB transmission is complex (Gandy & Zumla, 2002) and natural selection, the appearance of gene(s) resistant to TB, may require long period of time (Lipsitch & Sousa, 2002).

TB mortality and morbidity rates dropped during the 20th century aided by the development of a vaccine and the discovery of drugs to combat the disease. Albert Calmette and Camille Guérin developed an attenuated live vaccine from *M. bovis* by growing it in serial passage on culture medium for over 13 years leading to the creation of the globally-used attenuated *M. bovis* BCG vaccine (Doherty & Andersen, 2005). The discovery of streptomycin in 1944, *para*-Aminosalicylic acid in 1946, and isoniazid and pyrazinamide in 1952, led to effective chemotherapies that decreased TB globally (Zhang, 2005). This led to a decline in funding and interest in many TB research programs.

During the 1980s, TB was neglected as an international health issue since it was believed that the disease was under control due to advances in TB chemotherapy and only endemic regions, mainly in developing countries, were faced with TB (Raviglione, 2003). However, epidemiological studies in the United States of America showed that the new TB cases were increasing and diverse factors were attributed to explain this increase (Reichman,

1991). The rate of HIV was also increasing and it was associated with TB along with poverty in urban areas. Nevertheless, many of the cases of TB reported in USA came from immigrants from developing countries. This rise in newly detected cases of TB not only in USA, but also in European countries, such as the Netherlands, Switzerland, Sweden, Norway and Denmark. These cases of TB were attributed to local and international migration, and it became clear that TB was not longer only a problem in developing countries. Thus, TB became recognised as an international health emergency (Raviglione, 2003).

The status of the TB epidemic and progress in control of the disease has been assessed by the World Health Organization (WHO) since 1997. Much of the data for mortality and morbidity was predicted; however, estimations and reliable records begun in 1995 from analysis of case notifications due to programs implemented by the WHO. Hence, in 1990 there were an estimated 7.1 million cases of TB and 2.5 million deaths worldwide, the most affected countries were situated in Africa and Asia. An alarming number, 90 million cases and 30 million deaths were expected in the 1990s (Raviglione *et al*., 1995). However, the mathematical model of Murray and Salomon (1998) estimated 6.7 million new cases and 2.4 million deaths for 1998 alone. The model also predicted that death and new case rates would increase between 1998 and 2030, although the panorama might change with the implementation of diverse strategies to combat TB. However, based on a global epidemiologic study, in which the incidence of TB and mortality were determined in 212 countries revealed another scenario. Thus, in 1997, 7.96 million new cases of TB and 1.87 million deaths were reported (Dye *et al*., 1999). Since the introduction of the Directed Observed Treatment Short-Course Program (DOTS) by the WHO in the early 1990s, a steady decline in TB cases was observed. The new cases detected during this decade were mainly attributed to Multi-Drug Resistance-TB (MDR-TB) and the emergence of HIV-AIDS (Espinal *et al*., 2001; Heym *et al*., 1994; Raviglione, 2003).

In 2000 there were an estimated 8 to 9 million new cases of TB, fewer than half of which were reported; 3-4 million cases were sputum-smear positive, the most infectious form of the disease. Most cases (5-6 million) were in people aged 15-49 years (Frieden *et al*., 2003). The global incidence of TB *per* capita peaked in 2003 and apparently there was a decline of new TB cases reported. Nevertheless, the apparent decline of global TB was masked by the increase of the global growth population, and for 2005-2006, new TB cases incremented to 9.1-9.2 million (an increase of 0.6%). Furthermore, WHO epidemiological studies show that one third of the world's population may be latently infected by *M. tuberculosis* and these people could become ill or infectious at some time during their life (Corbett *et al*., 2003a). Despite various programs to control TB, this scenario has not changed and the WHO has reported 9.2 million new cases and 1.7 millions deaths from TB, of which 0.7 million cases and 0.2 million deaths were in HIV-positive individuals (WHO, 2008). Based on current data, the WHO has estimated that 55% of global TB cases (including all forms of TB) occur in the high burden countries (HBC) of South-East Asia and Western Pacific countries, whereas 31% cases arise in Africa. However, some African countries account for a strikingly large number of cases relative to their population. Among the 15 countries with the highest estimated TB incidence rates, 12 are in Africa. The high incidence rates correlate with high incidence of HIV co-infection (WHO, 2008). Figure 1 depicts new TB cases by country in 2006.

Diverse factors are involved in the increase of new TB cases worldwide; these include high population density, poor nutrition, poor sanitation, synergy between TB and HIV/AIDS due to immunosupression and the increase of strains resistant to drugs. This has ultimately lead to the emergence of multi-drug resistant strains of TB (MDR-TB) and, more recently, the appearance of extensively drug resistant-TB (XDR-TB), linked to the problems caused by international migration (Barnes *et al*., 1991; Gandy & Zumla, 2002). The global distribution of TB is skewed towards developing countries, mainly in Asia and Africa, due to HIV co-infection. The resurgence of MDR-TB strains in Eastern Europe and XDR-TB in some countries, will contribute to new cases and death rates and TB will continue to be a major health issue for the international community (Fatkenheuer *et al*., 1999; WHO, 2008).

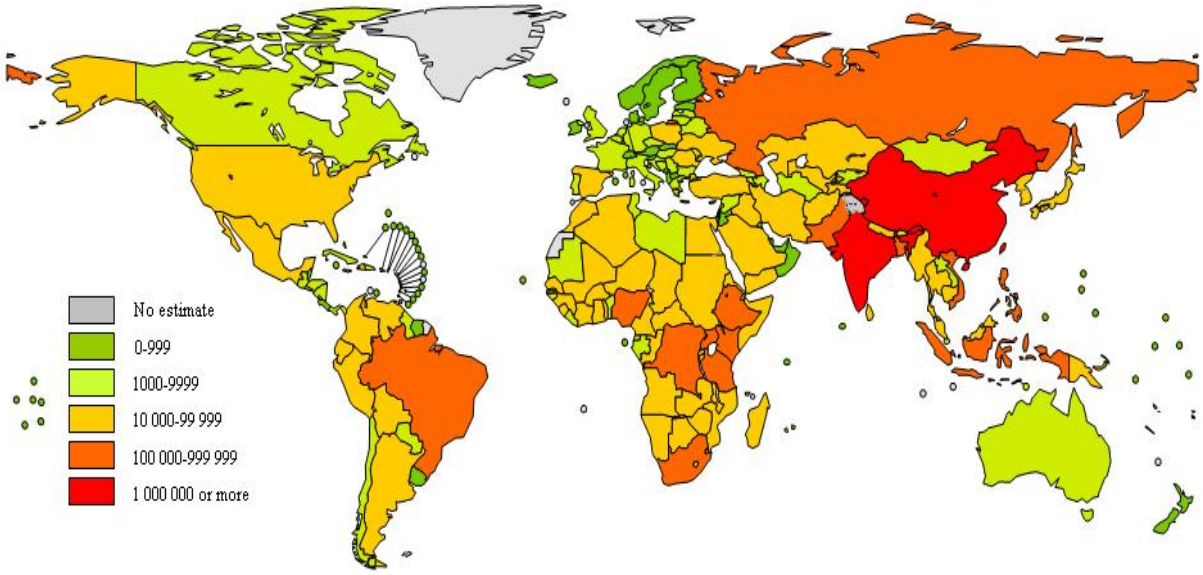


Figure 1. Estimated new TB cases (all forms) for 2006. Most high burden (coloured orange and red) countries are located in Africa and South-East Asia; however, some Western European countries have also high incidence of TB cases due to the emergence of MDR-TB (WHO, 2008).

1.3 Biology of *M. tuberculosis*: understanding the success of the bacillus.

Genotype and phenotype plasticity, slow growth, the unique and complex cell envelope, dormancy, intracellular pathogenesis, persistence, drug-tolerance (survival despite drug treatment), resistance (genetic mutations that result in heritable loss of susceptibility to antibiotics) and production of a range of virulence factors, are some of the biological features displayed by *M. tuberculosis* during infection. Understanding the basic biology of *M. tuberculosis* should aid in designing new vaccines and chemotherapy.

1.3.1 Taxonomy and classification.

Lehman and Neumann (1896) proposed the generic name *Mycobacterium*, from the Middle Latin noun meaning fungus rodlet, to include tubercle and leprosy bacilli. Historically, classification of mycobacteria represented a problem since only basic features, initially morphology then physiology, were considered to categorise related bacteria forming mycelia. The original description of the genus *Mycobacterium* was based mainly on morphological and staining properties, features then considered to be sufficiently diagnostic to distinguish mycobacteria from corynebacteria and other morphologically similar bacteria. However, the introduction of biochemical, chemical, genetical and numerical techniques have led to a re-grouping of the Class *Actinobacteria* in which the mycobacteria are grouped (Embley & Stackebrandt, 1994; Goodfellow & Wayne, 1982).

Although, modern techniques, currently used to group the *Actinobacteria*, such as sequence analysis of proteins and nucleic acids, has supported re-classification of this taxon, the traditional chemotaxonomic characters have defined specific artificial groups. The determination of key chemical structures of components of the cell wall, such as sugar, amino acids and lipids enabled the re-classification of bacteria sharing similar features with mycobacteria. Simple wall composition provided the first evidence of relationship between corynebacteria, mycobacteria and nocardiae, all of which contain large amounts of *meso*-diaminopimelic acid (*meso*-DAP), arabinose and galactose. However, the most predominant feature shared by these bacteria are the branched-chain fatty acids, termed mycolic acids, which have contributed to the differentiation of actinomycetes to a genus level (Minnikin & Goodfellow, 1980). Thus, the genera *Corynebacterium* (C), *Mycobacterium* (M)*, and Nocardia* (N) were grouped in a monophyletic taxon, the so-called CMN group, within the *Actinobacteria*, based on mycolic acids. The length and composition of these compounds have been used as chemotaxonomic markers for differentiating between the genera that produce them (Embley & Stackebrandt, 1994).

The current classification of mycobacteria is now based in chemotaxonomy markers, morphological and physiological characteristics, as well as molecular analysis, such as analyses of DNA-DNA re-association and determination of the 16S rRNA and rDNA sequence similarities. All these approaches have contributed to re-define the actinomycetes and therefore the genus *Mycobacterium* (Stackebrandt *et al*., 1997; Tortoli, 2003; Ventura *et al*., 2007). A phylogenetic tree of Actinobacteria is shown in Figure 2.

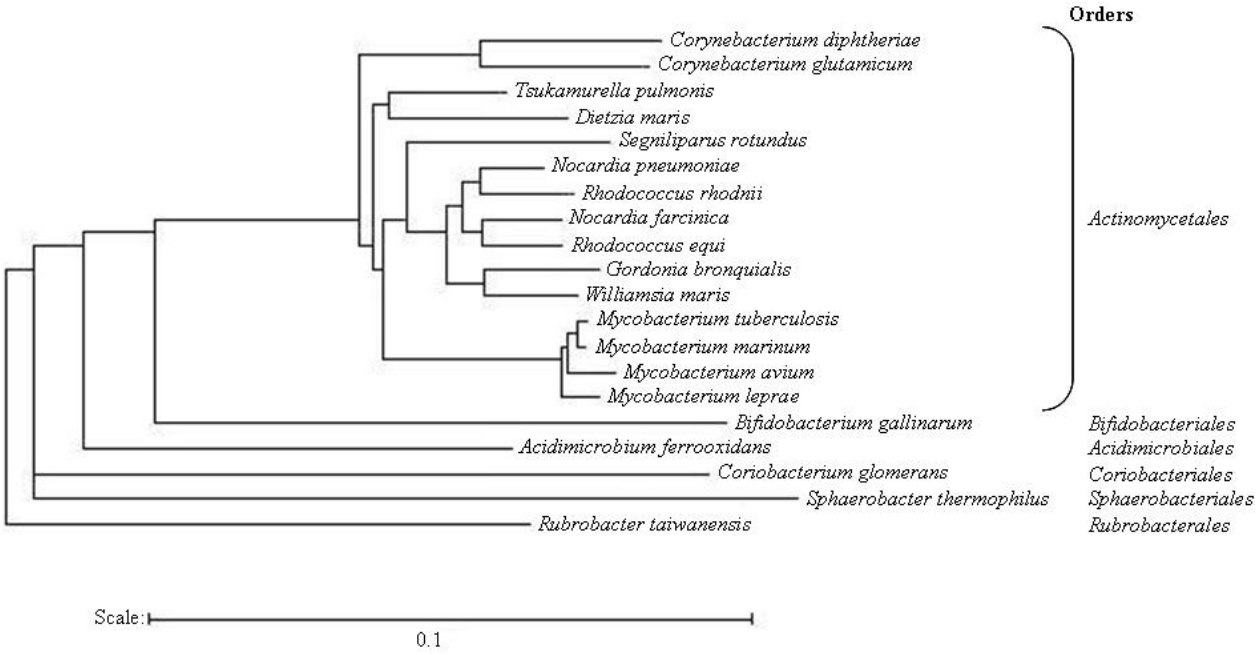


Figure 2. Intraclass relatedness of *Actinobacteria* showing the presence of six orders. The phylogenetic tree is based on >1,200 nucleotides of 16rRNA. The scale bar represents 10 nucleotide substitutions *per* 100 nucleotides. [Created in Tree Builder (Cole *et al*., 2009)].

Though, the defined *Mycobacterium* genus is highly diverse and comprises around 85 different species; the members share certain characteristics, especially the ability to form mycelia. Due to the diversity of the genus it is not easy to propose a simple definition, but in general mycobacteria are aerobic, acid-alcohol fast actinomycetes having a genome rich in guanidine and cytosine nucleotides. They are aerobic, acid-alcohol fast actinomycetes that

usually produce slightly curved or straight rods and coccoid elements, of around 0.2-0.7 X 1.0-10.0 μm in size (Wayne & Kubica, 1994).

1.3.2 Life style of mycobacteria.

The diversity of biological life style is immense in mycobacteria. Generally, mycobacteria are free-living saprophytes and are well adapted to different habitats, such as soil and aquatic environments, whereas other species are obligate intracellular pathogens that infect humans, birds and other animals (Goodfellow & Williams, 1983). Although, *M. ulcerans* has been isolated as a soil inhabitant in symbiosis with roots of certain plants present in tropical rain forests or similar environments (Hayman, 1991), this species is the third most common in mycobacterial disease mediating a chronic disease leading to expanding skin ulcers, the so-called Buruli ulcer (van der Werf *et al*., 2005).

In general, mycobacteria are the causative agents of a broad epidemiological, clinical, and pathological spectrum of diseases in humans. Mycobacterial diseases are very often associated with immunocompromised individuals, especially HIV-AIDS patients. Some pathogenic mycobacteria prefer a single host, such as *M. tuberculosis* and *M. leprae* being human pathogens, whereas others can infect different hosts, for instance *M. bovis* infecting cattle or humans, *M. microti* being able to infect wood mice, shrews, llamas, cats and very occasionally humans (Brosch *et al*., 2001).

1.3.3 Mycobacterial genomes: insight to the bacilli.

The clinical course and pattern of TB is highly variable, ranging from life long asymptomatic infection to rapidly progressive pulmonary or disseminated disease. This variability was assumed to be result of differences in individual responses to the disease. However, modern molecular biology techniques are revealing key biological features of *M. tuberculosis* at the genomic level, which are partially explaining the phenotype and genotype plasticity found in pathogenic mycobacteria (Brosch *et al*., 2001).

The architecture, size, and function of the genome within mycobacterial species are diverse. The genome of the *M. tuberculosis* H37Rv strain is 4.4 Mb and GC content about 65.6%, with a 91% coding capacity. More than 20% of the *M. tuberculosis* genome is devoted to genes encoding two different classes of proteins: enzymes involved in fatty acid metabolism and acidic, glycine-rich polypeptides of unknown function, the PE and PPE proteins (Cole *et al*., 1998). The remarkable number of genes encoding for fatty acid biosynthesis maybe related to the ability of the bacillus to grow in the host's tissue, where fatty acids maybe the major carbon source (Cole *et al*., 1998). Genome comparisons of the *M. tuberculosis* H37Rv and CDC1551 strains, the latter being a highly virulent strain, revealed interesting polymorphisms. The CDC1551 strain contains an extra gene that shares homology to the early secreted antigen of 6 kDa (ESAT-6), a major T-cell antigen (Betts *et al*., 2000).

The *M. tuberculosis* complex comprises *M. tuberculosis* together with *M. africanum*, *M. bovis*, *M. canettii*, *M. pinnipedii* and *M. microti*, which are closely related organism sharing >99% identity at the nucleotide level. They share 99.9% similarity at the nucleotide level and identical 16S RNA sequences, but differ in terms of their host tropisms, phenotypes and pathogenicity (Brosch *et al*., 2001). It is intriguing that some members are exclusively human pathogens, *M. tuberculosis*, *M. africanum*, *M. canettii*, whereas others have a wide host spectrum, such as *M. bovis*.

Although, human and bovine tubercle bacilli can be differentiated by host range, virulence and physiological features, the genetic basis for these differences are still unknown. The *M. bovis* AF2122/97 genome has been recently deciphered (Garnier *et al*., 2003). The genome sequence is 4.3 Mb in length, with an average GC content of 65.63%, which potentially encodes 3952 proteins (Garnier *et al*., 2003). The bovine bacillus genome shares >99.95% identity at the nucleotide level to that of *M. tuberculosis*. However, deletion of genetic information is the dominant trend in the *M. bovis* genome, which has many pseudogenes. These genes resemble intact genes that are involved in molecular transport, structure and biosynthesis of the cell surface, detoxification, intermediary metabolism, fatty acid metabolism and cofactor biosynthesis (Garnier *et al*., 2003).

Variation in genes encoding the PE-PGRS and PPE protein families, predicted to be involved in adhesion and immune modulation, may contribute to the different host ranges displayed by *M. bovis* and *M. tuberculosis*. In the *M. bovis* genome, the sequence sharing significant homology with *M. tuberculosis wag*22 (Rv1759c), which encodes a member of the PE-PGRS protein family that binds fibronectin, has been disrupted by the deletion of a single nucleotide. This, in turn, suggests that alterations to the PE-PGRS repertoire might influence host or tissue tropism (Espitia *et al*., 1999). Furthermore, six genes belonging to the ESAT-6 family are either missing or altered in the *M. bovis* genome and it is possible that these changes have an impact upon immune regulation in the host and thus host range (Garnier *et al*., 2003).

Likewise, the obligate intracellular pathogen, *M. leprae*, has the smallest genome sequenced among the mycobacteria. The complete genome of *M. leprae* TN strain contains 3.2 Mb, roughly 1.4 Mb smaller than that of *M. tuberculosis*, and has an average GC content of 57.8%. The *M. leprae* genome only encodes 49.5% of its coding capacity, whereas 27% contains pseudogenes, randomly distributed, and the remaining 23.5% of the genome does not appear to be coding (Cole *et al*., 2001a). The extensive genome downsizing and rearrangement must have occurred during evolution of the leprosy bacillus and this might be associated with the obligate intracellular habitat of the bacteria. Supporting this hypothesis, the prolyl-tRNA synthase, encoded by *proS*, is more similar to the enzymes of *Borrelia burdorferi* and eukaryotes. The *M. leprae proS* gene is both displaced and inverted with respect to the *M. tuberculosis* counterpart, consistent with a recent acquisition through horizontal gene transfer, from host to pathogen. Other interesting findings are the deletion of some polyketide synthases (PKS), involved in lipid biosynthesis, and the *mbt* operon required for the production of mycobactins (Cole *et al*., 2001a; b).

Since 1989, there has been an increase of cases of Buruli ulcer, a severe human skin disease caused by *M. ulcerans*. The genome of *M. ulcerans* strain Agy99 has recently been deciphered and it comprises two replicons: one corresponding to the chromosome which is 5.6 Mb and a plasmid (pMUM001) having 17.4 kbp in length (Stinear *et al*., 2007). Two

interesting observations are deduced from the genetic analysis to understand the pathology of the Buruli ulcer. Firstly, there is no formation of granulomas, which is typical in other mycobacterial infections. Secondly, *M. ulcerans* is mainly found extracelullarly in mammalian tissues. Despite their high abundance and the associated extensive tissue damage, there is no acute inflammatory response to the bacteria (van der Werf *et al*., 2005). This pathology is attributed to mycolactone, a macrolide toxin, which seems to have cytotoxic, analgesic and immunosuppressive activities. The mycolactone is encoded by the pMUM001 plasmid which bears three PKSs genes (*mlsA1*, *mlsA2* and *mlsB*) (Stinear *et al*., 2004). On the other hand, the reason that *M. ulcerans* does not form granulomas and occupies predominantly extracelullar location in infected tissues might be found in the deletion of the *esx1* and *espA* genes in its genome, both *M. tuberculosis* gene products trigger granuloma formation (Stinear *et al*., 2007).

The insight gained from the comparative genomic analyses not only leads the development of new hypotheses regarding the biology of mycobacteria and their virulence, but has also been useful in the development of new strategies to combat mycobacterial infections.

1.3.4 The cell wall.

M. tuberculosis possesses an unusual cell wall that provides resistance to many antibiotics and the components of the killing mechanisms of macrophages. Historically, the inhibition of the biosynthesis of this structure has proven useful in TB chemotherapy, with the frontline agents ethambutol (arabinan) and isonazid (mycolic acids) and second-line agents like D-cycloserine (peptidoglycan) and ethionamide (mycolic acids) inhibiting the production of its various components. A more complete understanding of its biosynthesis and structure has been a major research focus in order to define potential targets for the development of new drugs (Boshoff *et al*., 2004; Scherman *et al*., 2003).

The cell envelope of *M. tuberculosis* consists of three main structural components. The plasma membrane, cell wall and the capsule (Rastogi *et al*., 1986). The plasma membrane appears to be a typical bacterial membrane and perhaps contributes very little towards the pathology of the bacilli. *M. tuberculosis* and *M. leprae* possess a complex cell wall and its extensive hydrophobic domains contribute to poor permeability which, in turn, leads to an inherent resistance against many drugs (Minnikin *et al*., 2002). The cell wall is composed of two layers, upper and lower, both linked through different classes of lipids (Brennan, 2003). A proposed model of the mycolyl-arabinogalactan-peptidoglycan (mAGP) cell wall of *M. tuberculosis* and its components is represented in the Figure 3 (Dmitriev *et al*., 2000; Dover *et al*., 2004).

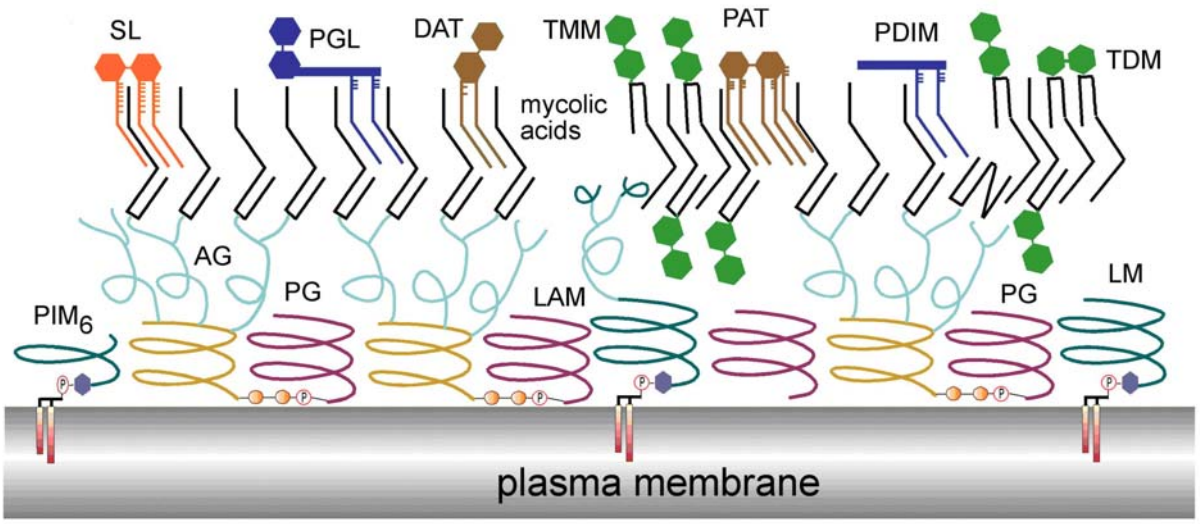


Figure 3. Structural components in the cell envelope of *M. tuberculosis*. Mycolic acids are sterified to arabinogalactan which in turn is connected to peptidoglycan (PG) to form the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex. Other complex free lipids (PGL, PDIM, DAT, PAT, SL) interact with mAG [Adapted from Dover *et al*. (2004) and Dmitriev *et al*. (2000)]. (AG) arabinogalactan, (DAT) diacyl trehalose, (LAM) lipoarabinomannan, (PAT) pentaacyl trehalose, (PG) peptidoglycan, (PGL) phenolic glycolipid, (PIM_6) phosphatidylinositol hexamannoside, (PDIM) phthiocerol dimycocerosate, (SL) sulphated tetra-acyl trehalose, (TMM) trehalose monomycolates, (TDM) trehalose dimycolates.

Beyond the membrane is peptidoglycan (PG) in covalent attachment to arabinogalactan (AG), which in turn is linked to the mycolic acids. All these molecules form the cell wall core, the mAGP complex (Brennan, 2003; Minnikin *et al*., 2002). The mycobacterial PG differs in two ways from that commonly found in other bacteria. Some or all of the muramic acid residues are N-glycolylated with glycolic acid (MurNGly) and the peptidic crosslinks can include bonds between two residues of diaminopimelic acid as well as between diaminopimelic acid and D-alanine (Lederer *et al*., 1975; Wietzerbin-Falszpan *et al*., 1970) in "stationary cultures".

The AG polysaccharide, unique to mycobacteria and other actinomycetes, is important for cell wall integrity and for anchoring the mycolic acids and to the PG layer. It is composed of D-arabinofuranose and D-galactofuranose residues. The arabinan chains are linked to the galactan chain toward the reducing end and are composed of linear α(1→5)-$_D$-arabinofuranosyl (*Araf*) residues with 3,5-branching. The non-reducing terminals of the arabinan are capped with a characteristic hexarabinose motif [t-β-$_D$-Araf-(1→2)-α-$_D$-Araf]2-3,5-α-$_D$-Araf-(1→5)-α-$_D$-Araf. The hexarabinose motifs are esterified at the C-5 positions, in clusters of four, with mycolic acids, although only two-thirds of these motifs are mycolated in AG (Brennan & Nikaido, 1995); together they produce the inner leaflet of a complex asymmetric lipid bilayer.

The mycolic acids are key lipid components in the cell wall of *M. tuberculosis*. These lipids are high molecular weight α-alkyl-β-hydroxy fatty acids, which are predominantly linked to AG, but some are found as extractable glycolipids such as trehalose monomycolates (TMM) and trehalose 6,6'-dimycolates (TDM). The main part of the branched chain is called "meromycolic acid" and the other part the α-branch (Minnikin *et al*., 2002). Mycolic acids from mycobacteria contain about 70-90 carbon atoms and 20-25 carbon atoms in the α-branch, while in *Corynebacterium diphtheriae*, corynomycolates have 30-36 C atoms in total (Dover *et al*., 2004a). The molecular organisation of mycolic acids plays an important role in nutrient uptake into the bacterium as well as conferring resistance to a wide range of antibacterial

drugs (Takayama *et al*., 2005). Moreover, the mycolic acids also play a role during cellular and innate immune responses during *M. tuberculosis* infection (Korf *et al*., 2005).

The outer leaflet of the bilayer and any gaps in the inner leaflet are most likely occupied by free lipids, some with longer fatty acids complementing the shorter α-chains of mycolic acids, and some with shorter fatty acids complementing the longer chains of mycolic acids (Dover *et al*., 2004). Other components of the cell envelope of *M. tuberculosis* are the lipoarabinomannan (LAM) and related lipomannan (LM); both lipoglycans are based on phosphatidylinositol mannoside (PIM) anchors, which may locate into the plasma membrane (Minnikin *et al*., 2002). It is speculated that LAM is linked to the interaction of the pathogen with the host, possibly leading to resistance mechanisms associated with macrophage killing (Chatterjee, 1997; Karakousis *et al*., 2004). On the other hand, a family of sulfated acyl trehaloses (SL) (Goren, 1970), and phenolic glycolipids (PGL) have been characterised from *M. tuberculosis* and *M. canettii*, respectively (Daffé *et al*., 1987; Watanabe *et al*., 1994). The sulfolipids of *M. tuberculosis* are five structurally related sulfatides, from which the sulfolipid-I (SL-I), the most abundant sulfatide, has been identified as a 2,3,6,6'-tetraacyl-a,a'-D-trehalose 2'-sulfate (Goren, 1970). The PGL consist of a lipid core formed by a long-chain β-diol, occurring naturally as diester of the polymethyl branched fatty acids (Malaga *et al*., 2008). Since both SLs and PGLs are probably located in the outer leaflet of the envelope, their location has prompted speculation that they may be virulence factors involved in host-pathogen interactions (Reed *et al*., 2004; Zhang *et al*., 1991).

1.3.5 Pathology of *M. tuberculosis*.

The success of *M. tuberculosis* is due to the adaptability that the bacterium has developed through evolution, i.e. resistance to drugs, multiplication in diverse organs of the host and evasion of the host's immune response. The pathogenesis of tuberculosis is complex and its manifestations diverse, which is reflected in a lifetime of dynamic interactions between mycobacterial virulence factors and the human immune system (Ellner, 1997; Hingley-Wilson *et al*., 2003; Russell, 2001). Based on Luries's studies (1964) in rabbits, four stages of

pulmonary TB have been distinguished. The first stage is related to inhalation of tubercle bacilli. TB is spread by airborne droplet nuclei, which are particles of 1-5 μm in diameter that contain one or two *M. tuberculosis* cells. Since, the particles are small they can remain in the air for several minutes after expectoration by people with pulmonary or laryngeal TB during coughing, sneezing, singing or talking, and when inhaled these nuclei can pass through to the alveoli of the lung (Riley *et al*., 1995).

Once the bacilli have been implanted in the alveoli, the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the primary or Ghon complex. The initial infection can initiate a cascade of events that results in either successful containment of the infection or progression to active disease (primary progressive TB) (Comstock *et al*., 1974; Sutherland, 1976). The destruction of mycobacteria depends on host phagocytes and virulence factors of the ingested mycobacteria. This stage lasts 3-8 weeks after *M. tuberculosis* cells have been inhaled (van Crevel *et al*., 2002).

In the second stage, which lasts around three months, tubercle bacilli are able to survive by intracellular destruction of macrophages. Mycobacterial multiplication leads to disruption of infected macrophages, ultimately attracting blood monocytes and other inflammatory cells to the lung. These monocytes differentiate into macrophages which again ingest but do not destroy the mycobacteria which are able to grow logarithmically. At this stage, dissemination of bacteria to many organs, including other parts of the lung, *via* the circulation might occur; at this time in some individuals, acute and fatal diseases can occur in the form of TB meningitis or miliary (disseminated) TB (Schluger & Rom, 1998; van Crevel *et al*., 2002). Also in the second stage, T-cell immunity develops. Antigenic-specific T lymphocytes arrive and proliferate within the early lesions or tubercles and then activate macrophages to kill the intracellular mycobacteria (Fenton & Vermeulen, 1996). Activated T-lymphocytes and macrophages form granulomas that limit *posteriori* replication of *M. tuberculosis*. Generally, the process of granuloma formation serves as a defence for containing pathogens and preventing their continued growth and dissemination (Soler & Bernaudin, 1993).

In the third stage, mycobacterial growth is inhibited by necrosis in primary lesions. As a result, infection may become stationary or dormant. Central solid necrosis in primary lesions inhibits extracellular growth of mycobacteria. Inflammation of the pleural surfaces can occur during the third stage with either hematogenous dissemination or the release of the bacteria into pleural space from subpleural concentrations of bacteria in the lung, but this can be retarded for two years (van Crevel *et al*., 2002). Severe chest pains also appear during this stage of infection.

The last stage or resolution of the primary complex, is where the disease is not continuous, and may last up to 3 years. At this stage, more slowly developing extrapulmonary lesions can appear. However, some individuals infected with TB do not exhibit progression of the disease. Nevertheless, 50% of those individuals infected by HIV/AIDS develop the reactivation of TB at some time in their lives (Garay, 1996).

Although, the most affected organ is the lung as well as other tissues, *M. tuberculosis* is able to replicate in many parts of human body. Disseminated TB infection is defined as involvement of many organs simultaneously and can occur as a result of primary progressive disease or reactivation of latent infection. TB can affect any bone or joint, but the spine is the most common structure invaded. Genitourinary TB is uncommon, but the most serious clinical manifestation of TB is involvement of the central nervous system which may cause meningitis (Brodie & Schluger, 2005).

1.4 Combating the bacillus: vaccines and the drug arsenal.

Historically, TB control started by simply isolating infected individuals in sanatoriums; however, this measure did not give effective control of the disease, since the bacillus is readily transmitted *via* the air. TB-associated mortality decreased due to the development and introduction of the BCG vaccine into Europe by the early 1920s, as a result of improved host-defence mechanisms, and especially by reduced transmission. Today, more than 3000 million people have received the BCG vaccine (Andersen & Doherty, 2005). In 1944, streptomycin

was discovered and subsequently introduced as the first antibiotic for the treatment of TB. Then, *p*-aminosalicylic acid (PAS) was introduced in 1946 and isoniazid in 1952. Further research lead to development of other drugs, such as cycloserine (1955), kanamycin (1957), ethambutol (1961), capreomycin (1961), rifampicin (1965), ethionamide (1966), ethambutol (1968) and pyrazinamide (1970) (Zhang, 2005).

1.4.1 Vaccines.

Active immunisation is one of the essential components of TB control, although vaccination is currently ineffective. The Bacilli Calmette-Guérin (BCG) live vaccine was successfully used to immunise billions of people since the last century. The BCG vaccine is an attenuated strain of *M. bovis* which was isolated by Albert Calmette and Camille Guérin (Andersen & Doherty, 2005; Fine, 1989). Despite this being the only vaccine, its efficacy has been in doubt because of its highly variable protective efficacy in controlled clinical trials (Brewer & Colditz, 1995). This variation might be due to the number of mutations and deletions that have occurred since its initial isolation in 1905 (Behr *et al*., 1999). Other reasons that can explain this variation are the previous exposure to environmental mycobacteria and genetic variation of the human population (Brewer & Colditz, 1995; Osborn, 1983; Springett & Sutherland, 1994). Due to this variation in BCG efficacy, a number of alternative living and non-living putative TB vaccines are currently being studied (Nor & Musa, 2004). In general, the efforts to make new vaccines are based in four strategies: (i) Rationally attenuated strains of *M. tuberculosis*, such as auxotrophic *M. tuberculosis* mutants (Collins, 2000; Guleria *et al*., 1996) or less virulent mycobacteria, such as *M. microti, M. habana, M. vaccae* or *M. smegmatis* that overproduce immunogenic antigens of *M. tuberculosis* (McMurray, 2000); (ii) The use of a recombinant BCG (Horwitz *et al*., 2000; Murray *et al*., 1996); (iii) Protein subunit vaccines (non-living vaccines). These vaccines represent good vaccine candidates because they seem safe and have no apparent side effects, which is very important when individuals are infected or exposed to HIV (Horwitz *et al*., 2000); (iv) The use of "Naked DNA vaccines" (plasmids containing genes encoding antigens of mycobacteria species) (Johansen *et al*., 2003).

1.4.2 Drug arsenal.

As outlined before, the 1950s and 60s represented a golden era of TB drug discovery. Despite the variety of drugs becoming available at that time, no biochemical characterisation was done and the activity of these antibiotics and their biosynthetic targets in *M. tuberculosis* were unknown. Complications soon arose after the introduction of streptomycin; patients improved their health, but they subsequently failed to fully recover and the bacteria isolated from these patients was resistant to streptomycin. This finding demonstrated that wild-strain populations of *M. tuberculosis* are not uniform in their susceptibility to drugs and to treat the disease it was necessary to use it in combination with another drug. Thus, streptomycin and *para*-Aminosalicylic acid (PAS) were used for the first time as a combination to combat TB (Hopewell, 2006).

The introduction of isoniazid (INH) in 1952 began a new era of TB chemotherapy, because it was highly affective, inexpensive and induced no significant side effects. Again, single-drug treatment with INH was not enough and *M tuberculosis* strains resistant to this drug were also observed. The combination of INH and PAS with or without streptomycin became the usual therapy for TB. Further investigations lead to the development of the core regimen of isoniazid and rifampicin (RIF), supplemented by pyrazinamide (PZA) and ethambutol (EMB); this is now recommended as standard treatment for both pulmonary and extrapulmonary TB, the so-called First Line anti-TB drugs. Since the introduction of the DOTS strategy in the early 1990s by the WHO, considerable progress has been made in controlling TB (Saltini, 2006).

However, even with the DOTS program, TB treatment is facing new challenges. Toward the end of the 1980s and the early 1990s, cases of patients, who did not respond to drug regimens were reported and this was attributed to the appearance of MDR-TB, defined as showing resistance to at least the first-line drugs INH and RIF (Crofton *et al*., 1997). The failure of these drugs requires the use of second line drugs which are more toxic, expensive and less effective. The scenario has worsened with the emergence of XDR-TB, which are

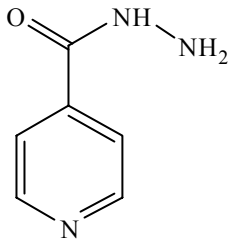
resistant to second line drugs (CDC, 2006). Thus, the emergence of MDR-TB and XDR-TB is threatening the public health and TB control worldwide.

Different molecular mechanisms have been presented to explain the causes of the development of resistance against existing drugs, such as impermeability of the highly hydrophobic cell envelope to many drugs, a well-developed-efflux system (Nguyen & Thompson, 2006), production of certain enzymes to inactivate drugs (β-lactamases, aminoglycoside acyltransferase) (Bush & Miller, 1998; Kwon *et al*., 1995) and, at the molecular level, acquisition of resistance due to mutational events in the chromosomes (Heym *et al*., 1994). On the other hand, others factors related to treatment of TB, i.e. patients not finishing their chemotherapy, administration of drugs without analysis of strains, have also increased the spread of *M. tuberculosis* strains resistant to front-line and second-line drugs (Snider & Castro, 1998).

Generally the targets against TB are based on the biosynthetic pathways which are involved in the production of macromolecules: proteins, nucleic acids or cell wall polymers (Rattan *et al*., 1998). The urgent need for new drugs has led researchers to combine genetic engineering techniques, genomics and proteomics tools, in a new approach to understand the biology of *M. tuberculosis* and to screen and develop new targets for drugs. Selecting targets for anti-TB therapy in this manner, is advantageous because one can avoid metabolic pathways occurring in mammalian cells.

First Line anti-TB drugs.

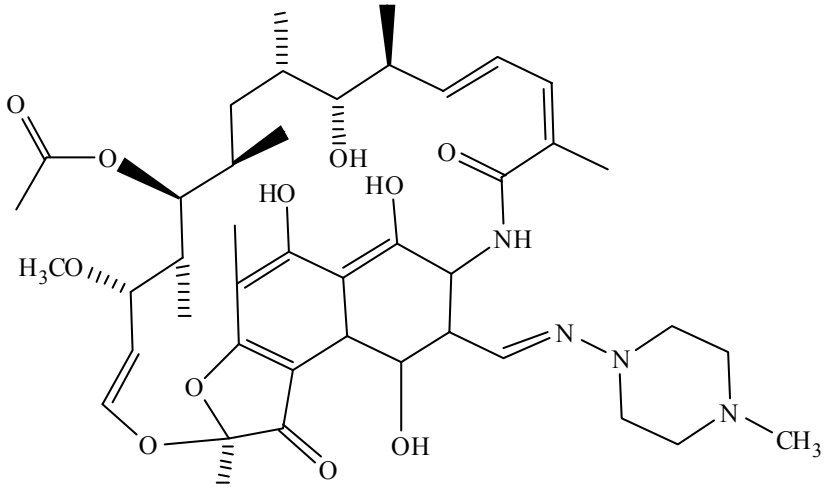
1.4.2.1 Isoniazid (INH).



INH acts best in slow-growing mycobacteria, having a minimum inhibitory concentration (MIC) of 0.01-0.2 μg/mL for *M. tuberculosis*, whereas *M. smegmatis* is 100-fold less sensitive (Zhang, 2005). Despite its effectiveness, the molecular mechanism of action was subject to debate until recently with several hypotheses suggested to explain its molecular activity. INH is a prodrug which requires activation by the *M. tuberculosis* catalase-peroxidase (KatG) (Zhang *et al*., 1992) to generate a range of reactive oxygen species and reactive organic radicals, which then attack multiple targets in the tubercle bacillus, probably DNA synthesis, carbohydrates and lipids (Broussy *et al*., 2003; Rozwarski *et al*., 1998).

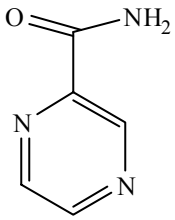
Subsequent biochemical studies demonstrated that KatG activates INH by converting the prodrug to a range of activated species, such as an isonicotinic acyl radical that can also acylate diverse compounds. Its interaction with NAD^+ results in the formation of an INH-NAD adduct, which inhibits the FASII enoyl ACP reductase (InhA), leading to the accumulation of long-chain fatty acids, inhibition of mycolic acid biosynthesis; this results in the loss of cellular integrity and ultimately cell death (Vilcheze & Jacobs, 2007). Mutations in *katG* and *inhA* leads to INH resistance (Rozwarski *et al*., 1998), but other mechanisms have also been postulated (Vilcheze & Jacobs, 2007).

1.4.2.2 Rifampicin (RIF).



RIF was introduced in 1972 as an anti-TB drug. The action of RIF in combination with PZA has allowed the shortening to TB treatment from 1 year to 9-6 months. The MIC for RIF is 0.05-0.5 μg/mL. The drug acts by interfering with the transcription *via* binding to DNA-dependent RNA polymerase. RNA polymerase has four different subunits (α, β, β', σ) encoded by *rpoA*, *rpoB*, *rpoC* and *rpoD* genes, respectively. RIF binds to the β subunit hindering transcription and therefore killing both active and slow growing bacilli (Jarvis & Lamb, 1998; Telenti *et al*., 1993; Williams *et al*., 1998). Mutation and short deletions in the *rpoB* gene confer resistance to *M. tuberculosis* (Herrera *et al*., 2003).

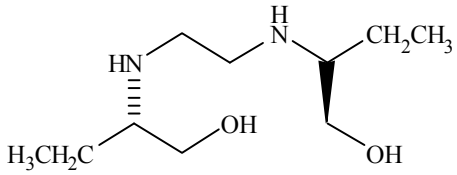
1.4.2.3 Pyrazinamide (PZA).



Although, PZA was synthesized in 1936, its activity as an anti-TB drug was not discovered until 1952 (Tarshis & Weed, 1953). PZA is an interesting and paradoxical drug since it has high *in vivo* but not *in vitro* activity. Structurally, PZA is an analogue of nicotinamide having

a MIC of 20-100 μg/mL (Zhang, 2005). PZA is a pro-drug, which is converted to its active form pyrazinoic acid (POA), by pyrazinamidase (PZase) encoded by *pncA*. The drug acts under certain conditions, being most active at acidic pH as well as in old cultures and at low oxygen levels or anaerobic conditions. The acidic pH facilitates the formation of uncharged protonated POA that permeates through the membrane and causes the accumulation of POA and reduces membrane potential in *M. tuberculosis*. As a consequence, protonated POA brings protons into the cell causing acidification and eventually inhibiting vital enzymes, although no specific target has been found (Zhang & Mitchison, 2003; Zhang *et al*., 2003). A derivative of PZA (5-chloro-pyrazinamide) inhibits fatty acid biosynthesis by targeting the fatty acid synthase I (FAS-I) (Boshoff *et al*., 2002), but PZA does not act on FAS-I. Work by Scorpio & Zhang (1996) and Scorpio *et al*. (1997) has shown that point mutations in the *pncA* gene confer PZA resistance to *M. tuberculosis*. The drug is only active against some *M. tuberculosis* complex organisms (*M. tuberculosis*, *M. microti* and *M. africanum*), but not against *M. bovis* due to the presence of point mutations in the *pncA* gene (Zhang & Mitchison, 2003).

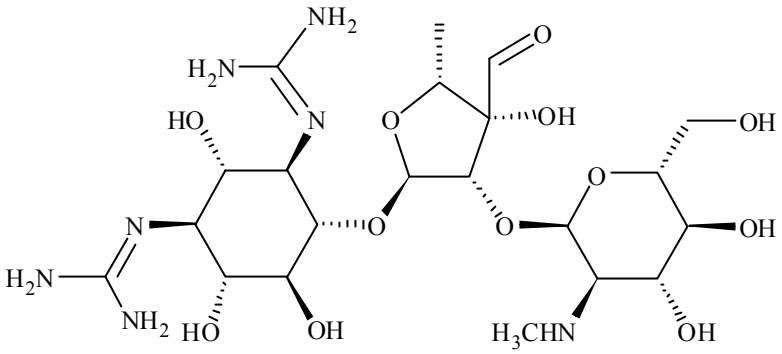
1.4.2.4 Ethambutol (EMB).



Ethambutol (S,S)-2,2'(ethylenediimino)di-1-butanol (EMB) was first identified as an anti-TB drug (Thomas *et al*., 1961). EMB is essential for TB treatment in combination with other drugs and its activity is restricted to mycobacterial strains. Early proposals for the mode of action of the drug ranged from trehalose, mycolate and glucose metabolism to spermidine biosynthesis. However, more recent studies have shown that the primary site of action is arabinan biosynthesis both in arabinogalactan (AG) and lipoarabinomannan (LAM) (Deng *et al*., 1995; Mikusova *et al*., 1995; Telenti *et al*., 1997). Since, AG and LAM are structurally essential for the mycobacterial cell wall, the destruction of these structures renders instability

of the mAGP complex and thus may lead to increased permeability of the cell wall. Belanger *et al*. (1996) proposed that the target for EMB is the arabinosyl transferase encoded by *emb* operon both in *M. avium* and *M. tuberculosis*. Supporting this hypothesis, various studies have identified five mutations in codon 306 in the *M. tuberculosis embB* gene (Ramaswamy *et al*., 2000; Sreevatsan *et al*., 1997b). Furthermore, two new arabinofuranosyl transferases, AftA and AftB, have been recently identified both in *M. tuberculosis* and *C. glutamicum* which are insensitive to EMB, suggesting that the drug acts only on the *emb* gene products (Alderwick *et al*., 2006; Seidel *et al*., 2007). Mutations in the *embCAB* operon are found in approximately 65% of clinical isolates of *M. tuberculosis* resistant to EMB (Sreevatsan *et al*., 1997b).

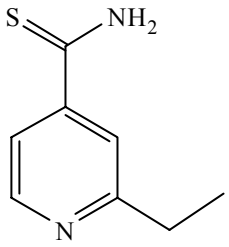
1.4.2.5 Streptomycin.



Streptomycin is an alternative first line anti-TB drug. The effect of streptomycin has been demonstrated to take place at the ribosomal level (Carter *et al*., 2000; Davies *et al*., 1965). It interferes with protein synthesis by inhibiting initiation of mRNA translation, facilitating misreading of the genetic code and damage to the cell membrane (Anand & Davis, 1960). The drug interferes specifically with the small 30S subunit of the ribosome interacting with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsL*), inducing ribosomal changes, which cause misreading of the mRNA and therefore inhibition of protein biosynthesis (Garvin *et al*., 1974). Point mutations in the *rrs* and *rpsL* genes are associated with *M. tuberculosis* resistant to streptomycin (Cooksey *et al*., 1996; Finken *et al*., 1993).

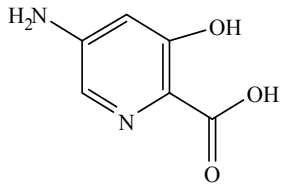
Second Line drugs.

1.4.2.6 Ethionamide (ETH).



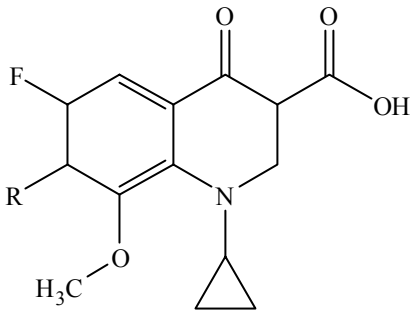
ETH is also a pro-drug which is activated by the monooxygenase enzyme EthA (also called EtaA) (Baulard *et al*., 2000; DeBarber *et al*., 2000; Vannelli *et al*., 2002). The drug is a structural and mechanistic an analogue of INH and therefore its target is proposed to be InhA causing inhibition of mycolic acids biosynthesis which consequently leads to disruption of the *M. tuberculosis* cell wall (Banerjee *et al*., 1994). The EthA was shown to be important in the activation and therefore efficacy of thiocarbamides anti-TB drugs, such as ethionamide (ETH), thiacetazone (TAZ) and isoaxyl (ISO) (Baulard *et al*., 2000; Dover *et al*., 2007; Vannelli *et al*., 2002). It has been shown that EthA mediates ETH and TAZ activation by sulfoxidation (Qian & Ortiz de Montellano, 2006). Mutations in the promoter of the *inhA* as well as in the *ethA* genes confer resistance to *M. tuberculosis* to ETH (Morlock *et al*., 2003; Vannelli *et al*., 2002). The expression of the *ethA* gene is under the control of the *ethR* gene encoding a repressor which is also implicated in resistance to ETH due to mutations in the DNA binding region of the protein (Dover *et al*., 2004b).

1.4.2.7 *para*-Aminosalicylic acid (PAS).



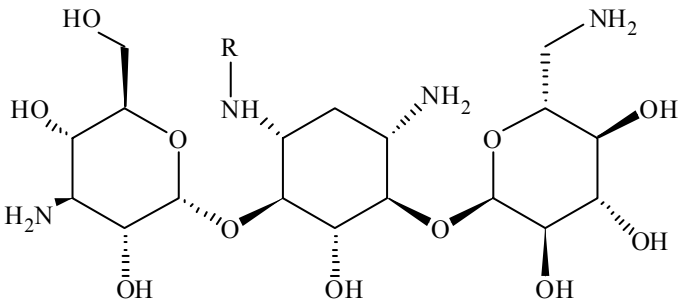
The anti-mycobacterial activity of PAS was first reported by Lehmann (1946). It has no effect on other bacteria, but it is highly effective against *M. tuberculosis*. The mode of action of PAS is still unclear, but it has been suggested to inhibit mycobactin biosynthesis by interfering with salicylate-dependent biosynthesis of these iron chelators (Brown & Ratledge, 1975a; Ratledge, 2004; Ratledge & Dover, 2000). However, another metabolic pathway has been suggested to be the target of PAS. Rengarajan *et al*. (2004) proposed that PAS also targets the thymidylate synthase (*thyA*), an enzyme involved in the folate biosynthesis pathway.

1.4.2.8 Fluoroquinolones.



The fluoroquinolones are derivatives of nalixidic acid which inhibit DNA synthesis by targeting bacterial topoisomerases II and IV (Drlica, 1999; Zhao *et al*., 1997). *M. tuberculosis* lacks topoisomerase IV but possesses topoisomerase II or DNA gyrase. The primary target of FQs in *Staphylococcus aureus* is topoisomerase IV, whereas in *E. coli* and *M. tuberculosis* it is DNA gyrase (Ginsburg *et al*., 2003). The mode of action of the fluoroquinolones is not fully elucidated, however, strand-breakage, SOS-mediated autolysis and blockade of replication by the gyrase-fluoroquinolone complex have been proposed (Ginsburg *et al*., 2003). Although there is structural diversity in fluoroquinolones, only two are used as second-line drugs in MDR-TB treatment, ciprofloxacin (CIP) and ofloxacin (OFL) (Ginsburg *et al*., 2003). The most common mutation in fluoroquinolone-resistant *M. tuberculosis* isolates involves a substitution at codon 94 of the *M. tuberculosis gyrA* gene (Takiff *et al*., 1994).

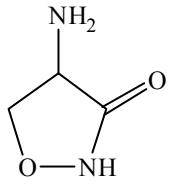
1.4.2.9 Aminoglycosides.



R=H= Kanamycin
R=$COCH(OH)CH_2CH_2NH_2$= Amikacin

Kanamycin (KAN) and amikacin (AMI) are aminoglycosides which inhibit protein synthesis (Magnet & Blanchard, 2005). Both drugs are commonly used for the treatment of MDR-TB. Aminoglycosides bind to bacterial ribosomes and perturb the elongation of the peptide chain in bacteria. Mutations in the *rrs* gene encoding for the 16S rRNA are associated with resistance to KAN and AMI (Alangaden *et al*., 1998; Susuki *et al*., 1998; Taniguchi *et al*., 1997). Cross-resistance has been observed between KAN and either AMI, capreomycin or viomycin, but a recent study found a little cross-resistance between KAN and AMI (Krüüner *et al*., 2003).

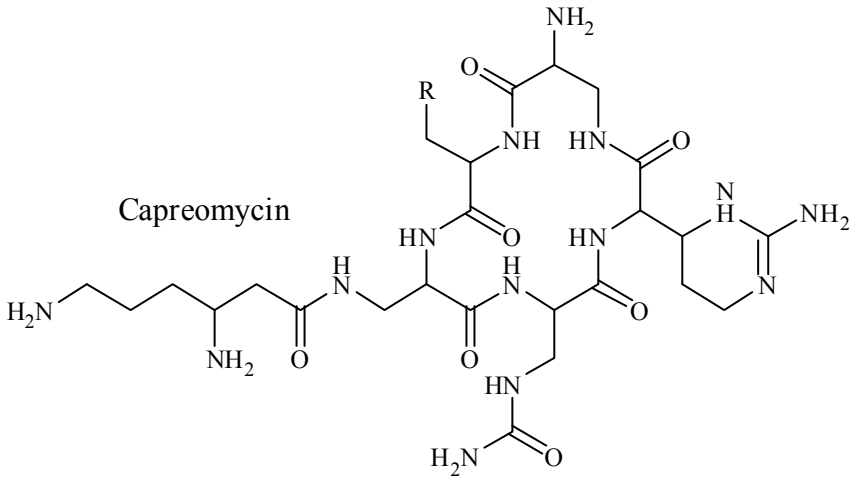
1.4.2.10 D-cycloserine.



D-cycloserine is a cyclic analogue of D-alanine which is one of the central molecules in the cross-linking step of peptidoglycan assembly (David *et al*., 1969). D-cycloserine is an effective antimycobacterial agent but is rarely prescribed and used in TB chemotherapy due to its severe secondary effects (Helmy, 1970). D-cycloserine inhibits cell wall synthesis by competing with D-alanine for the enzymes D-alanyl-D-alanine synthetase (Ddl) and D-alanine

racemase (Alr). The D-alanine-racemase gene has been cloned from *M. smegmatis* and its expression from a multicopy DNA vector in *M. smegmatis* and *M. bovis* BCG results in resistance to DCS (Cáceres *et al*., 1997), but the genetic basic for D-cycloserine resistance in *M. tuberculosis* has not yet been found.

1.4.2.11 Polypeptides.



Viomycin (VIO) and capreomycin (CAP) are both basic peptide antibiotics that inhibit protein synthesis. Both drugs are structurally related, their target has been linked to modification of ribosomal structures. VIO has been shown to bind both the 30S and the 50S ribosome subunits and to affect the dissociation of the 70S ribosome in *M. smegmatis* (Yamada, 1976; Yamada *et al*., 1978) and to inhibit ribosomal translocation by arresting peptidyl-tRNA in the ribosomal acceptor site (Modolell & Vazquez, 1977). CAP also interferes with ribosomal function yet no elucidated. The *tlyA* gene of *M. tuberculosis* encodes a putative methyltransferase for rRNA methylation which in turn may be required for CAP activity. This suggests that ribosomes lacking methylation would be CAP resistant (Maus *et al*., 2005).

1.5 Iron as an element.

Iron (Fe) is the second most abundant metal, after aluminium (Al), and the fourth most abundant element in the earth's crust. The core of the earth is believed to consist mainly of iron and nickel (Ni) (Cotton *et al*., 1995). Approximately one-third of the earth's mass is estimated to be iron and its concentration is relatively high in most crustal rocks (Theil & Raymond, 1994). In the oceans, the concentration of iron is low but increases with depth, since iron exists as suspended particulate matter rather than as soluble species (Fox, 1984, 1988). Iron belongs to the transition-metals. The elements of this group share the characteristic that their *d* orbitals are unfilled and, therefore, they may possess several stable oxidation states and most of the elements can form complexes (Cotton *et al*., 1995). Thus, iron is able to undergo changes in oxidation states, involving electron transfer, which allow it easy access to two oxidation states, Iron II (Fe^{2+}, ferrous iron) and Iron III (Fe^{3+}, ferric iron), participating in many reduction-oxidation (redox) processes (Fontecave & Pierre, 1991).

For iron, there are four features in its chemistry and biochemistry that have shaped the evolution and selection of iron uptake mechanisms. Firstly, the redox chemistry of iron is easily accomplished, due to the one-electron transfer between Fe^{2+} and Fe^{3+}. Common reducing agents will usually convert ferric iron to the ferrous form, while molecular dioxygen will promote the reverse reaction (Aisen *et al*., 2001; Cotton *et al*., 1995; Kosman, 2003). The reduction potential of the ferric/ferrous couple, $E^{\circ\prime}$ at neutral pH is +0.77 V (Loach, 1968). E° means standard reduction potential and measures, in volts (V), the velocity of electron flow from the donor to the acceptor (Nelson & Cox, 2004). This value indicates that this couple is reducing in comparison to dioxygen reduction to water (+0.82 V). Thus, under air iron is stable as Fe^{3+}, but Fe^{2+} will quickly auto-oxidize. For many biological iron-complexes whether in proteins or simpler molecules, redox chemistry is easy and reversible.

Secondly, ferric iron is insoluble in water at neutral pH. In aqueous solution, $Fe^{3+}(H_2O)_n$, hydrolyses, so, it loses protons to form a ferric hydroxide species that polymerises into an amorphous gel. Fe^{3+} is a strong Lewis acid and this relatively positive charge promotes

proton loss from the coordinated water molecules in the following hydrolytic deprotonations (Wilkins, 1991), all reactions take place when the pH is above 2:

$$[Fe(H_2O)_6]^{3+} \rightarrow [Fe(H_2O)_5(OH^-)]^{2+} + H_3O^+$$
$$[Fe(H_2O)_5(OH^-)]^{2+} \rightarrow [Fe(H_2O)_4(OH^-)_2]^{+} + H_3O^+$$
$$[Fe(H_2O)_4(OH^-)_2]^{+} \rightarrow [Fe(H_2O)_3(OH^-)_3] + H_3O^+$$

The latter species is extremely insoluble (K_{SP} 10^{-38} M), so that the maximum concentration of Fe^{+3} in aqueous solutions is only about 10^{-17} M (Aisen *et al*., 2001; Sylva, 1972). However, a recent study shows that the major form of iron at pH 7 is not $Fe(OH)_3$ but is instead $Fe(OH)_2$, which has a solubility of approximately 1.4 X 10^{-9} M (Chipperfield & Ratledge, 2000). On the other hand, Fe^{2+} is a weak Lewis acid because the nuclear charge is suppressed by an additional electron. The water molecules bound to Fe^{2+} do not ionise readily and therefore the aqua ferrous ion is relatively stable to hydrolysis and soluble at neutral pH. Thirdly, Fe^{3+} binds ligands more tightly, due in part to the charge difference, which makes ferric iron complexes exchange inert, that is, any ligand attached to Fe^{3+} in a complex can be replaced but with difficulty. In Fe^{2+}, H_2O exchange is ~ 10^4-fold faster than Fe^{3+} in otherwise similar ligand environments. Even, if the hydrolysis of $Fe^{3+}(H_2O)_n$ is suppressed, at acidic pH values, Fe^{3+} is still relatively inaccessible to biological ligands (Wilkins, 1991).

Fourthly, there is high biological competition between different ligands to bind iron (Kosman, 2003). Hydroxide complexes of iron readily polymerise by dehydration to form polynuclear complexes with iron atoms linked by oxo or hydroxo bridges as in the general reaction:

$$nFe^{+3}(OH)_3 \xrightarrow{-\ nH_2O} -(FeOOH)_n-$$

Iron polymers in biological systems may range from two iron atoms, as in the proteins with binuclear iron centres (e.g. ribonucleotide reductase), to three dimensional arrays of more than 4000 iron atoms as in the mineralized core of ferritins (Aisen *et al*., 2001).

1.5.1 Iron and its biological importance.

Both prokaryotic and eukaryotic cells acquire a variety of transition metals and employ them in structurally constrained binding sites, where they carry out regulatory or catalytic roles (Finney & O'Halloran, 2003). Many metals are essential for all living systems, since their deficiencies lead to disease, metabolic anomalies, or perturbations in their development (Fraústo da Silva & Williams, 2001). An element that is being used by any biological system must be abundant in the environment and must be in an easily extractable form, that is, it must be "biologically available" from the environment (Fraústo da Silva & Williams, 2001). Despite its abundance, iron is not readily available due to its insolubility at physiological pH. In the presence of oxygen, Fe^{2+} is rapidly oxidised to Fe^{3+}, which precipitates as polymeric oxyhydroxide. The resulting concentration of free Fe^{3+} in the environment is less than 10^{-17} M, a concentration too low to allow growth by aerobic microorganisms (Papanikolaou & Pantopoulos, 2005).

Free iron is toxic and it its redox capabilities that underlie its toxicity. The one-electron reduction of dioxygen by Fe^{2+}, acting as catalyst, results in superoxide formation. This in turn leads to a Haber-Weiss-Fenton sequence generating hydroxyl radical ($OH^{\bullet}$) and superoxide ($O_2^{-\bullet}$), collectively known as "reactive oxygen intermediates" (ROIs) (Table 1) (Halliwell & Gutteridge, 1990; Papanikolaou & Pantopoulos, 2005).

ROIs are produced in diverse compartments of the cell, in mitochondria during aerobic respiration by incomplete reduction of dioxygen, peroxisomes and the endoplasmic reticulum of the cytoplasm during enzymatic reactions (Papanikolaou & Pantopoulos, 2005), in the membrane-bound NADPH oxidase complex of phagocytic neutrophils and macrophages (Hampton *et al*., 1998). Redox activity of iron not only catalyzes the generation of hydroxyl

radicals, but also of organic reactive species, such as peroxyl (ROO^-), alkoxyl (RO^-), thioyl (RS), or thioyl-peroxyl ($RSOO^-$) radicals (Papanikolaou & Pantopoulos, 2005).

Table 1. **The toxicity of iron.** Iron catalyses the generation of hydroxyl radicals *via* the Fenton reaction and the net Haber-Weiss reaction.

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet}$
$2O_2^{-\bullet} + 2H \rightarrow H_2O_2^{+} + O_2$
$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$ (Fenton reaction)
Net reaction:
$H_2O_2 + O_2^{-\bullet} \xrightarrow{Fe} OH^- + OH^{\bullet} + O_2$ (Haber-Weiss reaction)

These hydroxyl and superoxide radicals are highly activated, therefore their biological lifetime is short. They will react with whatever biological molecule is in their vicinity (Aisen *et al*., 2001). The hydroxyl radical, perhaps the most powerful oxidant encountered in biological systems, will attack proteins, carbohydrates, nucleic acids and lipids initiating chain-propagating lipid peroxidation (Aisen *et al*., 2001; Valentine *et al*., 1998), while superoxide quickly reacts with [4Fe-4S] cluster-containing proteins (Fridovich, 1998; Liochev & Fridovich, 1994). However, aerobic organisms have evolved three enzymes, catalase, superoxide dismutase and glutathione peroxidase; the first two enzymes scavenge hydrogen peroxide (H_2O_2), whereas the latter superoxide anion, respectively (Aisen *et al*., 2001). The role of these enzymes, as antioxidants, is to protect biological systems against hydroxyl and oxygen radicals, being the first line of defence against ROIs (De Freitas & Meneghini, 2001). Despite their presence, free superoxide anion and hydrogen peroxide can still be detected during normal metabolism in most biological systems (Aisen *et al*., 2001). Due to this toxicity induced by iron, it must be bound in diverse molecules or ions, which are called ligands to avoid ROI production (Housecroft & Constable, 2002). As a consequence, cells and organisms have developed several mechanisms to reduce toxicity of iron, which include a

highly regulated transmembrane iron transport, intracellular chelators and enzymes that destroy ROI (Fridovich, 1998). Thus, the control of iron uptake and storage are primordial processes to avoid toxicity of iron (De Freitas & Meneghini, 2001).

At the same time, all organisms have to solve the problem of iron availability since it is strongly limited because of its dominant form (ferric iron) and the pervasive competition for this limited and essential nutrient (Kosman, 2003). Most of the organisms share the evolutionary strategy of how to scavenge iron. The reduction of Fe^{3+} for the purpose of intracellular incorporation into protein is called assimilatory iron reduction. The reduction of the complexed Fe^{3+} results in a weak Fe^{2+}-chelate complex allowing for dissociation and release of Fe^{2+} for transport or cellular incorporation into haem and non-haem Fe-containing proteins (Schröder *et al*., 2003). In contrast, dissimilatory iron reduction serves the generation of energy to fuel cell propagation. The reduction of Fe^{3+} is coupled to the generation of the proton motive force across the cytoplasmic membrane, which is then used to generate ATP by a membrane-bound ATP synthase, to fuel active transport of nutrients, or to drive motility (Schröder *et al*., 2003).

Once the organisms have solubilised Fe^{2+}, they must hold it tightly due to extreme competition between different ligands (Kosman, 2003). Successful organisms elaborate a high affinity system, one that binds iron with an association constant that can compete with the kinetic and equilibrium processes that lead to the precipitation of Fe^{3+}, with the auto-oxidation of Fe^{2+} and with the sequestration of iron by neighbours. Thus, some organisms have developed two types of system, (i) which is based on extracellular chelation of ferric iron, and (ii) based on ferric iron reduction and subsequent "free" iron permeation (assimilatory process) (Kosman, 2003). According to the prevailing iron concentration there are high- and low-affinity transport processes carried out by all organisms; both are controlled inversely by metal availability. The high affinity system is expressed when the iron concentration is low in the environment or media, while the low-affinity is utilised under iron sufficiency (Kosman, 2003).

The requirement for iron is almost universal for prokaryotes and eukaryotes, demonstrating its importance in cellular metabolism. All plants, animals, fungi and most bacteria use iron. The processes and reactions in which iron participates are crucial to the survival of terrestrial organisms, and include ribonucleotide reduction (DNA synthesis), energy production (respiration), energy conversion (photosynthesis), nitrogen reduction, oxygen transport (respiration, muscle contraction), and oxygenation (e.g. steroid synthesis solubilisation and detoxification of aromatic compounds) (Andrews & Schmidt, 2007; Braun, 2003). The molecules listed in Table 2 use iron as a cofactor or molecules which serve for iron uptake and storage.

Table 2. **The importance of iron in organisms.** Diverse molecules are produced by organisms to fulfil iron requirements as well as to avoid its toxicity. [1]Ferritin is among the most ancient proteins of iron metabolism. It is conserved from bacteria to plants to man.[2]Three classes of transferrin are recognised: serum transferrin, lactoferrin (Lf) or lactoferritin, and ovotransferrin, sometimes named conalbumin. [3]Ferric iron reductase is found in many prokaryotes and eukaryotes that live in aerobic and neutral environments. [4]The number of iron atoms being bound varies among organisms (Pierre *et al*., 2002; Schröder *et al*., 2003).

Ligand	Function of ligand	Iron form bound Ferric=Fe^{3+} Ferrous=Fe^{2+}	Iron atoms bound to ligand	Ligand producer
[1]Ferritin	Iron storage	Fe^{3+}	[4]2000-4500	Bacteria, plants, mammalian cells
[2]Transferrin (Tf)	Transfer of Fe^{3+}	Fe^{3+}	2	Human cells
[3]Ferric reductase	Reduction of Fe^{3+}	Fe^{3+}	2	Prokaryotic and Eukaryotic cells
Cytochromes	Electron transfer	Fe^{3+}, Fe^{2+}	6	Eukaryotic cells
Haemoglobin	Transport of oxygen	Fe^{2+}	5	Erythrocytes

Iron homeostasis is an important process in any cell, either prokaryotic or eukaryotic, and must be balanced to supply enough iron for cell metabolism and to avoid excessive toxic levels. Thus, the regulation of iron transportation is an important matter of iron homeostasis. In all organisms, iron homeostasis is achieved, in part, through the control of its transport across various membranes. These transport activities include iron uptake from the environment, distribution, and intracellular compartmentalisation, as will be outlined below for some groups of mammalian cells and bacteria (Andrews & Schmidt, 2007). Although iron has a vital role for almost all living organisms, *Lactobacillus* and some strains of *Bacillus* avoid the use of iron. Instead, they use manganese as a principal cofactor during their metabolism (Archibald, 1983; Archibald & Duong, 1984).

1.5.2 Iron in living organisms.

Although, the principal aim of this project is to investigate the potential for inhibiting the enzymes that produce mycobacterial siderophores as new targets for drugs, it is important to understand why iron is used in all organisms. Though many organisms use iron, this section will outline how mammals and bacteria have evolved systems to acquire, store and use iron for their metabolism.

1.5.3 Iron in mammals.

Most studies of mammalian iron homeostasis have been conducted in the mouse because it represents a good genetic model. Firstly, handlers in laboratories are able to recognise physical characteristics when mice are anaemic. Anaemic animals are pale, in comparison with healthy ones. Secondly, there are several mutant mouse strains with defects in iron metabolism. These strains have provided substantial information about defects in iron metabolism which have been useful in characterising iron-deficiency anaemia (Andrews, 2000). Despite most studies being carried out in mice, some investigators have employed human cells lines.

In humans, adults typically have a total body iron about 3-5 g which is maintained by a balancing adsorption and loss of iron (Conrad & Umbreit, 2002). The amount of iron that enters and leaves the body is between 0.5-2 mg *per* day. Humans conserve iron more efficiently than other animals. There is no regulated pathway for iron excretion, and normally iron leaves the body only through menstruation and sloughing of epithelial cells from the skin and from the mucosa of the gastrointestinal, biliary and urinary tracts (Andrews, 1999; 2000).

Iron absorption is normally carried out by mature enterocytes in the duodenum and upper part of the jejunum. In iron absorption, important proteins are expressed in the cell membranes for the optimal transportation of iron through the cell and into the blood (Morgan & Oates, 2002). Dietary iron compounds are divided in two types, haem and non haem (Bothwell *et al*., 1979) with most dietary iron present as Fe^{3+} salts (Riedel *et al*., 1995). Haem probably enters mucosal cells by endocytosis, as the intact iron-protoporphyrin complex (Conrad & Umbreit, 2002), and within the cells, iron is released by the action of the microsomal enzyme, haem oxygenase (Raffin *et al*., 1974). Normally non haem ferric iron is reduced to the ferrous form by protons from stomach acid (low pH) and by the duodenal ferric reductase (Dcytb) that is present on the surface of the intestinal wall (Riedel *et al*., 1995). Then, ferrous iron is transported in across the apical membrane into the absorptive enterocyte via the Divalent metal transporter 1 (DMT1). This protein is also known as Natural resistance associated macrophage protein 2 (Nramp2) or Divalent cation transporter I (DCT1) (Conrad & Umbreit, 2002), and is not specific to iron since it can transport other divalent metals such as manganese, cobalt, copper, zinc and cadmium (Fleming *et al*., 1997; Gunshin *et al*., 1997).

Inside the enterocytes, ferrous iron has two possible fates, either it may be stored in ferritin or transferred across the basolateral membrane to reach the plasma. Basolateral iron transport is mediated by ferroportin 1 (Donovan *et al*., 2000), named also as Iron-regulated transporter (IREG1) (McKie *et al*., 2000) or Metal transporter protein (MTP1) (Abboud & Haile, 2000), which probably works together with the membrane ferroxidase hephaestin, facilitating basolateral iron export (Vulpe *et al*., 1999).

Iron in its ferrous form is stored in ferritin, an important storage protein. Ferritin can store approximately 4,500 iron atoms. First, it acts enzymatically, converting Fe(II) to Fe(III) as iron is internalised and sequestered in the ferritin mineral core. Second, its critical role in cellular and organismal iron homeostasis is intimately linked to its primary and best-studied function iron sequestration, avoiding the generation of free radicals as discussed above (Torti & Torti, 2002). If it is not stored, the ferrous iron absorbed is then transferred into transferrin (Tf), an abundant plasma protein, which binds ferric iron with high-affinity, for instance human serum transferrin (K_d 10^{-22} M) (Aisen *et al*., 1978), Transferrin solubilises and attenuates iron reactivity and delivers it to the cells (Aisen, 2004). The transferrins are metal-binding glycoproteins that function in the transport of iron to cells through a cycle denominated the transferrin cycle and act as bacteriostatic agents in a variety of biological fluids as ferritin (Aisen *et al*., 2001). Transferrin is also important since it determines the distribution of iron through a cycle denominated the transferrin cycle (Aisen, 2004). There are two types of transferrins in mammalian cells. (i) Serum Tf (or serotransferrin) and (ii) lactoferrin (Lf) serve to transport iron in blood and mucosal secretions (including milk) (Aisen & Leibman, 1972).

During the transferrin cycle, holo-transferrin binds to transferrin receptors on the cell surface of erythroid precursors, forming complexes which localise the clathrin-coated pits (Aisen *et al*., 2001). Clathrin-coated pits (CCPs) are specialised plasma membrane proteins responsible for the selective recruitment of various lipids, proteins and other molecules which are transported from the plasma membrane (cell surface) to endosomes (Lodish *et al*., 2003). The CCPs invaginate to initiate endocytosis resulting in the formation of specialised endosomes. A proton pump decreases the pH within the endosomes, changing the conformation of Tf that results in the release of iron from transferrin. DMT1 removes iron across the endosomal membrane, to enter the cytoplasm (Andrews, 2000). *Apo*-transferrin and its receptors are recycled for additional cycles. Iron is distributed into different cell types, such as hepatocytes, lung cells, macrophages, neutrophils *via* this the transferrin cycle (Andrews, 2000; Turi *et al*., 2004).

In erythroid cells, most iron is transported into mitochondria, where it is incorporated into protoporphyrin to make haemoglobin. The majority of body iron (65-75%) is utilised within haemoglobin (Andrews, 1999). Each erythrocyte contains a billion atoms of iron. After red cells reach their lifespan, they are phagocytosed by reticuloendothelial macrophages, where haemoglobin is degraded and iron is released from haem. The enzyme haem oxygenase may participate in this process. Thus, iron is transported through the cell. The complete mechanism of macrophage iron export is still unknown all but ferroportin 1 and hephaestin are probably involved (Andrews, 2000).

Other cells that function in iron storage are the hepatocytes. They have mechanisms for acquiring both transferrin-bound iron, mediated by their own transferrin cycle, and free iron that circulates when transferrin-binding sites are fully occupied. Hepatocytes can store iron in ferritin. When iron is needed for other cells, hepatocytes release it to transferrin, and again ferroportin 1 and hephaestin may be involved. Both hepatocytes and reticuloendothelial macrophages retain 20-30% of body iron. The remaining body iron is localised in myoglobin cytochromes, and iron containing enzymes (Andrews, 1999; Andrews, 2000).

Mammals do not have any physiological pathway for iron excretion and the body iron homeostasis is maintained at the level of absorption. It is mainly dependent on regulatory feedback between body needs and intestinal iron absorption (Fleming & Bacon, 2005). Three general mechanisms have been found to influence the rate of iron homeostasis. Firstly, iron homeostasis can be modulated by the amount of iron recently consumed in the diet a mechanism known as dietary regulator. Secondly, a regulatory mechanism senses iron levels but responds to total body iron. This mechanism has been termed the stores regulator and it is capable of changing the amount of iron absorbed to a limited extent. Thirdly, erythropoiesis can modulate iron absorption, although a precise mechanism has not been found (Conrad & Umbreit, 2002; Fleming & Bacon, 2005). Iron not regulated leads to its deficiency and overload, causing anaemia and hereditary haemochromatosis, respectively (Aisen *et al*., 2001; Aisen *et al*., 1999).

1.5.4 Iron in bacteria.

As in other organisms, bacteria have developed strategies to acquire iron. *Escherichia coli* has been the model to study iron metabolism. However, with the deciphering of genomes of others species, there is an increased understanding of iron metabolism in other bacteria. Iron concentrations of 10^{-6} to 10^{-7} M are required by most microorganisms for various metabolic processes that are crucial for microbial replication, including electron transport, DNA synthesis and defence against toxic ROI (Weinberg, 1978). In *E. coli*, the levels of iron range 10^5 to 10^6 atoms per cell (Andrews *et al*., 2003).

In general, bacteria have adopted several strategies for the uptake and metabolism of iron. In most bacteria, there is a high-affinity iron transport, based on siderophores, which enables iron to be acquired from the environment. Another mechanism involves the direct binding of transferrin, lactoferrin, or haem-containing proteins to substrate-specific receptors in the outer membrane of Gram-negative bacteria. Once ferric iron is acquired from the environment, it must be reduced to the ferrous form. However, some bacteria can lower the external pH to make ferric iron available. In either strategy to acquire iron, it must be stored in specialised proteins and released when external supplies are limited, and also to avoid the toxicity of iron by producing resistance systems (Andrews *et al*., 2003).

1.5.4.1 Siderophores, iron and virulence.

During the course of infection, a pathogen might yield diverse factors, i.e. production of toxins and adhesion proteins, to establish an infection and their production might depend upon environmental conditions. Factors that might be expected to influence bacterial multiplication *in vivo* include temperature, pH, osmolarity, oxygen tension, resistance mechanisms of the host, and the availability of nutrients (Kuhn *et al*., 2002).

On the other hand, hosts have developed mechanisms to reduce or avoid invasion by pathogens, such as physical barriers, bacterial inactivation, producing antibiotics and withdrawal of certain nutrients (Sritharan, 2006). The withdrawal of nutrients, such as iron,

has been achieved by producing the glycoprotein transferrins (any of their classes) as transient storage and ferritin as final storage molecule of iron (Aisen *et al*., 2001; 1978). Thus, these molecules hold iron tightly and ensure that no free iron is available for pathogens. A general response to infection is to decrease the release of iron from tissues to circulation, with the result that iron is not available in extracellular fluid and serum (Wooldridge & Williams, 1993).

Along with transferrin and ferritin, the hosts have produced another mechanism to reduce the amount of iron by altering the status of their internal iron. Specialised cells, the macrophages, the principal cells that phagocytose invading bacteria, yield proteins that alter their own iron status generally by withholding iron (mediated by lactoferritin, ferritin and transferrin), regulating the iron pool *via* the transferrin receptors or transporting iron from endosome by specialised molecules (Agranoff & Krishna, 1998; Buschman *et al*., 1997; Cellier *et al*., 1996; Wardrop & Richardson, 1999). The importance of withholding iron in host cells is a crucial mechanism to reduce infection by pathogens. Three different compounds, desferrioxamine (DFO), N,N'-bis(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid (HBED) and a 1-amino-3-(2- bipyridyl)isoquinoline derivative (VUF8514), were tested as iron withholding molecules by adding these compounds either *in vivo* (macrophages infected with *M. avium*) or *in vitro* (media containing the same pathogen). It was found that these chelators inhibited the growth of *M. avium* as might occur with both transferrin and ferritin (Gomes *et al*., 1999). The molecules involved in iron transport and absorption are the natural resistance-associated macrophage proteins (Nramp). There are two, Nramp1 and Nramp2, but probably the former is involved during infection (Wardrop & Richardson, 1999).

The phenomenon of limiting iron availability to an invading pathogen has been considered as nutritional immunity, although there is no involvement of the immune system by the host in this process. The host limits the availability of free iron by decreasing the absorption from the intestine as well as by increasing the synthesis of transferrin (Means & Krantz, 1992). Haemochromatosis (HH) is a genetic disorder associated with progressive iron overload and deposition in multiple organs. Two mutations (C282Y, H63D) in the *hfE* gene

have been found to be associated with HH. Three consequences of the C282Y mutation are lack of expression of *hfE* on the cellular surface, a lowered iron level in macrophages and an increased rate of clearance of iron from the instestinal lumen (Moalem *et al*., 2002). Iron deficiency of macrophages in people with hereditary HH mutations is associated with increased resistance to infection by intracellular pathogens, such as *Yersinia, Coxiella, Francisella, Legionella, Mycobacterium and Salmonella*, a selection pressure which is called Epidemic Pathogenic Selection (EPS). Thus, people with hereditary HH mutations may result in increased resistance to these pathogens (Moalem *et al*., 2004).

Various intracellular bacteria, fungi and protozoa use distinct host-cell compartments as niches for survival, where access to nutrients is an important matter (Schaible & Kaufmann, 2004). *M. tuberculosis* blocks phagosome maturation to access cellular iron resources (Nguyen & Pieters, 2005). *Chlamydia trachomatis* forms a non-infectious reticulate body (RB) which replicates within a specialised vacuole, called inclusion. Its intracellular replication might need an intimate association with the host cell which allows it to scavenge for nutrients, including ATP (Stephens *et al*., 1998). *Toxoplasma gondii* replicates within the parasitophorous vacuole membrane (PVM) which forms tight associations with host mitochondria and the endoplasmic reticulum (ER) (Sinai *et al*., 1997). *Legionella pneumophila* creates an ER-derived organelle that supports bacterial growth by a process that involves intercepting early secretory vesicles (Kagan & Roy, 2002).

Iron is the only nutrient known to be generally growth-limiting and to play an important role in the virulence of bacteria and has lead pathogens to evolve and develop different mechanisms to compete with the host iron-bound molecules for its acquisition. For instance, some pathogens produce more than one siderophore in low iron conditions. *M. tuberculosis* produces mycobactin and carboxymycobactin (Ratledge & Dover, 2000), *P. aeruginosa* produces pyoverdin and pyochelin (Cox & Adams, 1985; Cox *et al*., 1981), species of enteric bacteria, for instance *E. coli*, produces enterochelin and aerobactin (Bagg & Neilands, 1987).

Within the hostile environment of the host, the availability of iron depends on two physical conditions, the oxidation-reduction potential (Eh) and the pH, both factors are crucial because they control the binding of iron to transferrin (Bullen *et al*., 2005; 1992). It has been stated that iron is insoluble at pH around neutrality and its concentration as Fe^{3+} is about 10^{-18} M that is still low for cells to be able to acquire it and therefore they need specialised molecules, such as the siderophores. For instance, in the phagocytic vacuoles of the macrophages, where *M. tuberculosis* grows, the pH is between 6.1 and 6.5 and the maximum concentration of Fe^{3+} is only between 1 and 10 ng/ml (Piddington *et al*., 2000).

Human plasma has an Eh around +200 mV, pH 7.5 and is strongly bactericidal against strains of *E. coli* and *Klebsiella pneumoniae* with about 99.9% of approximately 50,000 bacteria/mL being destroyed in 1h. When the Eh is lowered to -400 mV, pH 7.5, by adding a reducing agent the bactericidal effect was removed, and after 2 h, the remaining bacteria, about 50/mL, grew rapidly to reach over 10^6 bacteria/mL in a further 6 h. At this low Eh, about half of the ferric iron attached to the transferrin was reduced to the ferrous form, and ferrous form is no longer bound by transferrin and is freely available to bacteria (Bullen *et al*., 1992).

Diverse studies have demonstrated the relationship that there is between iron availability and pathogenicity of bacteria. The disruption of genes, which encode for enzymes involved in the biosynthesis of iron chelators, has shown that without these compounds pathogens are not able to grow or lose their virulence. On the other hand, in experimental studies iron enhances the virulence when it is added either *in vitro* or *in vivo* (Agranoff & Krishna, 1998; Brown & Holden, 2002; Schaible & Kaufmann, 2004).

The importance of iron to enhance infection by mycobacteria and for instance the production of its siderophores has been demonstrated in different ways. Addition of iron enhances the infection in macrophages by *M. tuberculosis*. A concentration of 50 μg ml^{-1} ferric ammonium citrate to co-cultures of *M. tuberculosis* and either murine or human macrophages for 4 days tended to enhance growth of the bacteria (Rook *et al*., 1986). In

another study, macrophages infected with mycobacteria were exposed to ^{59}Fe-transferrin. The study demonstrated that the bacteria could acquire iron delivered through the endocytic route (Wagner *et al*., 2005). Although Fe^{3+} was released from transferrin to bacteria, the mechanism is unknown, and perhaps due to similar association constant and higher affinity for iron, mycobactins were able to compete with transferrin for iron. Undoubtedly the interaction of many factors could explain that exchange of iron between molecules.

In a recent study, the deletion of the gene that encodes for MbtB in *M. tuberculosis* was shown to interrupt the biosynthesis of the mycobactins under conditions of iron limitation. In addition, this mutant was found to be impaired for growth in macrophage-like THP-1 cells, macrophages being the preferred cell type for growth of pathogenic mycobacteria in humans, which suggested that mycobactin was important as virulence factor for *M. tuberculosis* (De Voss *et al*., 2000). However, other studies have shown that the importance of mycobactin production and its relation with the growth of *M. tuberculosis* is important at short time intervals, but is not relevant over a longer period of growth (Sassetti *et al*., 2003; Sassetti & Rubin, 2003).

The relationship between iron availability and growth or virulence of *M. tuberculosis* is also supported further by clinical studies of dietary iron overload in Africa, where a strong association between elevated iron and tuberculosis was observed (Moyo *et al*., 1997). Experimental infections with *M. tuberculosis* and *M. avium* in mice are considerably worsened by iron supplementation with the clear implication that addition of iron to the diet of human sufferers, including AIDS patients suffering from *M. avium* infections, must be avoided (Gomes *et al*., 2001; Lounis *et al*., 2001).

Iron acquisition *via* yersiniabactin is also important for the virulence of *Yersinia pestis* during the early stages of infection in mice (Bearden & Perry, 1999). *Listeria monocytogenes*, a causative agent of foodborne illness, has been shown to increase its virulence in mice loaded with iron, and the effect was opposite when iron was sequestered from the host by administrating iron chelators (Cowart & Foster, 1985; Sword, 1966). A close association has

been observed between iron acquisition and expression of virulence factors/toxins like the diphtheria toxin by *Corynebacterium diphtheriae*, the shiga toxin by *Shigella sp*. and exotoxin A from *Pseudomonas aeruginosa* (Payne & Lawlor, 1990).

1.5.4.2 Siderophores in bacteria.

Although, the fungi also produce siderophores, here this subject will be emphasised in bacteria. Chemical and biological features of siderophores and their importance as molecular virulence factors for bacteria will be discussed.

Siderophores are produced by many microorganisms, in response to iron deficiency under aerobic environments. These molecules have low molecular weight and are considered as high-affinity extracellular ferric chelators to solubilise iron prior to transport (Andrews *et al*., 2003; Ratledge, 2004; Wandersman & Delepelaire, 2004). There are almost 500 compounds identified as siderophores. The selectivity of siderophores for Fe(III) is achieved through optimal selection of metal binding groups, the number of binding units, and their stereochemical arrangements (Boukhalfa & Crumbliss, 2002).

Siderophores must satisfy three criteria. Firstly, they must be iron-regulated biosynthesis, secondly, ferric ion binding capability, and thirdly, active transport through the cell membrane (Drechsel & Jung, 1998). The uptake of carboxymycobactins (siderophores of pathogenic mycobacteria) was thought to be not energy-linked. However recent data shows that IrtA, IrtB and Rv2895c participate in export and import of *M. tuberculosis* carboxymycobactins across the membrane and the consequent iron uptake (discussed in 1.7.1) (Farhana *et al*., 2008). Genes related to iron availability and therefore siderophore biosynthesis is regulated either positively or negatively. During iron uptake, the siderophore-Fe^{+3} complex is transported into the cell by diverse proteins (Andrews *et al*., 2003; Ratledge & Dover, 2000).

Not all bacteria can produce siderophores. For instance *Clostridium perfringens*, a Gram-positive bacterium, does not produce siderophores. Instead, they acidify their environments in the host tissue so that iron is acquired from molecules, such as transferrin or lactoferritin. These molecules are reduced to Fe (II) and then it is mobilised to the cell (Johnson, 2008). On the other hand, *Neisseria gonorrhoeae* and *N. meningiditis* do not produce their own siderophores, but they can use siderophores produced by other microorganisms as an iron source, e.g. aerobactin (Wandersman & Delepelaire, 2004).

1.5.4.3 Structure of siderophores.

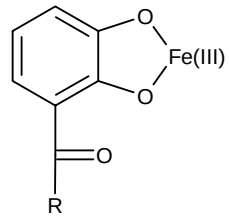
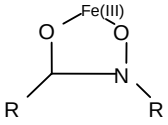
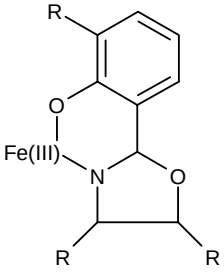
Despite their variety, most siderophores have a peptide backbone with several nonprotein amino acids including both modified and D-amino acids (Boukhalfa & Crumbliss, 2002). According to the iron coordination chemistry, various ligands which contain O, N and S atoms donate electrons to iron in order to form complexes, and this is reflected in the chelating groups of siderophores, having O and N as electron donors (Boukhalfa & Crumbliss, 2002).

There are three main types of iron-coordinating functional groups in siderophores. Firstly, there are the N-hydroxy amino acid side chains, with the oxygen atom as one of the ligands for Fe^{3+}. Secondly, there are adjacent hydroxyls of catechol rings, almost always derived from 2,3-dihydroxybenzoate (DHB). Variants are found, and instead of DHB, some siderophores have 2-hydroxybenzoate (salicylate) as ferric iron ligands. Thirdly, the nitrogen atoms of five thiazoline and oxazoline rings, resulting from enzymatic cyclisation of cysteinyl and seryl, or threonyl side chains, respectively, can also coordinate Fe^{3+} (Boukhalfa & Crumbliss, 2002; Crosa & Walsh, 2002).

Other authors have suggested classifiying siderophores considering only two categories of chelating groups. The first, the phenolic acid based structures or catechols, second is the hydroxamates in which the ω–N(HO)-CO group is used to chelate iron as N-hydroxy-N-acyl derivative of ornithine or lysine. A third type of chelating group, mixed ligands, has been suggested by combining the above types (Boukhalfa & Crumbliss, 2002). For convenience

this classification is adopted here. Table 3 illustrates these common ligand groups and Figure 4 shows examples of siderophore structures.

Table 3. Common ligand groups found in siderophore iron binding centres. Two types of chelating groups are considered, the catecholate rings and hydroxamates. A third type is considered when both types are mixed, the mixed ligands.

Chelating group	Structure	Occurrence in siderophores	Producing organisms	References
Catecholate		Enterochelin	*E. coli*	Raymond *et al*. (2003).
		Parabactin	*Paracoccus*	Bergeron *et al*. (1985).
		Yersiniabactin	*Yersinia*	Haag *et al*. (1993).
Hydroxamate		Coprogen	*Neurospora*	Drechsel & Jung (1998).
		Rhodotorulic acid	*Rhodotorula*	Atkin *et al*. (1970).
		Exochelin MS	*Mycobacterium*	Sharman *et al*. (1995b).
		Aerobactin	*Aerobacter*	Gibson & Magrath (1969).
Mixed ligands		Agrobactin	*Agrobacterium*	Bergeron & Ingeno (1987).
		Parabactin	*Paracoccus*	Bergeron & Ingeno (1987).
		Mycobactin	*Mycobacterium*	Snow (1970).
		Vulnibactin	*Vibrio*	Okujo *et al*. (1994).
		Pseudobactin	*Pseudomonas*	Teintze *et al*. (1981).

Independently of structural classification, the chelating groups are arranged in different architectures such as linear, tripodial, endocyclic or exocyclic (Boukhalfa & Crumbliss, 2002). Bacterial siderophores are structurally more diverse than fungal siderophores. Catecholate

siderophores, containing 2,3-dihydrobenzoic acid, are exclusively produced by bacteria. Bacteria also produce other structural classes of siderophores, hydroxamates and carboxylates (Drechsel & Jung, 1998).

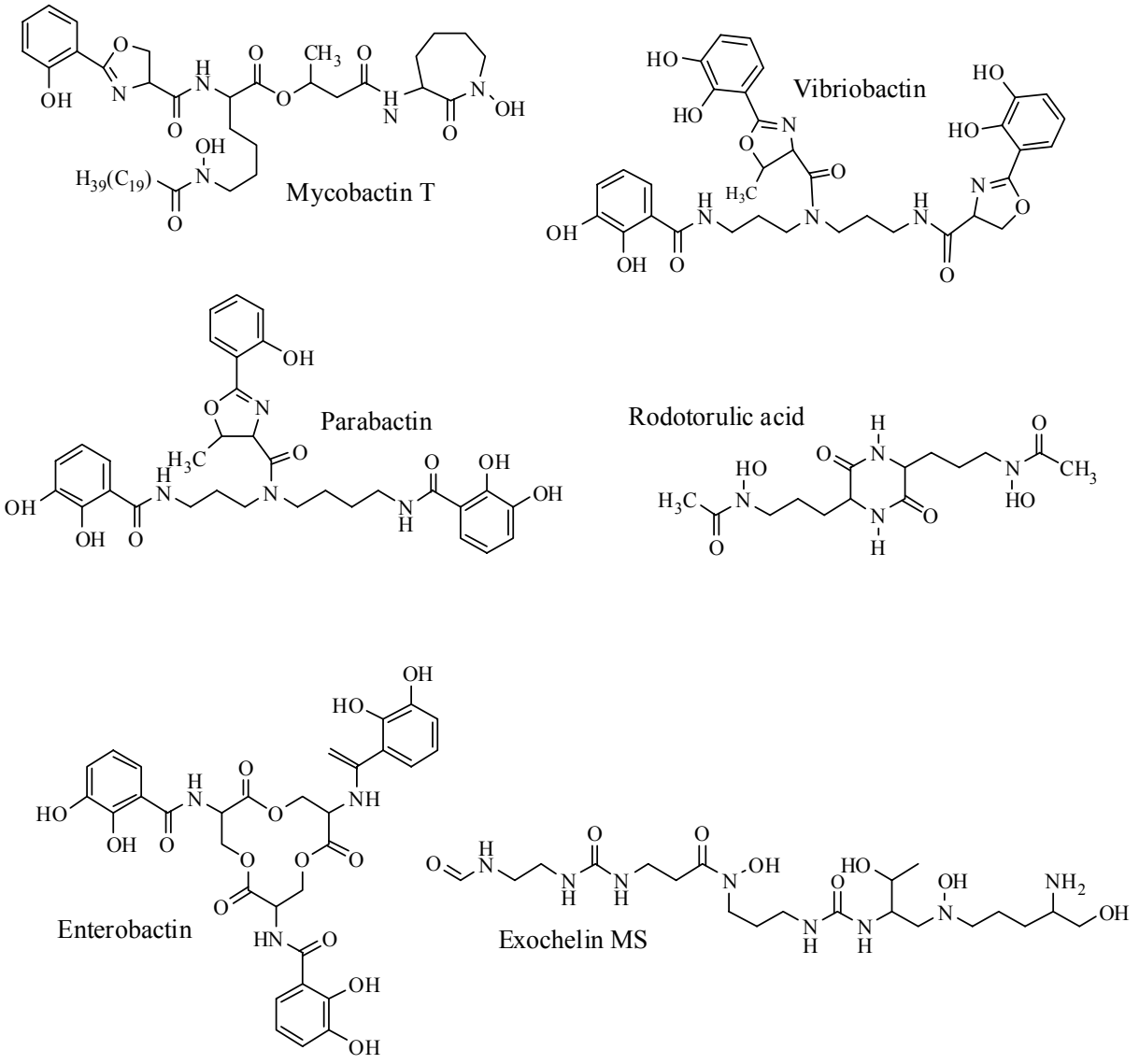


Figure 4. Structures of some siderophores that show the chelating groups. Enterochelin and parabactin have catecholate groups as iron-chelating. Rhodotorulic acid and exochelin MS possess hidroxamates as iron chelators. Vibriobactin and mycobactin T are mixed iron-ligand siderophores.

Three factors are important in order to bind ferric iron by siderophores. Firstly, denticity is the number of donor atoms that bind to the metal centre in a complex (Cotton *et al*., 1995). Thus, if one atom is bonded in the metal centre, the complex is named monodentate; two atoms, the complex is considered bidentate, and so on. Because ferric iron has six orbitals available for bonding these can form bonds in hexadentate complexes. Most siderophores are hexadentate, the optimal denticity to satisfy the six coordination sites of Fe(III). Although some siderophores are tetradentate, these need mulltimetallic assembly to ensure full ferric iron coordination. In general, hexadentate siderophores, based on hydroxamates and/or catecholates, have much higher affinity for ferric iron. These discriminate against ferrous form, compared to tetradentate siderophores, which in turn have a higher iron affinity compared to bidentate siderophores (Boukhalfa & Crumbliss, 2002). Secondly, the ligand structure affects the complex stability to bind iron. Cyclic structures such as ferrioxamine E and alcaligin show a higher Fe(III) affinity compared to their linear analogues ferrioxamine B and rhodotorulic acid, respectively. Thirdly, denticity also induces a concentration effect which favours ligands with high denticity (Boukhalfa & Crumbliss, 2002).

1.5.4.4 Nomenclature of siderophores.

Nomenclature of siderophores is problematic because researchers have denominated them according to various principles. In early work with siderophores, the colour of some siderophores played a major role in facilitating detection and study of siderophores. Thus, names were chosen for some siderophores: ferrichrome, ferrirubin, which only denotes a reddish-coloured, iron-containing substance. Since this nomenclature includes iron, the metal-free ligand has to be designated as desferri-siderophore, for example, desferri-ferrichrome (Drechsel & Jung, 1998).

Another characteristic considered to denominate a specific siderophore is naming it according to the producer. This was done firstly with enterobactin, isolated from *Enterobacteriaceae*. However, this principle has caused confusion, because for enterobactin

(Pollack & Neilands, 1970) there is another name, enterochelin, which was named by another research group (O'Brien & Gibson, 1970). Usually the name of siderophores is formed by two parts: the first part is related to the producer and the second part the chemical class of siderophore.

A reasonable siderophore nomenclature would be based upon their chemical structures, but there are reasons why this nomenclature has not been adopted. Siderophores are already named at the stage of isolation, purification and the optimisation of the fermentation procedure, when the chemical structure is still unknown or not fully characterised (Drechsel & Jung, 1998).

Another principle which causes a problem is the spelling of siderophores. Some siderophores, isolated and described in France, carried an "e" at the end, for example "pyoverdine". However, this nomenclature is being abandoned in the literature, thus the preferred name today is "pyoverdin".

1.5.4.5 Biosynthesis of siderophores: general mechanism.

Most of the siderophores in bacteria have a peptidic backbone, combined with a prevalence of nonproteinogenic amino acid units, which suggests that siderophores are made by nonribosomal peptide synthetases (NRPSs). Ribosomal peptide synthesis is normally restricted to a set of 20 amino acids for proteins, but in NRPS several hundred substrates have been identified. The presence of additional structures, such as fatty acids, heterocyclic rings, nonproteinogenic amino acids, carboxylic acids, and modified amino acids, is observed in siderophores. Thus, structural diversity is a predominant feature of nonribosomally produced peptides (Crosa & Walsh, 2002; Finking & Marahiel, 2004; Walsh *et al*., 1997).

The NRPSs were originally discovered as enzymes that catalyse the synthesis of antibiotics and other substances in Gram-positive bacteria. NRPSs are very large multimodular enzymes that produce peptide products of a particular sequence without mRNA

as a template. The NRPS enzymatic activity shares similarity with the polyketide synthases (PKSs) (Du & Shen, 2001); the latter produce several secondary metabolites, e.g. antibiotics and antitumor agents, from the same substrate used by fatty acid synthases (McDaniel *et al*., 2005). Both NRPSs and PKSs incorporate carrier domains which tether the growing chain *via* their post-translational modification by phosphopantetheine. A thioesterase (TE) domain is present in both systems, and this catalyses the release of the peptide or polyketide product. (Du & Shen, 2001; Quadri, 2000). Thus, these structural and catalytic similarities between NRPSs and PKSs have suggested that both might exist as hybrid enzymatic systems. This new enzymatic hybrid results in the production of diverse metabolites by incorporating amino acids and short carboxylic acids by NRSPs and PKSs, respectively. Based on the order in which either amino acid or carboxylic acid is incorporated, the hybrid NRSP-PKSs can be divided in two classes. Those whose biosynthesis do not involve functional interaction between NRPSs and PKSs modules and those whose biosynthesis are catalysed by hybrid NRPS-PKS systems, involving direct interactions between NRPSs and PKSs modules (Du & Shen, 2001). The order of activation and incorporation of the amino acids is specified by the order of NRPS domains (Crosa & Walsh, 2002; Finking & Marahiel, 2004; Quadri, 2000). NRPSs are structurally organised in modules. A module is defined as a section of the NRPS's polypeptide chain that is responsible for the incorporation of one building block into the growing polypeptide chain (Finking & Marahiel, 2004). Thus, NRPSs are used as a template (because amino acids to be incorporated are determined by the module) and biosynthetic machinery (it is the module that contains all necessary catalytic functions). Fungi use a single NRPS to synthesise a complete natural product and these multifunctional enzymes can reach remarkable sizes. In bacteria, modules are usually distributed in several NRPSs whose genes are organised in an operon (Finking & Marahiel, 2004).

The enzymatic units that reside within a module are called domains which carry out the steps of substrate activation, covalent binding, and peptide bond formation (Stachelhaus & Marahiel, 1995). The modules can be subdivided into initiation and elongation modules. Elongation modules contain at the minimum an adenylation domain (A-domain) for substrate recognition, a peptidyl carrier protein domain (PCP-domain, known also as thiolation domain)

that holds the activated substrate and a condensation domain (C-domain) for peptide bond formation. NRPSs initiation modules lack a C-domain. Additionally, a number of tailoring enzymes may be present to catalyse the maturation, modification reactions, such as *N*-methylation, amino acid epimerisation and heterocyclisation, of the NRPS product. These reactions generate the structural diversity of nonribosomal peptides (Figure 5) (Finking & Marahiel, 2004; Marahiel *et al*., 1997; Quadri, 2000).

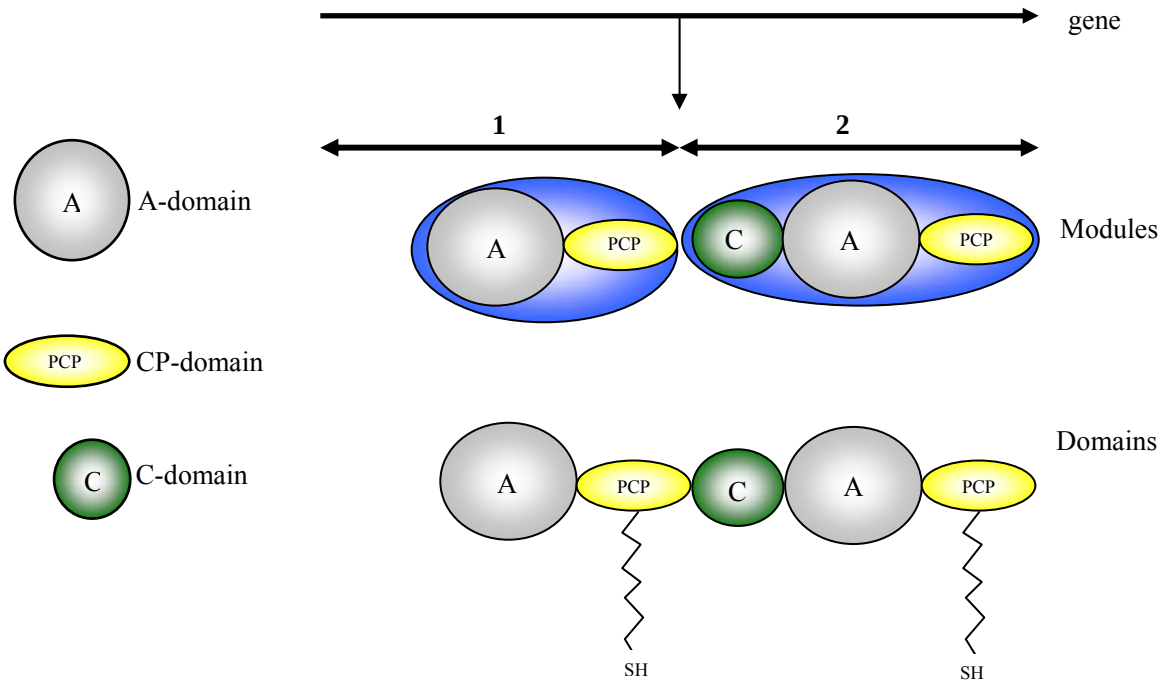


Figure 5. Genetic and protein organization of nonribosomal peptide synthetases (NRPSs). From gene to protein. Modules that are responsible for the incorporation of one amino acid can be identified on the protein level. Modules can be subdivided into domains that contain the catalytic activities for substrate activation (Adenylation domain, A-domain), covalent loading (carrier protein domain, CP-domain) with a phosphopantetheinyl group tailored (~SH), and peptide bond formation (condensation domain, C-domain). Modules which lack a C-domain are used to initiate nonribosomal peptide synthesis, while those that contain a C-domain are used for product elongation [Adapted from Finking and Marahiel (2004)].

1.5.4.5.1 Adenylation domain.

The adenylation domain (A-domain, ~550 aa), is responsible for the selection of the amino acids which will form the product and, therefore, these control its primary sequence. A-domains activate an amino or carboxy acid substrate as an amino acyl adenylate consuming ATP (Finking & Marahiel, 2004). Although, aa-tRNA synthetases and adenylation domain of NRPSs carry out similar enzymatic activities, they are structurally unrelated (Weber & Marahiel, 2001).

Also during initiation of siderophores biosynthesis, salicylate (2-hydroxybenzoate) and 2,3-dihydroxybenzoate (DHB) might be incorporated (Walsh *et al*., 2001; Walsh *et al*., 1997). These aryl acids act as aryl-*N*-caps and also incorporate iron ligands due to the presence of hydroxyl groups in their structure. To add salicyl and DHB groups at the start of siderophore synthesis, an A-domain for either salicyl-AMP or DHB-AMP formation and a carrier protein, the aryl carrier proteins (ArCPs), are required.

1.5.4.5.2 Carrier proteins.

The carrier protein domain (~80-100 aa) is the unit that accepts the activated amino acid, which becomes covalently bound to its 4'-phosphopantetheine cofactor as a thioester. These proteins belong to the carrier proteins superfamily, which consists a number of different members named according to the substrates that they carry during synthesis (Finking & Marahiel, 2004; Lambalot & Walsh, 1995).

Acyl carrier proteins (ACPs) of fatty acid and PKSs, carrying acetate, propionate, malonate units or fatty acyl substrates, and aryl carrier protein (ArCP) are involved in siderophores synthesis. Peptidyl carrier proteins (PCP) transfer amino acids or peptidyl substrates from one catalytic centre to the next (Linne *et al*., 2001; Linne & Marahiel, 2000). Although all carrier proteins carry out different roles within their biosynthetic machinery, they are structurally related. However, this class of proteins do not share sequence similarities when considering and comparing all members of the family. But the sequences around the

serine residue (core T-motif), which serves as attachment site for the phosphopantetheinyl arm, is similar within ACPs and PCPs but differs among species (Finking & Marahiel, 2004).

Erythromycin, an antibiotic yielded by *Saccharopolyspora erythraea*, is synthesised by a trimer of multidomain proteins, deoxyerythronolide B synthase (DEBS) I, II, III, which act sequentially, passing the growing chain from one ACP to the next. This process is a type I polyketide synthase (Leadlay *et al*., 1993). In the biosynthesis of siderophores, such as enterobactin, pyochelin, and yersiniabactin, peptidyl and aryl carriers are conjugated to produce those iron chelators (Quadri, 2000). A subunit of the synthetase, HMWP1, which makes the virulence-conferring siderophore yersiniabactin has a five domain PKS module followed by a four-domain NRPS module (Gehring *et al*., 1998b).

Although there are some biochemical gaps to be defined in mycobactin biosynthesis, it is probably produced by conjugating aryl, peptidyl carriers and polyketide synthases (De Voss *et al*., 1999; Quadri *et al*., 1998). Mycobactin biosynthesis is discussed in section 1.6.2.

1.5.4.5.3 Phosphopantetheinylation.

All carrier proteins require a post-translational modification in order to become active. Each carrier protein domain, ArCP, ACP, and PCP, must be converted from the *apo* form to the *holo* form, through a process called phosphopantetheinylation carried out by a phosphopantetheinyl transferase (PPTase). PPTases catalyse the attack of the side chain of a conserved serine residue of the carrier protein domain on the pyrophosphate linkage of coenzyme A (Figure 6A), transferring the 4'-phosphopantethenate (P-pant) moiety, producing a phosphodiester linkage to the carrier protein serine side chain and releasing 3',5'-ADP (Figure 6B). The reaction requires Mg^{2+} as a cofactor (Lambalot *et al*., 1996; Walsh *et al*., 1997).

Three types of PPTases are recognised according to their size and substrate selectivity (Lambalot *et al*., 1996). The first is the "Acyl Carrier Protein Synthase (ACPS)-type" from *E.*

coli, as the prototype. These PPTases are ~120 amino acids in size, and are very specific for CPs of primary metabolism in fatty acid biosynthesis (Chirgadze *et al*., 2000; Parris *et al*., 2000). The second type is the "Sfp-type" PPTases, denominated after Sfp (surfactin phosphopantetheinyl transferase), which is required for the production of the lipoheptapeptide antibiotic surfactin in *Bacillus subtilis* (Lambalot *et al*., 1996). The size of these enzymes is approximately twice the size of the ACPs-type. Although some Sfp-type PPTases seem to be specific enzymes, others are able to modify both ACPs and PCPs (Gehring *et al*., 1997b; Sánchez *et al*., 2001).

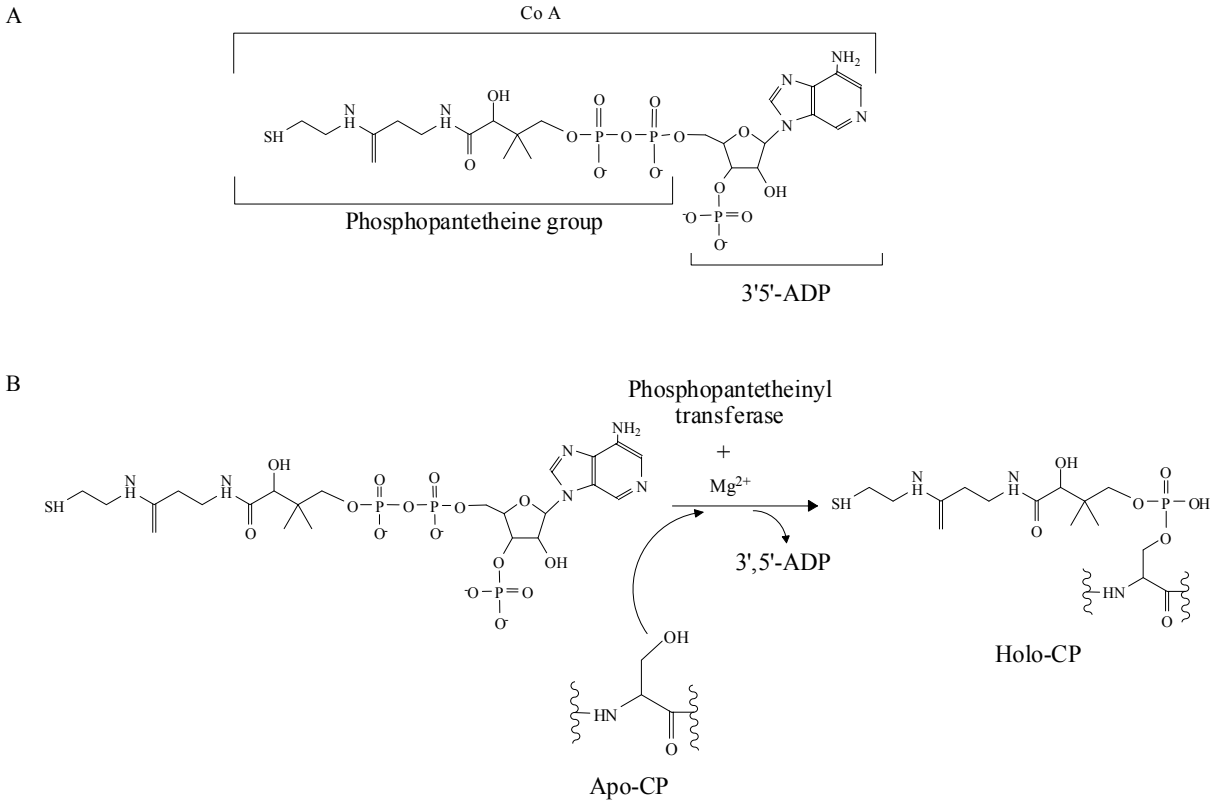


Figure 6. Catalytic function of phosphopantetheinyl transferases. A) Structure of coenzyme A from which the 4'-phosphopantetheine group is transferred onto a conserved serine residue within the carrier protein proteins of modular NRPSs and PKSs. B) Modification of carrier protein domains by post-translational phosphopantetheinylation catalysed by phosphopantetheinyl transferase. The modification converts inactive form *apo*-form to active *holo*-domains. The reaction is dependent on Mg^{2+} and yields 3',5'-ADP as a second product [Adapted from Lambalot *et al*. (1996)].

The third type of PPTase is incorporated as a domain at the carboxyl terminus of the α-subunit of fungal type I FASs, where it catalyses the *in cis* autophosphopantetheinylation of the ACP at the amino terminus of the same polypeptide (Fichtlscherer *et al*., 2000).

The P-pant introduced contains a terminal sulphydryl group (~SH) allowing biosynthetic intermediates to be bound as an energy-rich thioesters that facilitates hydrolysis during and after synthesis. Therefore, the P-pant groups act as a nucleophile for acylation by a substrate, either acyl-CoA or malonyl-CoA for the FASs and PKSs, or aminoacyl-AMPs for the peptide synthesis, for instance in siderophores (Figure 7) (Lambalot *et al*., 1996). During fatty acid biosynthesis, the carboxy-activated malonyl-ACP is produced and then it is decarboxylated, yielding a nucleophilic carbanion (negatively-charged carbon) that attacks a second acyl thioester to form a new carbon-carbon bond in one of the steps of PKSs. In peptides, the aminoacyl-S-PCPs or hydroxyacyl-S-PCPs serve as nucleophiles in amide and ester bond-formation (Figure 7) (Lambalot *et al*., 1996).

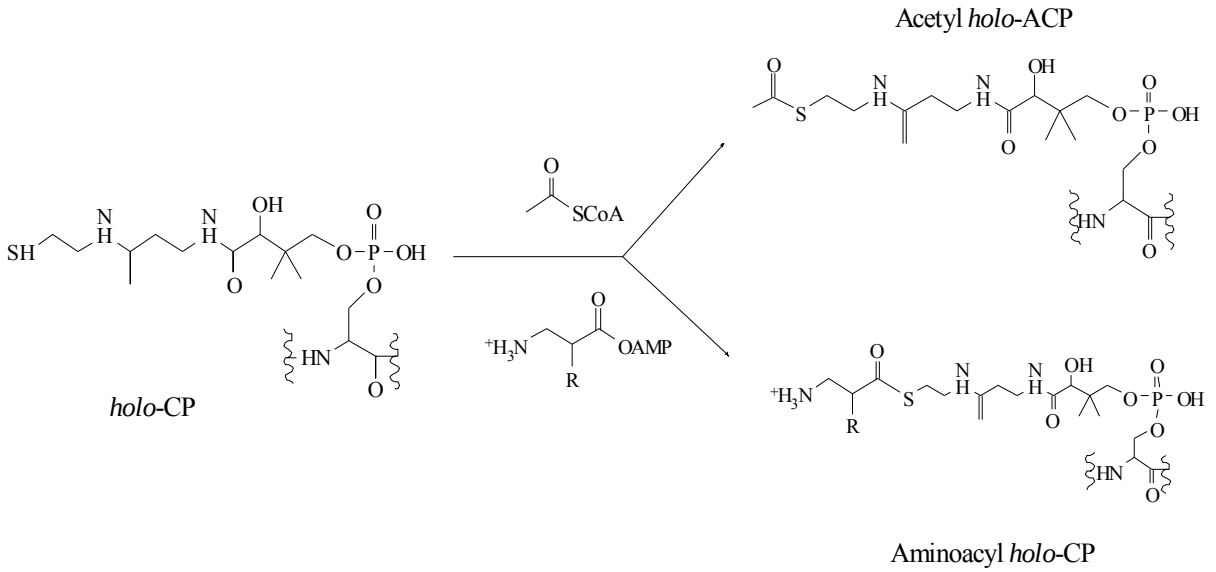


Figure 7. The terminal thiol of P-pant group acting as a nucleophile. The terminal cysteamine thiol of the phosphopantetheine cofactor acts as nucleophile for acyl activation. FASs and PKSs transfer acyl groups from acyl-CoA to the phosphopantetheine group attached to ACP. NRPSs first activate their aminoacyl or acyl substrates as acyl-adenylates before transfer to the phosphopantetheine tether of the carrier protein [Adapted from Lambalot *et al*. (1996)].

1.5.4.5.4 The condensation domain.

In NRPSs for siderophores, after activation of the carboxy groups as aminoacyl thioesters (aminoacyl-S-PCP), the next process is elongation. The siderophore chain growth is directional, from the most upstream, N-terminal PCP domains, to the most downstream, C-terminal PCP domains. The polypeptide synthetase catalytic domains are named condensation domains (C-domains). Basically, a core elongation module of the NRPSs assembly line has the three domains, C-A-PCP, as a functional module (Crosa & Walsh, 2002; Finking & Marahiel, 2004).

C-domains (~450 aa) are the central entity of NRPS because they are responsible for peptide bond formation between aminoacyl substrates bound to PCPs of adjacent modules. C-domains catalyse the nucleophilic attack of the amino (or imino, hydroxyl) group of the activated acid bound to the downstream (with respect to the C-domain) module onto acyl group of the amino acid tethered to the upstream module (Stachelhaus *et al*., 1996). These domains contain a site for the nucleophile (donor site, number two in Figure 8A) and a position for the electrophile (donor site, number one in Figure 8A).

One characteristic of siderophores, such as yersiniabactin, pyochelin and mycobactin, is the presence of thiazoline and oxazoline rings. These rings are formed by a C-domain variant, the cyclisation domain (Cy-domain). The Cy-domains catalyse the heterocyclisation of serine, threonine, or cysteine residues and possess a different active site to C-domains (Keating *et al*., 2000a; Keating *et al*., 2002; Quadri *et al*., 1998). Like C-domains, these extend the growing chain by condensation with the downstream Cysteine-S-PCP, Ser-S-PCP, and Thr-S-PCP but before the chain is transferred down, to the next elongation module, Cy-domains catalyse the ciclysation and dehydration of the side chain providing both metal chelating and intercalating groups to the modified peptides (Figure 8B) (Walsh *et al*., 2001).

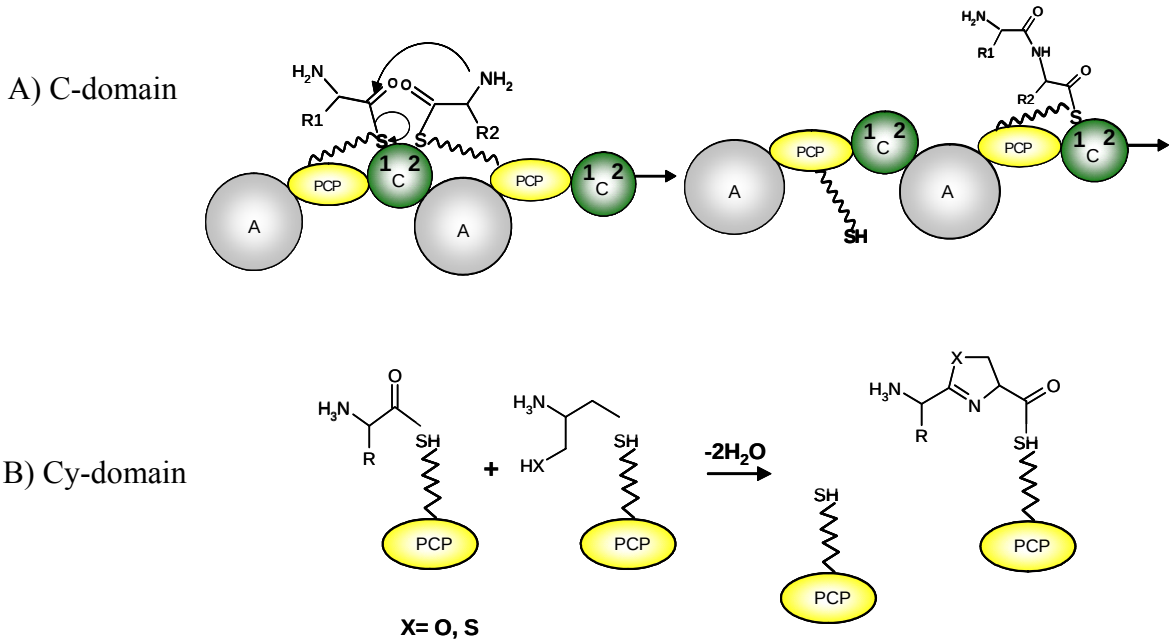


Figure 8. Domains which are activated during growth elongation of NRPSs products. A) In condensation (C)-domains two adjacent PCP-domains are loaded with their respective amino acid. The electrophile (acceptor) aminoacid enters the acceptor site (number 1) and the (donor) nucleophile amino acid is accommodated in the donor site (number 2) of the C-domain. After the peptide bond has formed, the resulting dipeptide can act as the electrophile in the active site of the next C-domain. B) Cy-domain catalyses the heterocyclisation of serine, threonine or cysteine residues which lead to the formation of thiazoline or oxazoline rings, which act as iron chelating groups [Adapted from Finking and Marahiel (2004)].

1.5.4.5.5 The thioesterase domain.

A final module is required to release from its phosphopantetheinyl tether. This module contains, in most cases, a thioesterase domain (TE-domain, ~ 250 aa), that is important for releasing the product (Figure 9) (Schneider & Marahiel, 1998).

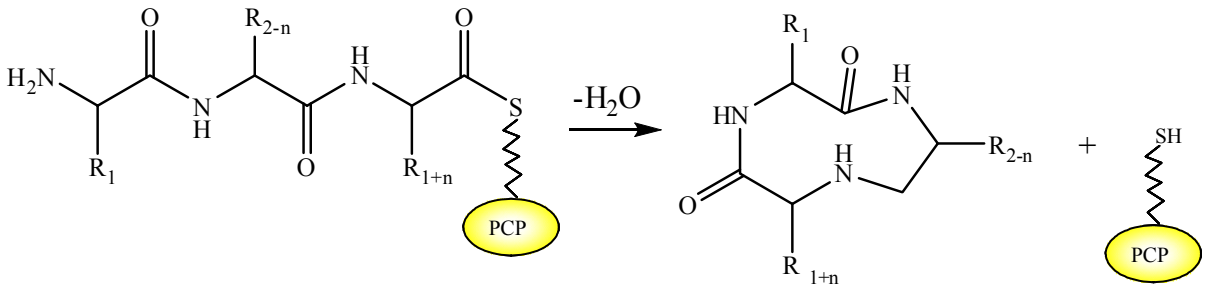


Figure 9. Catalytic activity of TE domain. TE-domain carries out the releasing of NRPS product [Adapted from Finking and Marahiel (2004)].

During the process, the release of the product is achieved by a two-step reaction that involves formation of an acyl-O-TE-enzyme intermediate that is subsequently attacked by either a peptide-internal nucleophile (intramolecular capture by an OH or NH_2) or water, which forms either a macrocyclic product (surfactin, an antibiotic produced by *Bacillus subtilis*) or a linear peptide like pyochelin (a siderophore produced by *Pseudomonas sp.*) (Crosa & Walsh, 2002; Finking & Marahiel, 2004). The TE-domains are also responsible for the structural diversification of NRPSs because these domains carry out some modifications such as oligomerisation of peptide units as has been observed in enterobactin synthesis.

Two types of TE-domains are recognised. Type I is usually found as the last domain of NRPS and catalyses cleavage of the assembled peptide product from the PCP domain in the last module. In this process the phosphopantetheinyl group is released to give an acyl TE intermediate that subsequently suffers a nucleophilic attack by an internal nucleophile (hydroxyl group). The second type, stand-alone TE, is often encoded within the gene cluster and possibly their role is to hydrolytically remove inactivating acetyl groups from the 4-phosphopantetheinyl thiols of PCP domains to regenerate active thiols (Challis & Naismith, 2004).

1.5.4.5.6 Diversity of siderophore produced by NRPS.

The great diversity of NRPS products, including siderophores, is due to additional enzymatic processes, called tailoring domains, which can be involved either during or after other biosyntheses. Some represent an integral part of the NRPS and act *in cis* (moving along in a single protein chain), and the others are distinct enzymes which act *in trans* (moving from one protein to chain to another) on the way to the mature NRPS product (Challis & Naismith, 2004; Finking & Marahiel, 2004).

One interesting feature of NRPS is the presence of D-amino acids. An Epimerisation domain (E-domain), is responsible for epimerisation of L-amino acids. In general, the A-domains of NRPS select L-amino acids for activation and the E-domains epimerise them when they are installed as aminoacyl or peptidyl-S-PCP acyl enzyme intermediates (Challis & Naismith, 2004; Walsh *et al*., 2001). In pyochelin and mycobactin synthesis there are E-domains which act *in cis*.

Some siderophores such as mycobactin, pyochelin and yersiniabactin contain N-methyl amino acids. These methyl groups are introduced by dedicated methyltransferases (MT), and it has been determined that N-methylation of the amino acid monomer occurs before the formation of the peptide bond. In pyochelin and yersiniabactin synthesis, the MT-domains are found closest to A-domain, acting *in cis* (Quadri, 2000), but in yersiniabactin there is an additional MT closest to Cy-domain in the HMWP1 unit (Brem *et al*., 2001).

As has been seen, Cy-domains catalyse the heterocyclisation of cysteine, serine and threonine residues to yield thiazoline or oxazoline rings, but their oxidative state is not always the final product. In pyochelin synthesis, a thiazolidine ring is the result of the *in trans* action of the NADPH-dependent reductase PchG that catalyses the two-electron transfer to thiazolinyl-S-PchF (Reimmann *et al*., 2001).

1.5.4.5.7 Siderophore biosynthesis independent of NRPSs.

Several bacterial siderophores are not polypeptides, and are assembled from alternating dicarboxylic acid and diamine or amino alcohol building blocks (which are nevertheless derived from amino acids) linked by amide or ester bonds. These NRPS-independent pathways lead to aerobactin, rhizobactin, achromobactin, vibrioferrin, alcaligin and desferrioxamine E biosynthesis (Table 4) (Challis, 2005).

Table 4. **Siderophores and their biosynthetic pathways.**

Siderophore	Producer	Pathway	Reference
Aerobactin	*Escherichia coli*	1	de Lorenzo & Neilands (1986).
Alcaligin	*Bordetella pertussis*	1	Moore *et al*., (1995).
Desferrioxamine	*Streptomyces coelicolor*	1	Barona-Gómez *et al*., (2004).
Vibrioferrin	*Vibrio parahaemolyticus*	1	Challis (2005).
Yersiniabactin	*Yersinia enterocolitica*	2	Brem *et al*., (2001).
Mycobactin	*M. tuberculosis*	2	Quadri *et al*., (1998).
Vibriobactin	*Vibrio cholerae*	2	Keating *et al*., (2000b).
Pyochelin	*Pseudomonas aeruginosa*	2	Quadri *et al*., (1999).

[1]Nonribosomal peptide synthases (NRPSs)-independent pathway.

[2]NRPSs pathway.

1.6 The discovery and structural elucidation of mycobactins.

Early attempts at *in vitro* culture of *M. avium* subsp. *paratuberculosis* (previously *M. johnei*) or Johne's bacillus, that is responsible for the condition of chronic enteritis in cattle, failed (Twort & Ingram, 1912). Studies were initiated to attempt to define a laboratory medium to culture the organism, which led to the observation that addition of dried and killed human tubercle bacilli as supplement promoted growth, presumably a growth factor was necessary (Twort & Ingram, 1912). However, the substance was not isolated and identified for almost 40 years.

Francis *et al*. (1949) isolated a similar growth promoting factor from *M. phlei* which could be crystallised in complex with aluminium, but not in a metal-free form. In 1953, the same authors were able to isolate the growth factor, named since then as mycobactin, in a metal-free form (Francis *et al*., 1953). The first structure of mycobactin P, i.e. the mycobactin from *M. phlei*, was determined and its constituent fragments identified by chemical degradation (Snow, 1954a). However, the structure proposed was tentative, and a further analysis determined the correct structure of the active ferric complex of mycobactin P in a crystalline form (Snow, 1965b). Mycobactin T was identified from *M. tuberculosis* by Marks (1953), but its structure was solved by Snow (1965a). Both mycobactin P and T were identified as very similar chemical entities, differing only in the chain length of a fatty acyl substituent. Subsequent structural analysis of other mycobactins from various mycobacteria, including *M. terrae*, *M. marinum* and *M. smegmatis*, revealed that all share the same core structure (Figure 8), but these are subject to species-specific differences at several sites (Table 5) (Snow & White, 1969).

The core structure was elucidated as the following. A 2-hydroxyphenyloxazoline moiety, arising from either salicylic acid or 6-methylsalicylate according to the specific mycobactin was linked *via* an amide bond to an acylated ε-N-hydroxylysine residue. This lysine was esterified at the α-carboxyl with a β-hydroxy acid that forms an amide link with a second ε-N-hydroxylysine which was cyclised to give a seven membered lactam. This basic structure serves to chelate ferric iron very effectively *via* the two hydroxamic acids of the ε-N-hydroxylysines, the N of the oxazoline ring, and the phenolate oxygen atom (Figure 10) (Snow, 1970). By X-ray analysis, the ferric iron coordination chemistry of ferrimycobactin P was determined. Six atoms, five oxygens and one nitrogen coordinate the Fe^{3+} atom and form an octahedron (Hough & Rogers, 1974).

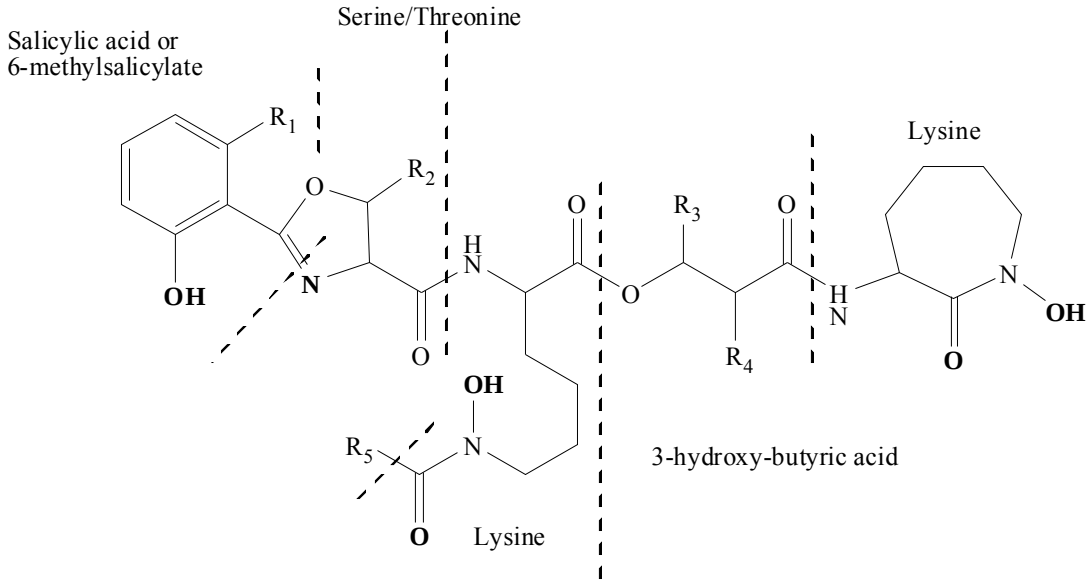


Figure 10. General structures of mycobactins and carboxymycobactins. All the known mycobactins have the same basic molecular pattern, the core structure which is formed by salicylic acid/6-methylsalicylate, serine/threonine, two lysines, and 3-hydroxy-butyric acid. Variation in mycobactins is due to changes at five positions in the molecule (R_1-R_5). The carboxymycobactins share the same core structure, but they differ by varying the long-chain fatty acid (R_5) for a short chain which terminates in either carboxyl or ester functionality. The three pairs of ferric iron-chelating groups are boldface [adapted from De Voss *et al*. (1999) and Snow (1970)].

A nomenclature was proposed to distinguish the mycobactins which have the same core structure are grouped in a family (Snow, 1970). Mycobactins have also been distinguished by a letter, and each letter represents a family of mycobactins that have the core structure. Individual members of a family are distinguished by adding the number of carbon atoms in the side chain (Table 5, Figure 10) (Snow, 1970; Snow & White, 1969). Great variation in the long-chain fatty acyl component has been observed in *M. smegmatis*; 17 different mycobactins have been detected varying only in the R_5 substituent (Ratledge & Ewing, 1996). A list of diverse mycobactins and their producers is shown in Table 5.

Table 5. Diversity of mycobactins (MBs). Within the core structure of mycobactins there are five main substitutions which lead to the diversity of these siderophores. The alkyl substituents of the hydroxy acid (R_3 and R_4) and the acyl moiety (R_5) are highly variable. However, the acyl moiety (R_5) is the source of most of the observed structural variation in the mycobactins.

MB type	Substituents					*Species*
	R_1	R_2	R_3	R_4	R_5	
A	CH_3	H	CH_3	H	C_{13}	*M. aurum*
Av	H	CH_3	CH_3CH_2	CH_3	$C1_{1-18}$	*M. avium*
F	CH_3/H	CH_3	CH_3	H	C_{9-17}	*M. fortuitum*
H	CH_3	CH_3	CH_3	H	C_{17-19}	*M. thermoresistible*
J	H	H	$(CH_3)_2CH_2$	H	C_{15}	*M. avium subsp. paratuberculosis*
P	CH_3	H	CH_3CH_2	CH_3	C_{15-19}	*M. phlei*
R	H	H	CH_3CH_2	CH_3	C_{19}	*M. terrae*
S	H	H	CH_3	H	C_{9-19}	*M. smegmatis*
T	H	H	CH_3	H	C_{17-20}	*M. tuberculosis*
M	H	CH3	C_{15-18}	CH_3	CH_3	*M. marinum*
N	H	CH3	C_{15-18}	CH_3	$(CH_3)_2CH_2$	*M. marinum*
Carboxy-MB Av	H	CH_3	CH_3CH_2	CH_3	C_{2-10}	*M. avium*
Carboxy-MB T	H	H/CH_3	CH_3	H	C_{2-8}	*M. tuberculosis*

According to the core structure, two types of mycobactins can be distinguished. Mycobactins P, T, A, R, F, H, S and J have the same core structure but differ only in details of substitution and absolute stereochemistry. Mycobactins M and N, from *M. marinum*, differ from the others by having a small acyl group at R_5, but having a long-chain substituent at R_3 (White & Snow, 1969). The acyl moiety (R_5) is the most variable structure in the mycobactins. The acyl group is a long-chain fatty acid (C_{10} to C_{21}) that is often unsaturated with an unusual *cis* double bond conjugated to the carbonyl group, but in mycobactin T in which some members of the family probably have saturated alkyl side chains (Snow, 1970).

Many mycobacteria are peculiar in their ability to produce both a cell-bound and a secreted siderophore, mycobactins and carboxymycobactins, respectively. Mycobactins, lipophilic siderophores, are insoluble in water because of the long alkyl chain attached to the molecule, but they are extremely soluble in chloroform, fairly soluble in ethanol, and less soluble in other alcoholic solvents, such as methanol or propylene glycol (Snow, 1970). It has been shown that the long chain is an important feature of the mycobactins not only increasing the hydrophobicity of the molecule, but also as a means of preventing siderophore diffusion due to its association with the cell membrane. However, mycobactins and other siderophores might become amphiphilic in their iron complex (Lin *et al*., 2001; Martinez *et al*., 2003).

On the other hand, carboxymycobactins are variations of mycobactins in which the long alkyl chain is replaced by a shorter chain terminating in a carboxylic acid, which might be further modified as a methyl ester (Figure 11); these modifications are responsible for their solubility in aqueous solutions (Lane *et al*., 1995; Wong *et al*., 1996). Since, both mycobactins and carboxymycobactins have the same core structure, it has been suggested that they are synthesised by the same set of enzymes and that only one of the final steps determines if a mycobactin or carboxymycobactin is produced (Gobin *et al*., 1995).

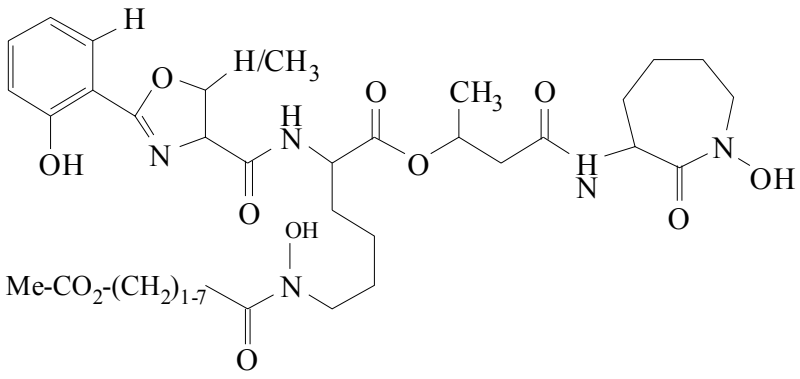


Figure 11. **Structure of *M. tuberculosis* carboxymycobactin T.** Both mycobactin and carboxymycobactin T have the same core structure, but they vary in their alkyl substituent where in the latter a carboxylic acid group acts as chain termination (Gobin *et al*., 1995). The side chain of carboxymycobactin Av, isolated from *M. avium*, can be saturated or unsaturated and terminates with either a carboxylate or methyl ester (Wong *et al*., 1996).

The presence of a second mycobacterial siderophore family has been recognised, the exochelins. Exochelins, which have been isolated from non-pathogenic mycobacteria, are water soluble. The structures of two exochelins, from *M. smegmatis* (Macham *et al*., 1975; Sharman *et al*., 1995b) and *M. neoaurum* (Sharman *et al*., 1995b) have been elucidated and their structures are as follows. They are linear penta and hexa-peptides in which the hydroxamate groups form the major iron binding centres (Figure 12). In exochelin MN from *M. neoaurum*, there is a unique β-hydroxyhistidine residue which forms one of the three iron binding pairs of ligands in the molecule (Sharman *et al*., 1995a).

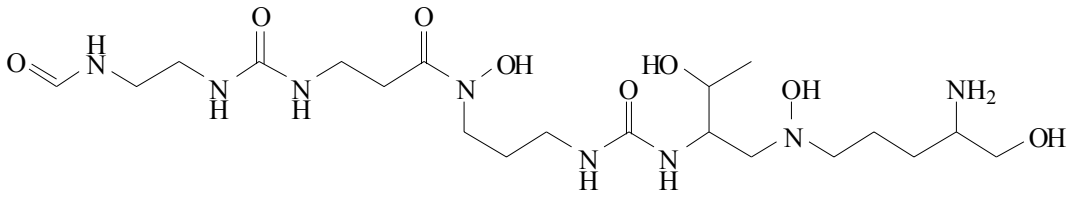


Figure 12. **Structure of exochelin MS from *M. smegmatis***. [adapted from Sharman *et al*. (1995a)]. Exochelin structures are based on modified peptides and are highly-water soluble molecules. The exochelins have been only isolated from non-pathogenic mycobacteria.

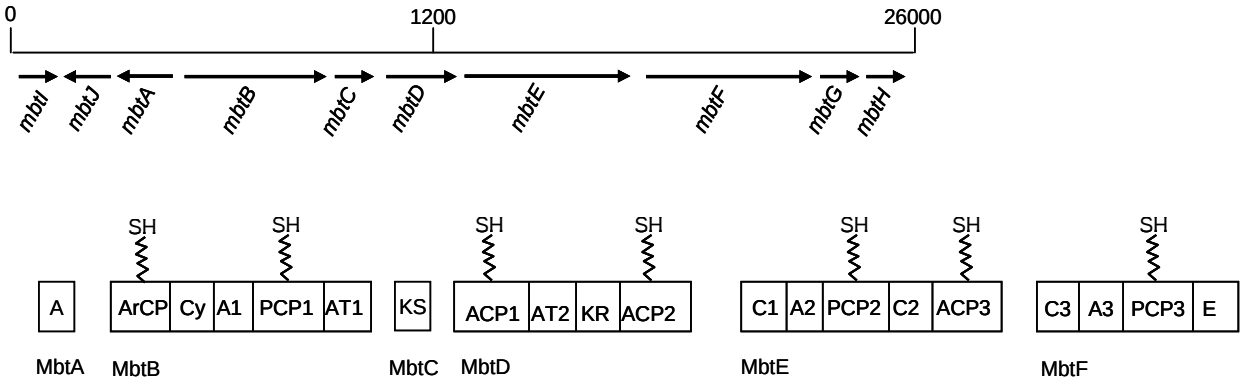
The nomenclature of carboxymycobactins has generated confusion since some authors have named them exochelins, water-soluble mycobactins or exomycobactins (Gobin *et al*., 1995; Wong *et al*., 1996; Zhu *et al*., 1998). Exochelins and carboxymycobactins share some physical characteristics in that they are extracellular soluble siderophores, although their chemical structure is different. Prior to the structural elucidation of the carboxymycobactin structure, all extracellular mycobacterial siderophores were denominated exochelins, independent of their solubility differences (Ratledge, 2004; Vergne *et al*., 2000). To avoid confusion the name carboxymycobactins is adopted here to distinguish these from exochelins. Although, the carboxymycobactins are produced by pathogenic mycobacteria, such as *M. avium* (Lane *et al*., 1995) and *M. tuberculosis* (Gobin *et al*., 1995), *M. smegmatis* also

produces a carboxymycobactin whose structure was confirmed as identical to the core structure of mycobactins (Ratledge & Ewing, 1996).

1.6.1 Organisation of the mycobactin-carboxymycobactin gene clusters.

In an attempt to elucidate mycobactin biosynthesis, early studies were restricted to experiments using an isotope-based approach and the identities of the basic core structure of mycobactins were clarified. The aromatic moiety of the mycobactins has two different biosynthetic sources according to the species. The aromatic moiety of mycobactin S, from *M. smegmatis*, arises from salicylate formed *via* the shikimic acid pathway (Hudson & Bentley, 1970). Addition of [G-^{14}C]-shikimic acid to the medium led to the radiolabeling of the mycobactic acid hydrolysis product of mycobactin and to the extracellular salicylic acid excreted by *M. smegmatis*. However, the aromatic moiety of mycobactin P was not derived from this pathway, as shown by the failure to incorporate radiolabel into the siderophore. Consistent with mycobactin P bearing a methyl function at R1, it was thought that the aromatic moiety was derived from 6-methylsalicylate, which is synthesised through the condensation of four acetate units and is not a shikimate metabolite (Hudson *et al*., 1970). Similarly, the presence of radioactivity from [U-^{14}C]-L-Lysine in the cobactin and mycobactic acid hydrolytic products showed that lysine was the precursor of both ε–N-hydroxylysines in *M. phlei* and *M. smegmatis* (Tateson, 1970). Incubation of *M. phlei* and *M. smegmatis* with [2-^{14}C]-propionate and [1-^{14}C]-acetate, respectively, yielded high radioactivity in the cobactin linker fragments of the two mycobactins.

Despite the availability of some data describing the provision of mycobactin precursors, the genetic determinants of mycobactin assembly remained unknown, until the genome sequence of *M. tuberculosis* H37Rv was completed (Cole *et al*., 1998). Initially twelve candidate genes for mycobactin biosynthesis were identified; a first cluster of ten genes (*mbtIJA-H*, occupying Rv2386c-Rv2377c) (Figure 13) was designated the *mbt* (mycobactin biosynthesis) locus along with two other genes encoding for phosphopantetheinyl transferases (*pptT* and *acpS*) (Figure 13).



Protein name	Proposed function	Domains within each module	Amino acids	Molecular weigth (Da)	Gene name
MbtA	Salicylate-AMP ligase (PAE)	A	565	59 279.7	*mbtA*
MbtB	NRPS/PKS hybrid system	ArCP, Cy, A1, PCP, T1/TE	1413	151 502.3	*mbtB*
MbtC	Polyketide synthase (PKS)	KS	444	46 623.4	*mbtC*
MbtD	Polyketide synthase (PKS)	ACP1, AT2, KR, ACP2	1004	105 591.5	*mbtC*
MbtE	Peptide synthetase (NRPS)	C1, A2, PCP2, C2, ACP3	1682	183 282.0	*mbtE*
MbtF	Peptide synthetase (NRSP)	C3, A3, PCP3, E	1461	156 747.7	*mbtF*
MbtG	Lysine-N-oxygenase		131	46 943	*mbtG*
MbtH	Unknown		57	6521.3	*mbtH*
MbtI	Isochorismate synthase		431	46 976.5	*mbtI*
MbtJ	Acetyl hydrolase		306	32 875.9	*mbtJ*
PptT	Phosphopantetheinyl transferase		227	24 707.5	*pptT*
AcpS	Phosphopantetheinyl transferase		130	14 001.8	*acps*

Figure 13. Locus involved (*mbt*-1) for assembly of the core structure of mycobactin/carboxymycobactins. Genes encoding for peptide arylation enzymes (PAES), non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), and non-ribosomal-peptide-polyketides (NRPSs/PKSs) hybrid synthetases are shown. Domain abbreviations are as follow: ArCP: aryl carrier protein, A:adenylation, C: cyclization, PCP= peptidyl carrier protein, TE, thioesterase, AT: acyl transferase, KS: ketoacyl synthase, KR: β-ketoreductase, ACP: acyl carrier protein, C: condensation, E: epimerase (Quadri, 2000; Quadri *et al*., 1998).

The gene products of this now renamed *mbt*-1 cluster encode proteins that resemble peptide arylation enzymes (PAEs), nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs). Based on analysis of their amino acid sequences, the catalytic function and domain organization of the enzymes could be predicted to include: a peptide arylation enzyme (MbtA), three nonribosomal peptide synthetases (NRPSs), MbtB, MbtE, MbtF and two polyketide synthases (MbtC, MbtD) (Figure 13). Hence, all the machinery necessary to produce the complex structure of the mycobactin core from simple building blocks is contained in these modular enzymes (Quadri, 2000; Quadri *et al*., 1998).

Because both mycobactin and carboxymycobactin share the same core structure, then both mycobacterial siderophores may be derived from a common precursor "pre-mycobactin". The deletion of the *mbtB* gene from the *mbt-1* cluster of *M. tuberculosis* resulted in a mutant unable to produce either mycobactin or carboxymycobactin, supporting the above hypothesis (De Voss *et al*., 2000). Clearly, the phenotype of the mutant linked the production of mycobactin and carboxymycobactin to the same enzymes. In addition to being impaired for growth in iron-deficient medium, this mutant did not grow in macrophage-like THP-1 cells, which suggested that mycobactins are important virulence factors for *M. tuberculosis* (De Voss *et al*., 2000).

Comparing the genome of *M. tuberculosis* and the sequenced genomes of other mycobacterial species revealed the presence of similar *mbt*-1 gene clusters for mycobactin-carboxymycobactin biosynthesis in *M. smegmatis* mc^2155, *M. bovis* AF2122/97, *M. bovis* BCG Pasteur 1173P2, *M. avium* subsp. *avium* strain 104, *M. avium* subsp. *paratuberculosis* K-10 and *M. marinum* M (Quadri & Ratledge, 2004). Mycobactin genes are not found in *M. leprae* TN (Cole *et al*., 2001a), therefore it is a pathogenic *Mycobacterium* lacking both mycobactin and carboxymycobactin siderophores, which is consistent with an early observation (Kato, 1985).

These comparisons of *mbt*-1 gene clusters indicated that there are significant differences in the organisation of the genes present in the cluster of different species. The

clusters of *M. tuberculosis* and *M. bovis* appears to be identical, however, in the genomes of *M. smegmatis*, *M. avium* and *M. avium* subsp. *paratuberculosis mbtJ* and *mbtI*, were not found at the 5' ends or in close proximity to the clusters. Likewise in *M. marinum*, *mbtI* is not present in the cluster (Quadri & Ratledge, 2004).

The observation that *M. avium* subsp. *paratuberculosis* cannot produce mycobactin is also explained by analysis of the *mbt*-1 gene cluster. Comparison of the *mbtA*-*mbtJ* cluster of *M. tuberculosis*, *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* show three significant differences. Firstly, in *M. tuberculosis*, *mbtI* and *mbtJ*, are adjacent to each other and immediately downstream of the mycobactin-carboxymycobactin biosynthesis gene cluster. However, in *M. avium* subsp. *paratuberculosis* and *M. avium*, there is a 6.6 and 5.7 kb gap, respectively, between the two genes. Moreover, there is 19.3 kb gap in *M. avium* subsp. *paratuberculosis* and 197.3 kb gap in *M. avium* between *mbtJ* and *mbtA*. The distance between these genes does not explain why *M. avium* subsp. *paratuberculosis* cannot produce mycobactin-carboxymycobactin, since *M. avium* subsp. *avium* is still able to make these siderophores. Secondly, a frame-shift mutation was identified in both *mbtB* and *mbtE* from both *M. avium* subspecies when compared to *M. tuberculosis*. The presence of these frame-shifts in *M. avium* subsp. *avium* suggests that this difference is not the limiting factor which affects mycobactin-carboxymycobactin production in *M. avium* subsp. *paratuberculosis*. However, the major difference between the *mbt*-1 gene clusters was found in the *mbtA* gene. The *mbtA* gene is shorter in *M. avium* subsp. *paratuberculosis*, encoding a 400 amino acid protein, compared with 565 and 551 amino acid residues in *M. avium* subsp. *avium* and *M. tuberculosis*. Hence, the abolition of mycobactin-carboxymycobactin production in *M. avium* subsp. *paratuberculosis* may be explained by the truncation of MbtA (Li *et al*., 2005; Semret *et al*., 2004).

1.6.2 Unified model for mycobactin-carboxymycobactin biosynthesis.

Several siderophore biosynthetic loci have been recently identified from diverse pathogenic bacteria and characterised both genetically and biochemically (Crosa & Walsh, 2002; Miethke

& Marahiel, 2007; Quadri, 2000). Analyses of the loci for pyochelin and pyoverdin (both produced by *P. aeruginosa*), yersiniabactin (*Yersinia pestis*), vibriobactin (*Vibrio cholera*) and enterobactin produced by diverse enterobacteria, such as *E. coli*, *Salmonella enterica*, *Klebsiella spp*. and *Shigella spp*., all encode PAESs, NRPSs, PKSs, and NRPSs/PKSs hybrid modular enzymes and are useful for comparison with the mycobacterial *mbt*-1 cluster. There are three features that unite the biosynthesis of all these siderophores. Firstly, they all incorporate aryl acids, 2,3-dihydroxybenzoate (DHB) or 2-hydroxybenzoate (salicylic acid), and are thus designated as aryl-*N*-capped siderophores. Secondly, they all contain thiazoline and oxazoline rings, which provide ligands for binding ferric iron (Crosa & Walsh, 2002; Miethke & Marahiel, 2007; Quadri, 2000).

Although, the genes required for the core mycobactin-carboxymycobactin core structure have been identified and their product function predicted, most of the models for the biosynthesis of these mycobacterial siderophores are still unknown; only the aryl carrier protein of MbtB, MbtA, MbtG, MbtI, and PptT for *mbt*-1 cluster, have been characterised (Harrison *et al*., 2006; Krithika *et al*., 2006; Quadri *et al*., 1998). Recently, a second cluster of genes, *mbt*-2, encoding the acyl chain transfer to the mycobactin core, were also identified (Krithika *et al*., 2006).

Despite the lack of genetic and biochemical characterisation, de Voss *et al*. (1999) proposed a biosynthetic model for salicylate-containing pre-mycobactin (Figure 14). The first committed biochemical reaction is the conversion of salicylic acid from chorismate by MbtI. Following salicylate loading of the aryl carrier protein domain of MbtB, by MbtA *via* an aryl adenylate, the adenylation domain of MbtB activates either serine or threonine, condenses it with the salicylate moiety, and cyclises this product to form hydroxyphenyloxazoline. MbtE and MbtF have the appropriate activation, condensation, and peptide carrier domains for donation of the two lysine-derived moieties of mycobactins.

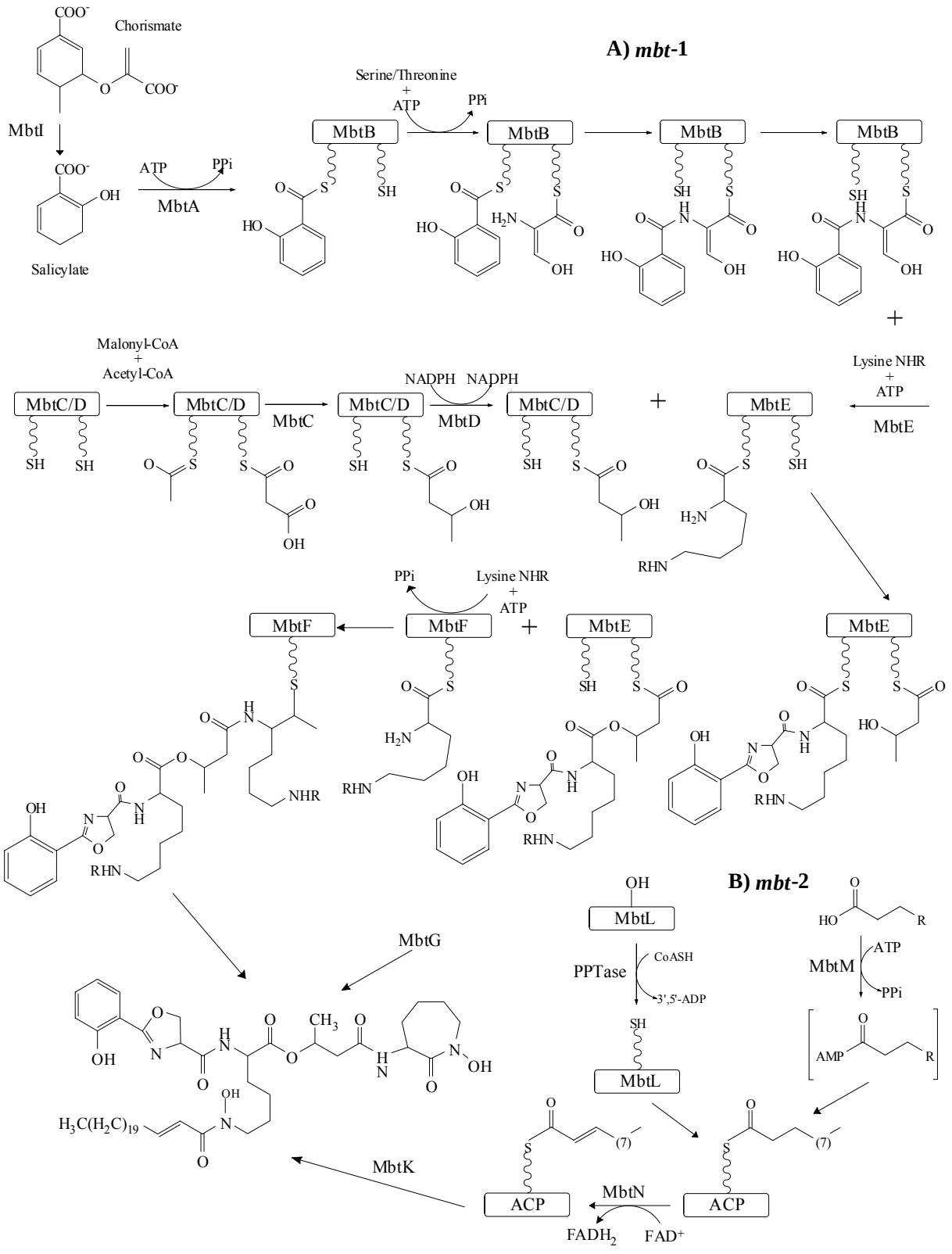

A) mbt-1
Chorismate
MbtI
Salicylate
ATP
PPi
MbtA
Serine/Threonine
+
ATP
PPi
MbtB
Malonyl-CoA
+
Acetyl-CoA
MbtC/D
MbtC
NADPH NADPH
MbtD
Lysine NHR
+
ATP
MbtE
PPi
Lysine NHR
+
ATP
MbtF
B) mbt-2
MbtL
PPTase
CoASH
3',5'-ADP
MbtM
ATP
PPi
MbtG
MbtK
MbtN
FADH2
FAD+
ACP

Figure 14. Proposed biosynthetic cascade catalysed by the mycobactin-carboxymycobactin gene clusters. A) The *mbt*-1 gene cluster produces the core structure (Quadri *et al*., 1998), whereas B) the *mbt*-2 produces acyl-S-ACP intermediates, which are subsequently transferred to the core structure to produce didehydroxymycobactin. The hydroxylation of the lysine residues is carried out by the MbtG to generate the functional amphiphilic mycobactin siderophore (Krithika *et al*., 2006). However, most of the enzymatic reactions to produce the core structure are still unknown and the putative function of the MbtC, MbtD, MbtE, MbtF, MbtH and MbtJ awaits genetic and biochemical characterisation.

MbtF has a terminal domain that was assigned a role either as an epimerisation domain or as thioesterase responsible for releasing the mycobactin from the enzyme by lactamisation of the terminal hydroxylysine residue. MbtC and MbtD are homologous proteins that might function as polyketide synthases and appear to contain the appropriate modules to donate the required β-hydroxybutyrate residue. The MbtG protein is a FAD-containing monooxygenase required for the production of the ε-N-hydroxylysines. MbtH and MbtJ are proposed to be involved in mycobactin biosynthesis, although their biochemical role is not yet clear (De Voss *et al*., 1999).

The biochemical characterization of some enzymes involved in mycobactin-carboxymycobactin supports the proposed model. The last gene in the *mbt*-1 cluster, Rv2386c, was originally annotated as *trpE2*; its product resembles the large subunit of anthranilate synthase, the enzyme that catalyses the first committed step in tryptophan biosynthesis (the conversion of chorismic acid to anthranilic acid). However, the expression of Rv2386c is up regulated under iron-low environments, and this indicated that its role could be in siderophore biosynthesis (Gold *et al*., 2001). Hence, the Rv2386c gene was re-annotated as *mbtI*, putatively encoding the enzyme that shares homology to a family of isochorismate synthase, anthranilate synthase and salicylate-forming enzymes, consistent with a role in the conversion of chorismate to salicylate, the starter unit of pre-mycobactin biosynthesis (Quadri *et al*.,

1998). Recently, the crystal structure and biochemical function of the MbtI protein have been characterised, both ring isomerisation and pyruvate lyase activities being demonstrated. This lead to the conversion of chorimate to salicylate demonstrating the first step in pre-mycobactin biosynthesis (Harrison *et al*., 2006). The same mechanistic conversion of chorismate to salicylic acid has also been reported in *Y. enterocolitica* Irp9 (Kerbarh *et al*., 2005; Pelludat *et al*., 2003). The second step is carried out by the lone-standing aryl acid adenylation (A) domain of MbtA, converting salicylate to an aryl adenylate. The function of MbtA as salicylate-AMP ligase was demonstrated using the classical ATP-[^{32}P]-PPi exchange assay. Next, the acyl-adenylate is selectively transfered to the N-terminal (holo-ArCP domain) of MbtB, which has been followed using [^{14}C] salicylate (Quadri *et al*., 1998). Clear similarities exist in the genes encoding homologous functions in the biosynthesis of other salicylate-capped siderophores, such as pyochelin produced by *P. aerugionsa* (Quadri *et al*., 1999) and yersiniabactin produced by *Y. pestis* (Gehring *et al*., 1998a). Significant homology is also apparent with the adenylation proteins participating in siderophore biosynthesis in *E. coli* (EntE) (Gehring *et al*., 1997a), DhbE from *Bacillus subtilis* (May *et al*., 2002) and VibE from *V. cholerae* (Keating *et al*., 2000b), which all activate dyhydroxylated aryl acid (DHB) instead.

In mycobactins and structurally-related mixed aryl-capped hydroxamate siderophores such as vibriobactin (Keating *et al*., 2000b), acinetobactin (Yamamoto *et al*., 1994) and frankobactin (Boyer *et al*., 1999), oxazoline rings are found as a result of serine or threonine cyclisation. In vibriobactin bioynthesis, the oxazoline ring formation is catalysed by a cyclisation domain (Cy) of VibF, a NRPS. Here DHB is first adenylated by VibE and then transferred to VibB, an aryl carrier protein, to form the aryl N-cap donor DHB-S-pantetheinyl-VibB. The VibF protein provides two cyclisation domains, two condensation domains, an adenylation domain and a peptidyl carrier protein (PCP), the domains required for condensation, heterocyclisation and dehydration, leading to oxazoline ring formation. The A domain of VibF activates L-threonine and attaches to the HS-pantetheinylated PCP to yield the threonyl-S-PCP substrate for condensation/heterocyclisation by one of the two Cy domains with the aryl moiety derived from the DHB-S-VibB donor to form the oxazoline ring

in vibriobacin (Keating *et al*., 2000b). Similar biochemical reactions might occur for the formation of the oxazoline ring during pre-mycobactin biosynthesis, in which the interaction of the MbtA and MbtB proteins are involved, but this scenario awaits further investigation.

As was outlined before, each of the carrier domains must be converted from the *apo* form to the *holo* form by adding the phosphopantetheinyl arm by a dedicated phosphopantetheinyl transferase (PPTase) (Lambalot *et al*., 1996). In *E. coli* there are two types of PPTases, one is specific for *apo*-ACP domains in fatty acid and polyketide synthases, CP-specific PPTase (AcpS), and the second specific for modification of *apo*-PCP and *apo*-ArCP domains (EntD domain) in peptide synthetases (Gehring *et al*., 1997a). Using these PPTases as models, homologues were identified in the *M. tuberculosis* genome. One of these homologues (33% similar to *E. coli* AcpS) was designated as mycobacterial AcpS (*Rv2523c*) (Cole *et al*., 1998) and the second homologue had similarity to EntD (30%) and was designated PptT (Rv2794c) (Quadri *et al*., 1998). Normally, a PPTase is encoded in a siderophore biosynthetic gene cluster (Lambalot *et al*., 1996). Interestingly, neither the mycobacterial *acpS* nor *pptT* genes are encoded near the *mbt*-1 gene cluster.

Recently the function of the MbtG protein was investigated (Krithika *et al*., 2006). This protein is homologous to ornithine and lysine oxygenases, which are responsible for the production of ε-hydroxylysines to generate functionally important hydroxamate moietes in siderophores. *In vitro*, the protein was able to carry out the hydroxylation of both lysine and an acylated lysine in presence of NADPH and FAD with a clear preference for the latter (Krithika *et al*., 2006). This preference might support a hypothesis in which the oxygenation of the two lysines of pre-mycobactin occurs after their incorporation into the siderophore precursor and is consistent with the isolation of a proposed mycobactin precursor didehydroxymycobactin that has been taken to suggest that hydroxylation might be the last step in mycobactin biosynthesis (Moody *et al*., 2004). However, this model must be considered with some caution until a more complex study of MbtG function is completed: the two lysinyl residues are diverse structurally, one being *N*-acylated and the other being cyclised, and thus it is implied that MbtG has a very relaxed substrate specificity.

Alternatively, the preference exhibited for the acylated lysine could be artefactual in that the effective concentration of the substrate might be exaggerated by the recruitment of the enzyme to vesicles of this amphiphilic substrate, a property not shared with the unmodified amino acid.

In order to reconcile the annotation of MbtJ as acetyl hydrolase, Ratledge (2004) has suggested that the "pre-mycobactin" nucleus might be synthesized with an acetyl group attached to both N^6 atoms of the lysine residues. Both groups will remain until the hydroxylation occur in both lysyl-N^6 atoms is required to yield the two hydroxamate ligands (Ratledge, 2004). The hydroxylation events and the subsequent cyclisation of the terminal lysine residue and acylation of the central residue would thus be facilitated by the deacetylation activity of MbtJ.

These issues will be clearly resolved by the construction and careful analysis of knock-out mutants in *mbtG* and *mbtJ*, but a comparison of the kinetics of aminoacyl adenylation of the A domains of MbtE and MbtF, the NRPSs proposed to incorporate the pre-mycobactin lysines, using lysine, N-acetyl and N-hydroxy-lysine, would also provide further mechanistic insight.

1.6.3 The *mbt*-2 gene cluster.

Because mycobactin and carboxymycobactin share the same core structure, it has been suggested that both siderophores are synthesised by the same multienzymatic reactions to yield a common precursor "pre-mycobactin", but the final step must be different due to the different alkyl groups in these mycobacterial iron chelators. In both mycobacterial siderophores the long chain is usually ligated to the central lysine residue but no candidate acyltransferase encoding genes were identified or near the *mbt*-1 cluster. However, good gene candidates for the addition of the long-chain were suggested using transposon mutagenesis (LaMarca *et al*., 2004). Insertion of Tn611 into *M. smegmatis* LR222 yielded, two potential mycobactins mutants, designated LUN8 and LUN9. LUN8 produced a ferric-siderophore

whereas LUN9 failed to yield siderophore. The mycobactins produced by the wild type and LUN8 strains were compared by an incomplete NMR analysis, the latter produced a modified mycobactin, in which the long acyl-chain was abnormal (LaMarca *et al*., 2004).

Nucleotide sequencing showed that the insertion-flanking region from LUN9 shared homology with the mycobactin biosynthesis genes *mbtD* and *mbtE* of *M. tuberculosis*, consistent with disruption of pre-mycobactin biosynthesis and the lack of mycobactin-like siderophores. The transposon insertion in LUN8 was located in a region that encoded orthologues of *M. tuberculosis* FadD33 (Rv1345, an acyl-CoA synthase), AcpA (Rv1344, an acyl carrier protein) and FadE14 (Rv1346, an acyl CoA-dehydrogenase) and specifically disrupted the sequence encoding FadD33. Complementation of LUN8 with plasmids encoding native FadD33 and FadE14 restored mycobactin biosynthesis, the resulting transformant produced the typical mycobactin S. Based on sequence, FadD33 and FadE14 are the enzymes required to initiate modification of a pre-existing lipid with subsequent attachment of the lipid to an acyl carrier protein with final transfer to the mycobactin core by an acyl-transferase (LaMarca *et al*., 2004).

The Rv1347c gene of *M. tuberculosis* was predicted to encode for an aminoglycoside 6'-N-acetyltransferase, however the crystal determination of its product suggested a different biochemical function (Card *et al*., 2005). Rv1347c is a member of the GCN5 related N-acetyltransferase (GNAT) family of enzymes. Bioinformatics analysis and modelling experiments suggested that Rv1347c functions in the acylation of the *N*-hydroxylysine residues of mycobactin. An indication that Rv1347c may have the ability to transfer long acyl groups necessary for mycobactin-carboxymycobactin biosynthesis was provided by the observation that the enzyme showed thioesterase activity (an essential part-reaction in acyl transfer) with a range of acyl CoA substrates with a clear preference for longer acyl chains (Card *et al*., 2005).

Recently, the biochemical function of those putative genes was elucidated and another cluster for mycobactin biosynthesis was proposed, *mbt*-2. The cluster was identified by

computational analysis of iron-dependent genes regulated by IdeR repressor with subsequent biochemical characterisation of the proteins (Krithika *et al*., 2006). These studies confirmed that this second gene cluster is responsible for the acyl substitution of different aliphatic chain lengths and for introducing the unusal *cis* double bond conjugated to a carbonyl group that distinguish mycobactin and carboxymycobactin from other amphiphilic siderophores, such as marinobactin, aquachelin and amphibactin (Krithika *et al*., 2006). This second cluster is formed by a fatty acyl-AMP ligase (*fadD33*= Rv1345), an acyl carrier protein (ACP= Rv1344), *fadE14* (Rv1346) homologous to acyl-CoA dehydrogenase and the acetyl transferase (Rv1347c) which is transcribed in the opposite direction (Krithika *et al*., 2006).

The enzyme FadD33 (Rv1345) was characterised as a fatty acyl-AMP ligase (FAAL) which is responsible for converting fatty acids into acyl adenylates. This mode of activation and transfer of fatty acids is contrary to the previously described universal mechanism, involving the formation of acyl-coenzyme A thioesters (Trivedi *et al*., 2004). The enzyme is able to use a variety of medium and long chain fatty acids to form acyl adenylates. Then, the activated acyl adenylates are transferred onto the phosphopantetheinyl group of AcpA (Rv1344) (Krithika *et al*., 2006).

The fatty acid chain of mycobactin contains an unusual double bond. The formation of double bonds is normally catalysed by an acyl-CoA dehydrogenase during β oxidation of saturated fatty acids where FAD is the electron acceptor (Nelson & Cox, 2004). The *fadE14* gene, a proposed acyl-ACP dehydrogenase, could catalyse this reaction. The next step in the assembly of the amphiphilic mycobactin would involve *N*-acylation of these ACP tethered long-chain fatty acids directly onto the ε-amino group of lysine. This reaction is catalysed by the novel proposed acetyl-transferase (Rv1347c) using a wide range of medium to long chain fatty acids (C_6-C_{16}) (Krithika *et al*., 2006). Whether this single enzyme participates in the acylation of pre-mycobactin toward the synthesis of both mycobactin and carboxymycobactin is yet to be established.

Hence, the genes described above carry out the incorporation of an unsaturated fatty acid chain in to the core structure of the cell-bound mycobactins. Following the nomenclature for the existing mycobactin cluster for the biosynthesis of the core structure, the four genes are annotated as *mbtK*, *mbtL*, *mbtM*, and *mbtN* for Rv1347c, Rv1344, fadD33 (Rv1345), and fadE14 (Rv1346), respectively (Table 6) (Krithika *et al*., 2006).

Probably, after the synthesis of the core structure, "pre-mycobactin" is transferred from the cytoplasm, where its biosynthesis may occur, and it is finally positioned in the cell envelope (Ratledge, 2004). Carboxylic and methyl ester groups, positioned in carboxymycobactins, suggest that the final biosynthetic steps must be different. A dicarboxylic acid has been proposed as precursor for the carboxylic acid end, but nothing is known about this step and the genome does not indicate how it might be accomplished (Ratledge, 2004).

Table 6. Characteristics and designation of proteins proposed for *mbt*-2 in *M. tuberculosis* (Krithika *et al*., 2006).

Protein name	Proposed function	Amino acids	Molecular weight (Da)	Gene name
MbtK	Acetyl transferase	210	23 798.94	*mbtK*
MbtL	Acyl carrier protein	106	11 399.93	*mbtL*
MbtM	Fatty acyl-AMP ligase	521	53 957.26	*mbtM*
MbtN	Acyl-ACP dehydrogenase	386	41 180	*mbtN*

1.7 Transport of ferri-siderophore across the membrane.

Differences in the complexity of ferri-siderophore uptake between Gram-negative and Gram-positive bacteria are necessitated by the former's possession of an outer membrane (OM), essentially transport requires additional proteins (receptors and accessory proteins). It is the challenge of crossing this outer membrane that shall be considered first; the elements of the subsequent steps in Gram-negative bacteria are very similar to the processes employed by Gram-positive bacteria. The uptake of ferri-siderophore requires their efficient capture at the cell surface. Gram-negative bacteria synthesise a range of specific high-affinity receptor proteins that effectively concentrate these ligands at the cell surface (Ratledge & Dover, 2000).

Porins are membrane transporters that allow energy-independent passage of solutes of molecular weight ≤ 600 Da across the membrane. These proteins are found in the outer membrane of Gram-negative bacteria, acid fast Gram-positive bacteria, plastids, chloroplasts and mitochondria (Saier, 2000). Siderophores and haem are too big to cross the outer membrane through porins and are also present at low concentrations in the extracellular medium, making necessary the production of high affinity protein receptors (Wandersman & Delepelaire, 2004). For this reason, the first step in the internalisation process, for Gram-negative bacteria, requires that outer membrane receptor proteins bind ferri-siderophores with high specificity (Andrews *et al*., 2003; Ratledge & Dover, 2000; Wandersman & Delepelaire, 2004).

Currently, the structures of four metal-chelate receptors are known. FepA (Buchanan *et al*., 1999), FhuA (Endriss *et al*., 2003; Locher *et al*., 1998) and FecA (Ferguson *et al*., 2002; Yue *et al*., 2003) are the receptors for enterobactin, ferrichrome, and ferric dicitrate, respectively, and BtuB is the receptor for vitamin B12. The latter though, is not a siderophore, but is also transported through a TonB-dependent receptor (Chimento *et al*., 2003). These structures show remarkable similarities. All of them consist of 22 anti parallel β strand barrels formed by ~600 C-terminal amino residues and a plug formed by ~150 N-terminal residues. The β strands are connected on the extracellular side by loops of variable length while in the

periplasmic side they are connected by shorter loops. The plug or cork domain consists of multiple β short strands connected *via* loops and α helices. Mutagenesis studies of the FepA and FhuA plug domains suggest that conformational rearrangements of this domain are responsible for substrate passage rather than complete displacement of the plug (Chakraborty *et al*., 2007). A representative of this type of receptor is shown in Figure 15.

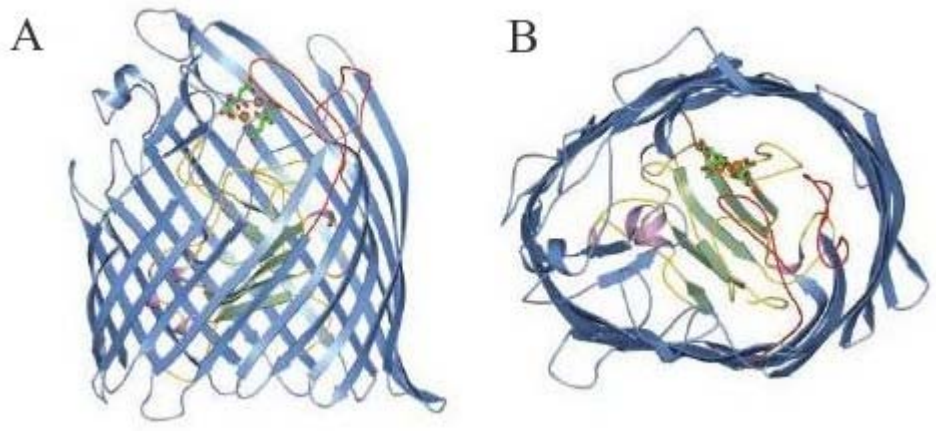


Figure 15. Crystallographic structure of FecA. A) The 22-stranded β barrel is shown in blue. The molecule is presented as found in the outer membrane with extracellular loops extending into the solvent (top) and periplasmic turns oriented toward the periplasm (bottom). The plug domain is shown in green, with helices in purple and loops in yellow. B) Dinuclear ferric citrate is represented as a bond model with oxygen atoms in red and carbon atoms in green; both ferric ions are shown as orange spheres [Adapted from Ferguson *et al*., (2002)].

Once these receptors bind ferri-siderophore complexes, they change their conformation in the external loop and periplasmic pocket; this is believed to prepare the complex and receptor for the next stage in the transport process. The outer membrane siderophore receptors are induced by low concentrations of iron and are not present under iron-sufficient conditions (Andrews *et al*., 2003). In *E. coli* K-12, there are at least six OM receptors which acquire iron *via* eight different iron-chelate complexes, either produced by itself (enterobactin) or using other siderophores produced by other organisms (coprogen, ferrichrome, ferrioxamine and rhodotorulic acid).

Although binding of ferri-siderophores to OM receptors is independent of other factors, transport of ferri-siderophores through OM receptors requires energy. This energy is provided by the electrochemical charge gradient of the cytoplasmic membrane (CM) and is delivered by the energy-transducing Ton protein complex (Higgs *et al*., 1998; Larsen *et al*., 1994). The Ton system consists of three proteins, TonB, ExbB and ExbD that interact at the cytoplasmic membrane (CM) (Bradbeer, 1993; Braun, 2003; Cadieux *et al*., 2002; Faraldo-Gómez & Sansom, 2003). ExbB and ExbD are integral CM proteins, while TonB is periplasmic and anchored to the CM by its hydrophobic N-terminal domain (Letain & Postle, 1997). The energy transduction requires direct contact between TonB and receptors.

In *E. coli*, there is a conserved hydrophobic seven-amino acid segment, the Ton box, at the N terminus of TonB-dependent OM receptors and has been found to be required for TonB-mediated uptake of ferri-siderophores (Postle, 1993). A mutation study showed that substitution in this region inactivated the transport capability of the receptors, but complementary TonB substitutions reversed the effect, suggesting that the TonB box region interacts physically with the TonB protein (Bell *et al*., 1990; Moeck *et al*., 1997; Schöffler & Braun, 1989; Tuckman & Osburne, 1992). The interaction of TonB with the TonB box of OM receptors, including FhuA, may be only an initial event in the transport of siderophores, and further interaction between TonB and the various siderophores receptors may be necessary to effect siderophore uptake (Braun, 1997). It is believed that ExbB and ExbD use the membrane electro-chemical charge gradient to produce an "energised" form of TonB that mediates a conformational change in the liganded OM receptor (Reynolds *et al*., 1980; Wooldridge & Williams, 1993). In some bacteria, there is more than one TonB-ExbB-ExbD system. *Vibrio cholerae* contains two TonB proteins, TonB1 and TonB2, which appear to have specificities for different OM receptors (Mey & Payne, 2001). TonB1 is associated with ExbB1, ExbD1 and haem-specific permease, HutBCD, on chromosome II, and TonB2 is associated with ExbB2, ExbD2 on chromosome I. *V. cholerae* produces haemoglobin receptors: HutA, HutR, and HasR, and several other receptors for siderophores, including ferrichrome, vibriobactin (ViuA), and enterobactin (IrgA, VctA) (Occhino *et al*., 1998).

1.7.1 Transport across the periplasm and cytoplasmic membrane.

Transport of ferri-siderophore complexes across the periplasmic space and cytoplasmic membrane is mediated by periplasmic binding protein-dependent transport (PBT) systems and associated CM transporters (Nikaido & Saier, 1992).

Although Gram-positive bacteria lack an OM, binding protein-uptake systems occur, and these proteins must be anchored to the cell to concentrate nutrients at the cell surface. Such binding proteins found in Gram-positive bacteria are similar in structure and organization to the periplasmic binding-dependent systems of Gram-negative bacteria (Nikaido & Saier, 1992).

Each binding protein accepts one ferri-siderophore complex at a time. It is unclear if the binding proteins of Gram-negative systems collect the ferri-siderophore directly from the OM receptors, or if the binding proteins only pick up the ferri-siderophore from the periplasm (Köster, 2001). The PBT belongs to the subclass of the ATP Binding Cassette (ABC) superfamily of transport proteins which are energised by ATP (van der Does & Tampe, 2004). The ABC transporters are a class of proteins that use energy from ATP hydrolysis to pump a diverse set of compounds across biological membranes. They require a minimum of four domains. Two transmembrane domains (TMDs) form the ligand binding sites and provide specificity. The TMDs span the membrane multiple times *via* putative α-helices, usually six predicted *per* domain. The other two domains are the nucleotide binding domains (NBDs) that bind and hydrolyze ATP to drive the tranlocation of the bound ligand. The NBDs are hydrophilic and periferically associated with the cytoplasmic face of the membrane (Davidson & Maloney, 2007).

Although, most eukaryotic ABC transporters export hydrophobic molecules from the cytoplasm (Dean & Allikmets, 2001), bacterial ABC transporters predominantly import essential nutrients that are delivered to them by specific binding proteins (Nikaido & Hall, 1998). The binding proteins act as a shuttle, collecting the ferri-siderophore released from the

OM receptor and delivering it to a specific permease in the inner membrane (Andrews *et al*., 2003).

Although, *E. coli* K-12 possesses six iron-transporting OM receptors, it only contains three associated binding-protein dependent ABC systems implicated in iron transport (Köster, 2001). The FecB protein of the Fe(III)-dicitrate transport system (Staudenmaier *et al*., 1989), the FepB protein of the ferric-enterochelin transport system (Elkins & Earhart, 1989), and the FhuD protein which binds ferric-hydroxamates, such as ferrichrome, coprogen, rhodotorulic acid, ferrioxamine A, B, and E, and aerobactin (Clarke *et al*., 2002; Clarke *et al*., 2000; Köster & Braun, 1990). While Fe-siderophores that bind to OM receptors are completely buried in their external cavities prior transport, there are fewer residues involved in the recognition of ligand interaction; this is consistent and therefore there is a need for several OM receptors. On the other hand, the ABC transporters show higher ligand promiscuity for substrate binding compared to that of OM receptors (Davidson & Maloney, 2007).

Although, Gram-positive bacteria lack an OM, the existence of permeases and their associated ATP-binding, they share similarities with Gram-negative bacteria systems. In Gram-positive bacteria, the binding proteins must be attached to the cell to concentrate nutrients at the cell surface (Nikaido & Hall, 1998).

Similarly, a periplasmic Fe(III) binding protein (FbpA) has been identified for both *Haemophilus* and *Neisseria*, and are associated with receptors for iron binding proteins, such as TbpAB (Adhikari *et al*., 1995; Dhungana *et al*., 2003; Taboy *et al*., 2001). In *Staphylococcus aureus*, a Gram-positive bacterium, two FhuD homologues, FhuD1 and FhuD2 have been found. The protein FhuD2 is involved in the transport of iron(III) in complex with ferrichrome, ferrioxamine B, aerobactin, and coprogen, whereas FhuD1 shows a more limited substrate range, capable of only iron(III)-ferrichrome and iron(III)-ferrioxamine B transport in *S. aureus* (Sebulsky & Heinrichs, 2001).

Iron uptake in mycobacteria is more complex since, the production of siderophores depends whether the strain is pathogenic or saprophytic. *M. tuberculosis* produces two types of mycobactins, the cell-bound mycobactin (intracellular) and the extracellular carboxymycobactin, whereas *M. smegmatis* can produce both mycobactins as well as the extracellular exochelins (Ratledge, 2004). The uptake of ferric exochelins into saprophytic mycobacteria, involves the transfer of the ferric-exochelin complex which requires the input of metabolic energy (Ratledge, 2004). There are various proteins involved in the uptake process. Ferric-exochelin is probably recognised by its receptor, a 29 kDa protein (Dover & Ratledge, 1996) and then the complex is taken across the cell envelope, possibly in association with the FxuD protein. The participation of the ferric-exochelin uptake proteins, FxuA, FxuB and FxuC, which share amino-acid sequence similar to the ABC transporters, might be required for translocation of the complex across the membrane (Fiss *et al*., 1994). To release the iron from the exochelins to the cytoplasm, a reductase is involved in which the ferric iron is reduced in the presence of NAD(P)H to ferrous iron (Brown & Ratledge, 1975b). After releasing iron, the exochelin is transferred back into the extracellular environment of the cells mediated by the Exit protein, in conjunction with other proteins (Zhu *et al*., 1998).

On the other hand, two genes encoding for ABC transporters, *irtA and irtB*, as well as the function of Rv2895c, have been identified and characterised to mediate siderophore transport both in *M. tuberculosis* and *M. smegmatis* (Farhana *et al*., 2008; Rodriguez & Smith, 2006). IrtA and IrtB have motifs typical of integral membrane ABC transporters, and they closely resemble the *Y. pestis* iron transporter YbtPQ (Rodriguez & Smith, 2006). Rv2895c is also predicted to be extracytoplasmic (Farhana *et al*., 2008). Inactivation of *irtA* and *irtB* has no effect on growth of *M. tuberculosis* in high-iron conditions, but it does affect the ability of the bacilli to multiply under iron-low conditions. The IrtAB system is important for replication not only under iron-deficient conditions *in vitro*, but also in human macrophages and mouse lungs. Based on amino-acid sequence, the N-terminal of IrtA was suggested to act as substrate-binding protein (SBP), as well as a translocation system, features which are required for recognition and transport of the substrate into the cell (Rodriguez & Smith, 2006).

The current model for iron transport in *M. tuberculosis* suggests a transfer of iron from Fe-carboxymycobactin to mycobactin in the cell surface, the latter functioning as an iron storage molecule in the cell envelope (Ratledge, 2004). However, results shown in the study conducted by Rodriguez and Smith (2006) and Farhana *et al*. (2008) suggest a different pathway. In the proposed model, the carboxymycobactin is transported outside the cytoplasm in an energy dependent manner by means of the IrtA exporter protein. Then, the carboxymycobactin extracts iron from host molecules, such as transferrin and ferritin. The Fe^{3+}-carboxymycobactin complex is subsequently internalised and assimilated into the mycobacterial cytoplasm in which the interplay of the IrtB-Rv2895c importer system is involved (Farhana *et al*., 2008). Since only carboxymycobactin was tested during these experiments, there is still controversy how both mycobactins contribute exactly during iron uptake and storage in *M. tuberculosis*.

1.7.2 Intracellular release of iron into bacteria cell.

Once internalised, the ferri-siderophore complex must be dissociated to liberate the complexed iron for use in cellular metabolism (Andrews *et al*., 2003). There are two processes suggested for iron release from siderophores after passing through the OM and CM.

In the first hypothesis, siderophores have a much lower affinity for ferrous iron than for ferric iron, and iron release may therefore be spontaneous after iron reduction by free intracellular electron donors or by more specific siderophore reductases (Pierre *et al*., 2002; Schröder *et al*., 2003). After iron is released, the siderophore may be recycled for another cycle ferric iron uptake. This hypothesis has been controversial because specific ferri-siderophore reductases have not been isolated, efflux of unloaded siderophores has not been studied in detail, and siderophores have such a low redox potential that iron reduction by intracellular electrons donors is only likely if coupled with efficient Fe(II) trapping (Müller *et al*., 1998). In addition, purified bacterial ferri-siderophore reductases are neither substrate specific nor subjected to iron-dependent regulation (Halle & Meyer, 1992).

Only two classes of ferric reductases are known. The soluble prokaryotic flavin reductases and the membrane cytochrome β-like reductases, found in eukaryotes (Pierre *et al*., 2002). All soluble ferric reductases seem to be biochemically similar and the majority use NADH or NADPH as the electron donor for ferric iron reduction (Schröder *et al*., 2003). The next general reaction occurs in all cases:

$$\text{Fe(III)-siderophore} + \text{NAD (P) H} \rightarrow \text{Fe(II)-siderophore} + \text{NAD (P)}^{+}$$

and thus, Fe(II) is now available to be removed from siderophore and enter cytoplasm, and therefore it is able to be incorporated into porphyrins or into apoproteins.

Many bacterial ferric iron reductases have been described from a variety of different bacteria including *E. coli, Bacillus subtilis, Mycobacterium paratuberculosis, M. smegmatis, Neisseria gonorrhoeae, Pseudomonas aeruginosa,* and *Vibrio vulnificus* (Schröder *et al*., 2003). The localisation of ferric reductases, in the cytoplasm, periplasm and cytoplasmic membrane, depends on the bacterial species and the strategy for iron uptake (Schröder *et al*., 2003). The majority of the ferric reductases are either localised in the bacterial cytoplasm or in the periplasm of Gram-negative bacteria. *P. aeruginosa* has been described to contain a periplasmic ferri-pyochelin reductase (Cox, 1980). Other pathogens like *Listeria monocyto-genes*, a Gram- positive, produce a surface-bound ferric reductase that may also be secreted into the culture medium (Barchini & Cowart, 1996). Membrane-bound reductases have been found in *Spirillum itersonii* (Dailey & Lascelles, 1977), *E. coli* (Fischer *et al*., 1990) and *S. aureus* (Lascelles & Burke, 1978).

A new type of ferrisiderophore reductase was discovered with the *E. coli* FhuF protein that shows for the first time both an iron-dependent regulation mediated by the Fur repressor and substrate specificity to a set of Fe-hydroxamate siderophores including ferrioxamine B, Fe-coprogen and ferrichrome (Matzanke *et al*., 2004). However, the iron of some ferri-siderophores can not be reduced because their redox potentials are too negative and thermodynamically unfavourable (Pierre *et al*., 2002). For instance, the redox potential of

ferri-enterochelin/ferro-enterochelin is -750 mV at pH 7 and is the lowest of all siderophores. It has been considered that neither NAD nor NADP could ever act as reductants for ferric-enterochelin, because the redox potential for those reductants is -324 mV, and another mechanism would be required to release iron from the siderophore (Ratledge & Dover, 2000).

The second mechanism involves the intracellular breakdown of the siderophore and thus implies that some siderophores are used once without recycling them. In *E. coli* an esterase encoded by *fes* (located within a cluster of genes (*ent-fec*) involved in enterobactin biosynthesis) hydrolyses the ester bonds of internalised ferri-enterobactin producing dihydroxybenzoyl serine (Earhart, 1996). However, the function of the enzyme is unclear because *in vitro* the esterase can hydrolyze enterobactin as well as ferric-enterobactin. Its activity was four-fold higher in catalysing the hydrolysis of free enterobactin than ferric-enterobactin, indicating that the protein possesses a distinct esterase activity that is independent of any potential reductive mechanism of iron removal from the ferric enterobactin complex (Brickman & McIntosh, 1992).

Although the equilibrium of reaction is in favour of Fe(III)-enterochelin its reduction might proceed as long as the Fe(II) can be withdrawn from the reduced siderophore and effectively removed from participating in the back reaction, since the reactions are never in equilibrium (Ratledge & Dover, 2000). The function of the esterase may simply be to inactivate the potent chelator in the bacterial cytoplasm in order to minimise any adverse effects it might generate in bacterial iron homeostasis (Ratledge & Dover, 2000).

Recently, two other hydrolases have been found in pathogenic enteric bacteria, which are encoded by the *iroD* and *iroE* genes of the virulence-associated *iroA* gene cluster in *E. coli* and *Salmonella enterica* (Lin *et al*., 2005). The product encoded by these genes showed different substrate preference as well as cellular localization. IroE was predicted to be membrane-tethered and only hydrolyses cyclic salmochelins only once in the periplasm, leading to the release of linear salmochelin trimer. IroD, located in the cytosol, mediated iron release from the enterobactin and salmochelin complexes (Lin *et al*., 2005). However, another

study has shown different substrate specificities for these esterases: IroD preferred free iron enterobactin and salmochelin substrates over the ferri-siderophores, while IroE hydrolased both free iron-siderophores and iron-charged diglucosylated enterobactin (Zhu *et al*., 2005).

Another hydrolase has been characterised in *B. subtilis*, the Fe-bacillabactin esterase BesA (YuiL). The esterase was found to hydrolyse Fe-bacillabactin into monomers without generating detectable amounts of hydrolysis intermediates; iron-free bacillabactin used as substrate led to intermediate accumulation. Furthermore, the catalytic efficiency of BesA was 25-fold higher for ferri-bacillabactin than for iron-free siderophore (Miethke *et al*., 2006b).

1.7.3 Iron storage in bacteria.

After uptake, iron can be stored for eventual use when external iron supplies are restricted. Three types of iron storage proteins are recognised in bacteria. The archetypal ferritins which are also found in eukaryotes, the haem-containing bacterioferritins found only in eubacteria and the smaller Dps proteins present only in prokaryotes. All types can exist in the same bacterium and multiple ferritin or bacterioferritin genes are common (Andrews *et al*., 2003; Wandersman & Delepelaire, 2004). Dps proteins, ~ 250 kDa, have a lower storage capacity around 500 iron atoms (Zhao *et al*., 2002), while ferritins and bacterioferritins, ~ 500 kDa, can bind between 2000-5000 iron atoms (Carrondo, 2003). Ferritins and bacterioferritins use oxygen and peroxide, respectively, to bind soluble Fe^{+2}. Both ferritin and bacterioferritin are composed of 24 identical subunits, but Dps contains only 12 monomers. Specific sites, called ferroxidase centres, are found in the central region of individual subunits and catalyses the redox process in ferritin and bacterioferritins.

The X-ray structures of bacterioferritins from *M. smegmatis* and *M. tuberculosis* have recently been solved (Gupta *et al*., 2008; Janowski *et al*., 2008). Unexpectedly, the metal binding centre of bacterioferritin from *M. smegmatis* contains zinc instead of the typically observed iron ions in other bacterioferritins (Janowski *et al*., 2008).

1.7.4 Gene regulation by iron in bacteria.

The importance of iron as a cofactor in many proteins and as a virulence factor has been highlighted, and its regulation is a transcendental process in bacteria. Many enzymes and other proteins change their activities according to iron concentration within the cell. Such changes might result in an increase of a particular protein when the cell is deprived of iron, but at the same time there are decreases in the production of other proteins. Thus, the response for iron concentration suggests control at the gene level (McHugh *et al*., 2003; Rodriguez, 2006).

The Fur protein (ferric-uptake regulator), which is widely distributed among the Gram-negative bacteria, controls iron-dependent expression of more than 90 genes in *E. coli* (Hantke, 2001). These genes are involved in diverse functions including iron and haem acquisition, virulence factor production and acid resistance (McHugh *et al*., 2003). Some genes are positively regulated by Fur. An iron-containing superoxide dismutase, SodB, increases its activity in presence of iron and Fur (Dubrac & Touati, 2000). Also genes which encode for ferritin, bacterioferritin, and aconitase A, are positively regulated. On the other hand, the Fur gene can also be autoregulated in a negative way. During oxidative stress the Fur gene is turned on by peroxide *via* OxyR regulation, allowing a decrease of iron uptake (Zheng *et al*., 1999).

Fur protein forms a complex with Fe^{+2} when iron is available, and that complex, Fur-Fe^{+2}, binds to a number of specific operator sequences which block the transcription of functional genes (Saito & Williams, 1991; Saito *et al*., 1991). Also, the complex is mediated by other divalent metals such as Co^{+2} and Mn^{+2}.

Fur acts positively on the expression of a number of proteins. It acts often indirect mediated by Fur-dependent repression of an antisense regulatory small RNA (sRNA) (Masse & Arguin, 2005). This RNA acts at the post-transcriptional level to repress translation of target genes. For instance, the *E. coli* RyhB, a 90 nt sRNA is repressed by Fur in an iron-dependent manner. In an iron-defficient environment, or in a *fur* mutant, expression of RyhB is derepressed thereby causing a decrease in the level of target mRNAs. Several target genes

are regulated this way such as *acnA* (aconitase A), *fumA* (fumarase A), *sdhCDAB* (succinate dehydrogenase), *bfr* (bacterioferritin) and *ftnA* (ferritin) and *sodB* (Masse & Arguin, 2005).

Another iron regulation protein is found in *Corynebacterium diphtheriae*. The diphtheria toxin repressor protein, DtxR, regulates genes related to iron uptake and virulence (Yellaboina *et al*., 2004), in particular the prophage-encoded diphtheria toxin gene (Tao *et al*., 1992). DtxR also requires Fe^{+2} as a co-repressor.

M. tuberculosis contains two Fur-like proteins (FurA and FurB) and two members of the DtxR family, the iron-dependent regulator protein (IdeR) and SirR (designated after staphylococcal iron regulator repressor) (Rodriguez, 2006). IdeR is closely related to DtxR. As for Fur and DtxR, IdeR needs Fe^{+2} to form a complex and subsequently binds to DNA and that complex interacts with "iron-box" operator sequences (Dussurget *et al*., 1996; Dussurget & Smith, 1998; Schmitt *et al*., 1995). IdeR regulates about 40 *M. tuberculosis* genes involved in iron uptake and metabolism by binding to operator regions of these genes and usually repressing transcription, although the transcription of some genes are enhanced (Gold *et al*., 2001). Many of these genes are involved in the synthesis of the mycobacterial siderophores, the mycobactins and exochelins (Dussurget & Smith, 1998; Manabe *et al*., 1999). On the other hand, the metal binding site of *M. tuberculosis* SirR might be an orthologue of *S. epidermidis* SirR which has both iron and manganese-dependent regulatory activity (Hill *et al*., 1998).

M. tuberculosis contains two genes, *furA* and *furB*, which encode proteins more similar to *E. coli* Fur than to IdeR/DtxR (De Voss *et al*., 1999). FurA negatively regulates the expression of *katG* gene, thereby modulating the response against oxidative stress (Pym *et al*., 2001; Zahrt *et al*., 2001), whereas FurB might be involved in sensing and controlling Zn-sensitive genes (Canneva *et al*., 2005).

1.8 Aims of the project.

Iron homeostasis is essential for the growth and survival of mycobacteria, as well as being involved in host macrophage defence by catalysing the production of toxic hydroxyl radicals via the Haber-Weiss-Fenton reaction. *M. tuberculosis* resides within the macrophage phagosome and prevents phagosome-lysosome fusion by an unclear mechanism. During infection, a competition for iron occurs between the host macrophage and the pathogen. The macrophage attempts to suppress pathogen proliferation by complex iron-withholding mechanisms. However, *M. tuberculosis* perceives the phagosome as iron-low environment, and in response the bacterium needs to compete for iron by producing the high affinity iron-chelators, the siderophores.

The importance of mycobacterial siderophores as virulence factors during infection of macrophages has been demonstrated by a *M. tuberculosis* H37Rv mutant lacking the *mbtB* gene, which encodes a NRPS for the initial step in mycobactin biosynthesis (De Voss *et al*., 2000). Furthermore, a structure related to mycobactins, the didehydroxymycobactin (DDM), functions as an antigen by activating CD1 proteins which ultimately stimulates T cells, highlighting the importance of the molecule during host-*M. tuberculosis* interaction (Moody *et al*., 2004).

New anti-TB drugs must act on a target that is essential for bacterial survival, be specific, and without affecting the mammalian cells. Snow (1970) proposed that most of the enzymes involved in the synthesis of mycobactin may not have an equivalent in animals, which do not synthesise siderophores, and therefore would be safe targets for any drug as adverse inhibitory effects would be avoided. Thus, salicylic acid synthesis, the formation of N-hydroxylysine and the final acyltransferase involved in assembling the long chain acyl group are considered to be attractive targets for new drugs (Ratledge, 2004; Ratledge & Dover, 2000).

PAS has been demonstrated to inhibit the biosynthesis of mycobactin (Brown & Ratledge, 1975a; Ratledge & Brown, 1972). The authors suggested that PAS could inhibit the conversion of salicylic acid to mycobactin, and therefore carboxymycobactin, probably in the first reaction that involves its interaction with ATP or its subsequent condensation with serine/threonine. PAS is an analogous of salicylic acid and therefore blocks the biosynthesis of mycobactin, probing its activity as an anti-TB drug by preventing the bacteria to acquire iron from the host.

The inhibition of the aryl-capped siderophore biosynthesis in pathogenic bacteria such as *Y. pestis*, *M. tuberculosis* and *P. aeruginosa* has been recently demonstrated (Ferreras *et al*., 2005; Miethke *et al*., 2006a; Somu *et al*., 2006a). The first step during aryl-capped siderophore biosynthesis is the activation of either salicylic acid or 2,3-dihydroxybenzoate (DHB), depending on the enzymatic equipment of the microorganism, by an adenylation domain. In this reaction the α-phosphate of ATP is charged by the substrate to generate a tightly bound acyl adenylate, salicyl-AMP for instance, with subsequent release of pyrophosphate. This biosynthetic step resembles a similar mechanistic reaction in all members of the adenylate-forming enzyme superfamily and the functionally related aminoacyl tRNA synthetases (Schimmel *et al*., 1998). Analogues of the acyl adenylate have been developed and tested *in vitro* for the depletion of siderophore biosynthesis. Those analogues were designed to act as intermediate mimics that could inhibit the adenylate-forming and thus stop the initial siderophore biosynthesis (Ferreras *et al*., 2005; Miethke *et al*., 2006a; Somu *et al*., 2006a).

The elucidation of the chemical structures of mycobacterial siderophores was an important step forward, however, the mechanism of their biosynthesis is still not fully resolved. Since, siderophores biosynthesis, as well as secretion and uptake or transport after iron chelation represents outstanding candidates as targets for the development of new antibiotics for the treatment of tuberculosis and other mycobacterial diseases, the aims of this study are to investigate:

1. The expression, post-translational activation and biochemical characterisation of *M. tuberculosis* MbtB protein.

2. Potential inhibitors of enzymes during siderophore biosynthesis, specifically MbtA, by testing a series of SAL and PAS analogues *in vitro* and *in vivo*.

3. The expression and biochemical characterisation of *M. tuberculosis* MbtG and the adenylation protein domains of MbtE and MbtF during hydroxylation of lysine residues.

2. Post-translational modification of MbtB.

2.1 Post-translational modification of MbtB.

Numerous microorganisms utilise a nonribosomal process for the biosynthesis of small peptides that are mainly products of secondary metabolic pathways, such as the siderophores. Domains within each NRPS module provide sites for the recognition and activation of a substrate amino acid (adenylation, or, A domain); transfer of this activated intermediate to a phosphopantetheinyl residue (bound by a thiolation domain, or T, domain); and a peptide bond formation (catalysed by a condensation domain, or C domain), so that the activated residue is incorporated into the growing peptide chain. A final thioesterase (T) domain might be present in this multienzyme complex that cleaves the growing molecule from the terminal NRPS. This domain may also serve a further catalytic process such as the internal cyclisation of the residue (Challis & Naismith, 2004).

In the original *M. tuberculosis* genome annotation, only *mbtB*, *mbtE*, *mbtF*, which participate in pre-mycobactin biosynthesis (Quadri *et al*., 1998), along with Rv0101, to which no function has yet been assigned, were annotated to encode potential NRPSs (Cole *et al*., 1998). However, in recent studies other *M. tuberculosis* genes were ascribed with putative features of NRPSs. By mimicking the acidic phagosomal environment, in which *M. tuberculosis* resides during infection, and using Microarray and Real Time-Reverse Transcription-PCR (RT-R T- PCR) techniques, genes involved in fatty acid metabolism, as well as the Rv3083-Rv3089 operon were positively regulated by the acidic environment (Fisher *et al*., 2002). Additional, *in silico* analysis of that operon showed that the genes displayed NRPSs features: Rv3089 contains an AMP-binding domain, as occurs in the A domains of NRPSs; Rv3087 and Rv3088 possess a partial condensation domain, which includes the HHXXXDG catalytic site to the C domains; Rv3084 contains a partial thioesterase domain. The Rv3083 and Rv3086 genes resemble genes encoding the organisation of a NRPS/PKS hybrid system for rapamycin biosynthesis in *Streptomyces hygroscopicus* (Fisher *et al*., 2002). In another study, microarray analysis of the over-expression *M. tuberculosis* sigma factor (SigM) positively regulated two gene operons (Raman *et al*., 2006). The first operon carries a gene of unknown function, PPE19, whereas the second operon, Rv0096-Rv0101, contains the PPE1 and NRPSs genes along with other

three genes of unknown function and a putative fatty acid biosynthetic gene. The Rv0096-Rv0101 operon is found highly conserved in *M. bovis* and *M. leprae*, but not in *M. smegmatis*. Further analysis of this operon found that the Rv0099 and Rv0100 have NRPSs domains, the adenylation and peptidyl carrier protein, respectively. The authors of this study suggested that the Rv0099, Rv0100, and Rv0101 might function as three separate NRPS entities to produce a bioactive molecule to modulate host-pathogen interactions (Raman *et al*., 2006). The analysis of a *M. bovis* mutant in which the Mb100, the homologue of Rv0097, was disrupted by transposon mutagenesis, supports this hypothesis (Hotter *et al*., 2005). Disruption of the Mab100 gene had polar effects in the Mb101-Mb104 genes, generating phenotypes unable to produce phthiocerol and phthiodiolone dimycocerosate esters (PDIMs) and glycosylphenol-PDIMs, suggesting a role involved in the biosynthesis of these molecules (Hotter *et al*., 2005). However, this finding waits further genetic and biochemical analysis of these genes, specifically the role of Rv0101.

On the other hand, the recently sequenced *M. marinum* genome shows the presence of 23 NRPS-encoding genes, 18 of which cannot be ascribed a precise function since there are no orthologues of genes in other mycobacteria (Stinear *et al*., 2008). This finding suggests the existence of complex and diverse novel secondary metabolites produced by *M. marinum* compared to that of *M. tuberculosis*.

The MbtB, MbtE, and MbtF proteins are conserved among mycobacterial species that produce the mycobactins, but not in *M. leprae*. However, there are differences in the domain organisation of these modular enzymes. The MbtB in *M. tuberculosis* and *M. bovis* share high similarity internal domain organisation, however the T domain (originally annotated as an acyl-transferase), predicted at the C-terminus of the protein is replaced by a non-integrated stand-alone predicted T, MbtT, encoded downstream of MbtB in the clusters of *M. avium subsp. avium*, *M. avium subsp. paratuberculosis*, and *M. smegmatis* or upstream of MbtE in *M. marinum* (Quadri & Ratledge, 2004).

The *M. tuberculosis* MbtB protein possesses the following predicted domains: ArCP, Cy, A1, PCP and T/AT1. Those integrated domains lead to the formation of the oxazoline/thiazoline rings like those also found in vibriobactin and pyoverdin biosynthesis. The remarkable catalytic activity of the Cy domains represents an attractive target for new anti-TB drugs. Thus, dissecting the activity MbtB domains not only would aid in fully understanding mycobactin-carboxymycobactin biosynthesis, but also in rational design of new drugs.

In order to be active, the carrier protein domains of the MbtB must be post-translationally modified by the dedicated phosphopantetheinyl transferase. Two candidates for phosphopanthethienylation of MbtB were apparent on analysis of the *M. tuberculosis* genome (Quadri *et al*., 1998). The mycobacterial *acpS* encodes a 14 kDa protein and *pptT* a 25 kDa protein. *In vitro* biochemical studies showed that PptT carried out post-translational modification of both carrier proteins, the ArCP domain in the peptide synthetase, MbtB, and the PCP domain of MbtE, respectively (Quadri *et al*., 1998). In the genome of *M. tuberculosis* there are more than 18 type-I PKSs and two fatty acid synthase (FAS) systems (Cole *et al*., 1998). The function of those enzymes depends of a post-translational modification of the acyl carrier proteins of FAS and PKSs by phosphopantetheinyl transferase. Recently, it has been demonstrated that both of the PPTases of *M. tuberculosis* are essential for mycobacterial viability. The protein AcpS was shown to be responsible for the post-translational modification of Fatty acid synthase-I and AcpM subunit of Fatty acid synthase-II, whereas the protein PptT activates the condensing enzyme Pks13 and various type-I Pks required for the formation of lipid (Chalut *et al*., 2006). Bioinformatic analyses led to the finding of orthologues of *acpS* and *pptT* genes in both mycobacterial and corynebacterial species. The amino acid sequences of the encoded proteins were > 80% similar for the mycobacterial proteins, with ~ 40% similarity between corynebacterial proteins and their *M. tuberculosis* counterparts (Chalut *et al*., 2006).

Previously other researchers had not achieved the molecular cloning of full length MbtB. Here, the isolated ArCP and the full *mbtB* genes have been cloned, expressed and successfully post-translationally modified in *E. coli* by a heterologous "broad substrate phosphopantetheinyl transferase". Subsequent purification of the protein has allowed the confirmation of these goals by molecular analyses and thus facilitates further biochemical characterisation of the protein toward mechanistic studies and the testing of novel compounds that might target the initial step in mycobactin-carboxymycobactin biosynthesis (Chapter 3).

2.2 Material and methods.

A range of approaches was tested to achieve the post-translational modification of the carrier proteins of MbtB. In this study, the first approach was to clone both the full-length *mbtB* (Rv2383c) and the intrinsic phosphopantetheinyl transferase (*pptT*, Rv2794c) of *M. tuberculosis* to carry out either *in vivo* or *in vitro* post-translational modification of the carrier proteins. Nevertheless, non of these approaches were successful and other ways were attempted to achieve this aim. Secondly, the *ArCP* domain of MbtB was cloned and successfully expressed but, as for the MbtB, no evidence of modification was found. Thirdly, the use of a "broad substrate phosphopantetheinyl transferase" rather than the intrinsic PPTase of *M. tuberculosis*, afforded the phosphopantetheinylation in the carrier proteins for both the ArCP domain as well as the full-length of MbtB. Material and methods will be described in the next pages in the order outlined above.

2.2.1 *In vivo* post-translational modification of the MbtB protein.

A method to achieve post-translational modification of carrier proteins is to co-express both the *mbtB* and the intrinsic phosphopantetheinyl transferase *pptT*, involved in mycobactin-carboxymycobactin biosynthesis, in suitable heterologous expression host and using compatible vectors. To achieve this, two expression vectors must be used, each of them being controlled under different replicons and resistance genetic markers. Therefore, pET23b and pCDFDuet1 (Novagen) expression vectors were used and features of each expression vector are summarised in Table 7.

Table 7. Genetic features of the vectors used in expression of the *mbtB* and *pptT* genes.

Vector	Origin of replication	Resistance marker	Promoter
pET23B	ColE1	Ampicillin	T7
pCDFDuet-1	CloDF13	Streptomycin/Spectinomycin	T7

2.2.2 Molecular cloning and expression of the *mbtB* and *pptT* genes of *M. tuberculosis*.

The recipe and the typical conditions for PCR reactions to clone the genes of interest were derived from the following generic conditions:

A) Recipe

2 µL	5' Cloning primer (100 pmole)
2 µL	3' Cloning primer (100 pmole)
1 µL	Genomic DNA (H37Rv) (1 µg/µL)
2 µL	dNTP's
10 µL	Thermostable buffer (10X)
8 µL	DMSO (optional)
2 µL	$MgSO_4$ (optional) (100 mM)
1 µL	DNA polymerase[1]
72 µL	ddH_20 (for molecular biology)

[1]Deep Vent™ DNA polymerase was used for cloning both *mbtB* and *pptT* genes.

B) Thermal cycling conditions

95ºC	3 minutes	
95ºC	1 second	
60-70ºC	30 seconds	35 cycles
68ºC	0.5 minutes *per* kb	
68ºC	10 minutes	
4ºC	∞	

For each gene, the conditions were adjusted to yield the desired PCR product. The PCR products were electrophorosed on agarose gel, DNA was visualised using Ethidium bromide (EtBr). The appropriate DNA fragment was excised from the gel and extracted using the Qiagen Gel extraction Kit. The purified PCR products were digested with appropriate restriction enzymes according to the plasmid in which they were inserted. The cut PCR product and similarly digested plasmid DNA were assessed by DNA electrophoresis and EtBr staining. The cut PCR product was ligated with the cut vector by T4 DNA Ligase (New England Biolabs) at 16ºC for 16 hours. The ligation mix was used to transform the corresponding *E. coli* heterologous cloning host for selecting the right genetic construct. Screening was done by double digestion followed by electrophoresis analysis on agarose gel and staining with EtBr. All genetic constructs generated were verified by sequencing by means of ABI PRISM Byg DyeTM Terminator v3.0 kit (Applied Biosystems) and an ABI PRISM 3700 sequencer (Applied Biosystems).

Amplifcation of the *mbtB* and *pptT* genes was achieved using Deep Vent^{TM} DNA Polymerase (New England Biolabs) and *M. tuberculosis* H37Rv chromosomal DNA. Primers for each gene are listed in Table 8. After digestion with NdeI and NotI (New England Biolabs), the *mbtB* amplicon was then directly ligated into pET23b vector (Novagen), which was cut in the same manner as the PCR product, to give the *mbtB*-pET23b construct. The design of the primers is such that recombinant MbtB product was modified with a C-terminal hexahistide tag, derived from pET23b, to facilitate its purification by metal chelate affinity chromatography; the stop codon of the gene was therefore excluded from the *mbtB* down primer design. The ligation mix was used to transform *E. coli* TOP 10 (Invitrogen) cells for screening under Ampicillin (Amp, purchased from Sigma Aldrich) resistance marker. Similarly, after amplification, the *pptT* PCR product was digested with NcoI and NotI (New England Biolabs), which was then ligated into the NcoI/NotI sites of the pCDFDuet1 plasmid (Novagen), thus giving rise to *pptT*-pCDFDuet-1. The ligation mixture was used to transform *E. coli* XL1 Blue (Strategene) cells for screening under Streptomycin (Str, purchased from Sigma Aldrich) resistance marker. Both genetic constructs were subjected to nucleotide sequencing to verify their integrity. The *pptT*-pCDFDuet1 was then subsequently used to

make *E. coli* C41 (DE3) (OverExpress) chemical competent cells to carry out *in vivo* post-translational modification of the MbtB.

Table 8. Primers used for cloning the *mbtB* and *pptT* genes into pET23b and pCDFDuet-1, respectively. Restriction sites are underlined.

mbtB up	5'-GATCGATCCATATGGTGCATGCTACGGCGTGC-3'
mbtB down	5'-GATCGATCGCGGCCGCTCGGACATCGGCACTCACC-3'
pptT up	5'-GATCGATCCATGG ACGGTAGGCACGCTGGTG-3'
pptT dwn	5'-GATCGATCGCGGCCGCTTATAGCACGATCGCGGTCAGCAC-3'

2.2.3 Determination of expression levels of the *mbtB*-pET23b in *E. coli* C41 (DE3).

Expression levels of the *mbtB*-pET23b were determined by testing different temperatures, incubation time and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations using *E. coli* C41 (DE3) (OverExpress) as a heterologous host. A stock of 50 µL *E. coli* C41 (DE3) (OverExpress) chemical competent cells was transformed with 1 µL *mbtB*-pET23b by heat shock at 42ºC for two minutes. Cells were incubated with 150 µL of Luria Bertoni (LB) broth medium at 37ºC for 1 hour, followed by selection in LB agar plates supplemented with Amp (100 µg/mL). Cells forming single colonies were used to inoculate 5 mL of LB broth supplemented with Amp (100 µg/mL) and were cultured overnight at 37 ºC with shaking (160 rpm). From this starting culture, 1 mL was transferred to fresh LB broth medium (100 mL) supplemented with Amp (100 µg/mL) and this was incubated until the optical density (OD) at 600 nm reached 0.5-0.6 when, where appropriate a subsequently shifted to 30ºC or 16ºC was affected. Cells incubated at 37ºC and 30ºC were allowed to grow for an additional 4 hours, whereas cells growing at 16ºC were incubated for an additional 16 hours. At each temperature, various concentrations of IPTG (1 mM, 0.5 mM, 0.1 mM) were tested to induce gene expression. A negative control (no addition of IPTG) was performed along with the experimental conditions for comparison.

2.2.4 Preparation of *E. coli* C41 (DE3) chemical competent cells harbouring *pptT*-pCDFDuet1.

A stock of 50 µL *E. coli* C41 (DE3) (OverExpress) chemical competent cells was transformed with 1 µL *pptT*-pCDFDuet by heat shock at 42ºC for two minutes. Cells were incubated with 150 µL of LB broth at 37ºC for 1 hour, which were then selected on LB agar supplemented with Str (50 µg/mL). Cells from a single colony on a LB agar plate were then used to inoculate 50 mL of LB Broth supplemented with Str (50 µg/mL) and cultured overnight at 37ºC with shaking (180 rpm). From this starting culture, 5 mL was transferred to fresh LB broth medium (100 mL) supplemented with Str (50 µg/mL) and this was incubated until the OD_{600} reached 0.5-0.6. Cells were centrifuged (3000 rpm) for 5 minutes at 4ºC. Cell pellets were resuspended with 20 mL of ice cold buffer TFB1 (40%) and incubated on ice for 30 minutes, followed by centrifugation (3, 000 rpm) for 5 minutes at 4ºC. Then, cells were resuspended with 2 mL of ice-cold buffer TFB2 (40%). *E. coli* C41(DE3)-*pptT*-pCDFDuet1 chemical competent cells were flash frozen in 50 µL aliquots using liquid nitrogen and stored at -80ºC until required.

2.2.5 Co-overexpression of *mbtB*-pET23b and *pptT*-pCDFDuet1 in *E. coli* C41(DE3).

A stock of 50 µL *E. coli* C41(DE3)-*pptT*-pCDFDuet-1 was transformed with 1 µl of *mbtB*-pET23b by heat shock at 42°C for two minutes. Cells then were incubated with 150 µL of LB broth at 37ºC for 1 hour followed by selection on LB agar supplemented with Str (50 µg/mL) and Amp (100 µg/mL). Cells from a single colony were used to inoculate 50 mL of LB Broth supplemented with the above antibiotics and cultured overnight at 37ºC with shaking (180 rpm). From this starting culture, 5 mL was transferred to 1 L of Terrific Broth (TB) media (solution 1) supplemented with salts (solution 2) and both antibiotics. The large-scale culture was initially incubated at 37ºC until the OD_{600} reached 0.5-0.6 and recombinant gene expression induced with 1 mM IPTG and culture incubated for 16 hours at 16ºC followed by centrifugation at 8000 rpm for 5 minutes at 4ºC.

The cell pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.9, 500 mM NaCl) and broken by passing through a French Pressure cell twice (3000 psi) in the presence of a cocktail of serine protease inhibitors (PMSF, benzamidine, and DNAase at 1 mM final concentration). The extract was clarified by centrifugation at 15 000 rpm for 30 minutes at 4ºC. The supernatant was collected and loaded onto a Ni^{2+}pre-charged and equilibrated His-Trap column (5 mL) following the manufacturer's instructions (Amersham). The clarified extract was applied to the column (50 mL maximum volume for 5 mL column capacity) and non-binding material collected for analysis. The column was then washed with 50 mL of binding buffer A (50 mM Tris-HCl pH 7.9 and 500 mM NaCl) and the protein eluted with a step gradient (10-500 mM) of increasing imidazole concentrations in buffer A. The purity of the protein was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and Coomasie blue staining. Purified protein fractions were pooled and dialysed against buffer B (10 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10 % glycerol, pH 8.6) and concentrated using a Centricon YM-50 filter unit (Millipore) and stored at -80C ºC until use. Protein concentration was estimated by the Bicinchoninic acid protein (BCA) assay (Pierce) and by absorbance at 280 nm with the predicted molar extinction coefficient (ε= 151 601.9 M^{-1} cm^{-1}).

2.2.5.1 Screening for *in vivo* post-translational modification of the carrier proteins by MALDI-Mass Spectrometry.

To verify *in vivo* modification, proteins were separated by gel electrophoresis, followed by in-gel trypsin digestion and peptide mass fingerprint analysis by positive ion mode matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). The recombinant protein was separated by 12% SDS-PAGE and bands of the expected size of MbtB (151 kDa) were excised from the gel, eliminating as much polyacrylamide as possible. These bands were transferred to microtubes and treated with 200 µL of 100 mM NH_4HCO_3 (pH 7.8)/50% acetonitrile (ACN) and incubated at 37ºC for 30 minutes. The supernatant was removed and 100 µL 100% ACN was added to the gel slices for 5 minutes. At this stage, the slices became whitish and much smaller due to dehydration; the gel slices were dried for further analysis.

Since trypsin is a serine protease that specifically cleaves at the carboxylic acid side of lysine and arginine residues, it was possible to generate peptides in order to analyse them by mass spectrometry, and determine potential modification of MbtB. Trypsin Gold Mass Spectrometry Grade (Promega) was used for this purpose. Trypsin Gold was resuspended at 1 μg/μL in 50 mM acetic acid, 5 mM β-mercaptoethanol, 0.1% SDS as was indicated by the manufacturer. Gel slices were initially incubated with 50 μL of trypsin (100 μg/mL in 40 mM NH_4HCO_3/10% ACN) at room temperature for 1 hour. Then, another 25 μL of tryspin was added (same concentration as above) to the gel slices and incubated at 37ºC overnight. Digests were stopped by adding 5μl of 0.1 % trifluoroacetic Acid (TFA).

Since MS analyses do not tolerate salts, two desalting methods were tested to determine which one could allow the analysis of peptides by using Zip Tip C_{18} (Millipore). In the first method, the preparation of tips was as follows: tips were hydrated and washed with 10 μL of 100% ACN, twice, and then equilibrated with 10 μL of 0.1 % TFA (three times). After preparation of tips, 10 μL of the sample (peptides generated from in-gel digestion) was taken and aspirated 10 times by pipetting fully into and out of tips and the liquid was expelled. Tips were washed in 5% methanol/0.1% TFA. To elute the peptides from tips, 10 μL of 1% formic acid/50% methanol solution was used. Samples were then ready for mass spectrometry analysis. The second method was as follows: tips were hydrated and washed with 10 μL of 50% ACN three times, and then equilibrated with 1% acetic acid for three times. After preparation of tips, 10 μL of the sample were aspirated by pippeting fully with the tips. To elute the peptides from tips, 10 μL of 50% ACN/1% acetic acid solution was used, then the sample was ready for analysis.

For screening both small and large peptides, two matrices were used. Sinapinic acid (SA, at 1 mg/mL) allowed detection of peptides > 2500 Da and α-cyano-4-hydroxycinnamic acid (CCA, at 2.5 mg/mL) permitting screening of peptides < 2500 Da. Virtual post-translational modification of MbtB was performed in Peptide Mass Software (Expasy Tools) to compare to those generated in-gel digestion. An aliquot (1-0.7 μL) of the purified digest was spotted into the matrices and the prepared plates were analysed in the mass analyzer 4800

MALDI TOF/TOF (Applied Biosystems) and carried out in the Pharmaceutical Biotechnology Department, University of Saarland, Saarbrücken, Germany. Proteins were identified by a combination of the Peptide Mass fingerprint and the data for the tandem mass measurement of digested peptides. Evidence of post-translational modification of the carrier proteins fragments was sought by manually inspection of the generated masses in the spectra and compared with the predicted modified peptides.

2.2.5.2 Screening for *in vivo* post-translational modification of carrier proteins by HPLC.

The post-translational modification of MbtB by PptT involves the transfer of the phosphopantetheine which presents a thiol group that serves as the linkage point for the carrier proteins during mycobactin assembly. By using a fluorescent dye, Alexa Fluor® 488 C_5-maleimide (Molecular Probes Invitrogen), a reagent that reacts with thiol groups on proteins to give thioether-coupled products, post-translational modification can be monitored. Thus, the modification of MbtB were detected by HPLC by labeling with a fluorophore.

Co-overexpression and purification of the MbtB recombinant protein was carried out as described in section 2.2.5. In-solution trypsin digestion was performed to generate peptides from MbtB. Trypsin Gold Mass Spectrometry Grade (Promega) was resuspended at 1 μg/uL in 50 mM acetic acid, 5 mM β-mercaptoethanol, 0.1 % SDS as was indicated by the manufacturer. A concentration of trypsin 1:20 (w/w) (protease: protein ratio) was added to the protein solution, and the reaction was incubated for 20 minutes at 37ºC. Solution was chilled on ice for 30 minutes to stop the reaction. Then, labeling was performed by conjugating with a 10-fold molar excess of the dye in 50 mM Tris-HCl-pH 7.9-500 mM NaCl for 4 hours at room temperature in the dark. Labelled peptides were purified by Reverse Phase-HPLC at A=493 and analysed by MALDI-MS.

2.2.6 *In vitro* post-translational modification of MbtB.

In vitro phosphopantetheinylation by PptT was attempted by molecular cloning, expression and purification of the PptT as well as MbtB. For cloning *pptT* the following primers were used: up 5'-GATCGATCCATATGACGGTAGGCACGCTGG-3' down 5'-GATCGATCGCGGCCGCTCATAGCACGATCGCGGTCAGCAC-3' (NdeI and NotI restriction sites are underlined) and the PCR product was inserted into pET28b to create *pptT*-pET28b which featured an in-frame fusion with an N-terminal hexa-histidine tag to facilitate protein purification using metal chelate affinity chromatography. The ligation mixture was used to transform *E. coli* TOP10 (Invitrogen) for screening under Kanamycin (Kan, purchased from Sigma Aldrich) resistance marker. Molecular cloning of the *mbtB* gene is described in section 1.5.1.1. Nucleotide sequence of DNA plasmids from recombinant transformants were confirmed by double digest and their integrity verified by sequencing.

The examine expression of *pptT*-pET28b, an aliquot of 50 μL *E. coli* C41 (DE3) (OverExpress) chemical competent cells was transformed with 1 μL *pptT*-pET28b by heat shock at 42ºC for two minutes. Conditions were tested as previously detailed in section 1.5.1.4. Cells were grown at 37ºC followed by the addition of 1 mM of IPTG to induce overproduction of the recombinant gene. To scale up protein production, cells from a single colony were used to inoculate 50 mL of LB Broth supplemented with Kan (25 μg/mL) and cultured overnight at 37ºC with shaking (180 rpm). From this starting culture, 5 mL was transferred to 1 L of TB media (solution 1) supplemented with salts (solution 2) and Kan (25 μg/mL). The large-scale culture was incubated at 37ºC until the OD_{600} reached 0.5-0.6 and recombinant gene expression induced with 1 mM IPTG. The culture was further incubated for 4 hours followed by harvesting by centrifugation at 8, 000 rpm for 5 minutes at 4ºC.

Cells were resuspended in 200 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4 as described by Quadri *et al*., (1998). Following resuspension cells were disrupted by two passages through a French Press cell (3000 psi) and serine protease inhibitors added to the cell debris. The extract was clarified by centrifugation at 15,000 for 30 minutes at 4ºC. Supernatant was collected and the recombinant protein purified by affinity chromatography

using a Ni^{2+}pre-charged and equilibrated His-Trap column (5 mL) following the manufacturer's instructions (Amersham). A step gradient of 50-500 mM imidazole was used to elute the protein. The purity of the protein was assessed by 12 % SDS-PAGE and Coomasie blue stain staining. Purified protein fractions were pooled and dialysed against 10 mM Tris-HCl, pH 8.0, 1 mm EDTA, 1 mM DTT and 5% glycerol, and concentrated using a Centricon YM-10 filter unit (Millipore) and stored at -80C ºC until use. The protein concentration was estimated by the BCA assay (Pierce) and by absorbance at 280 nm with the predicted molar extinction coefficient (ε= 24 708.44 M^{-1} cm^{-1}).

2.2.6.1 Screening for *in vitro* post-translational modification of MbtB.

In vitro assays were performed in an attempt to modify MbtB. The typical biochemical assay for determining the conversion from *apo*-protein to *holo*-protein was carried out in 100 µL reaction following previous method reported by Quadri *et al*., (1998) with minor modifications: 75 mM Tris-HCl (pH 7), *apo*-protein (~100 µM), PptT (25-55 nM), 1 mM CoA, and 10 mM $MgCl_2$. The reaction mixtures were incubated at 37ºC and samples were taken at different time points (0, 30, 60, 120, and 240 minutes) to analyse them by MALDI-MS.

Lanes on a 12% SDS-PAGE gel that contained the protein of interest (about 151 kDa) were excised and treated in-gel trypsin digestion as was described above. Samples were concentrated and desalted by using Zip Tip (section 2.2.5.1). Once the samples were purified, peptides were analysed by MALDI-MS (section 2.2.5.1). In addition, both MbtB and PptT proteins were separately loaded on gels and treated for mass spectrometry analyses to compare the results from the reactions carried out to modify MbtB.

2.2.7 Molecular cloning and expression of the ArCP domain of MbtB.

Other research groups have been successful in achieving ArCP phosphopantetheinylation allowing study of the initial transfer reaction, but study of further biochemical reactions by MbtB requires the modification of a further peptidyl carrier domain. However, cloning only the *ArCP* domain (the amino terminus of MbtB) would simplify the phosphopantetheinylation. For this purpose, expression vectors from IMPACT-CN system (New England Biolabs) were tested. This system allows protein purification that utilises the inducible self-cleavage activity of a protein splicing element (termed intein) to separate the target protein from the affinity tag. A target protein is fused to a self-cleavable intein tag in which a chitin binding domain allows affinity purification of the fusion precursor on a chitin column. In the presence of thiols such as DTT, β-mercaptoethanol or cysteine, the intein undergoes specific self-cleavage which releases the target protein from the chitin-bound intein tag resulting in a single-column purification of the target protein (Chong *et al*., 1998) with gentle and efficient post-column processing to achieve a protein product of near native sequence. The pTYB expression vectors allow the fusion of the cleavable intein tag to either the C-terminus (pTYB1 and pTYB2, C-terminal fusion) or N-terminus (pTYB11 and pTYB12, N-terminal fusion) of a target protein (New England Biolabs). pTYB1 and pTYB2 allow cloning the interest gene in fusion with intein tag (and the cleavage site) without adding extra amino acid residues to the recombinant protein, whereas pTYB11 and pTYB12 will add a small peptide that is cleaved and co-eluted with recombinant protein during purification. The pTYB vectors use a T7/lac promoter to provide stringent control of the fusion protein. Another feature of the pTYB vectors is that they carry an ampicillin gene marker.

PCR amplification of the ArCP truncated gene from *M. tuberculosis* H37Rv was performed with Vent^{TM} DNA polymerase (New England Biolabs). Primers used to clone the ArCP gene into pTYB vectors are listed in Table 9. PCR reactions were attempted as was described in section 2.5.1.1 Amplicons were obtained for all pTYB vectors and the PCR products were then directly ligated according to their respective vectors. Ligations mixes were used to transform *E. coli* TOP 10 cells (Invitrogen). However, only the following genetic constructs were created: ArCP-pTYB1, ArCP-pTYB2 and ArCP-pTYB12. The nucleotide

sequences of plasmid DNA from recombinant transformants were confirmed by sequencing as described above. The genetic constructs were used to attempt both *in vivo* and *in vitro* modification of the ArCP protein.

Seeking optimal over-expression conditions of the ArCP protein, using *E. coli* ER2566 (New England Biolabs) as described in section 2.2.3, no expression was found. However, further analysis of the genetic constructs by Western Blot and Immunodetection were carried out to determine overexpression.

Table 9. **Primers used to clone ArCP in pTYB vectors.** Restrictions sites are underlined and showed on the right side of the table.

Vector	Primer	Restriction site (underlined)
pTYB1	Up 5'-GATCGATCCATATGGTGCATGCTACGGCGTGCTCGGAGATC-3'	Nde I
	Down 5'-GATCGATCTGCTCTTCCGCACCCTGGTGCGGCAACTGCCGTGGGCGC-3'	Sap I
pTYB2	Up 5'-GATCGATCCATATGGTGCATGCTACGGCGTGCTCGGAGATC-3'	Nde I
	Down 5'-GATCGATCCCCGGGCCCTGGTGCGGCAACTGCCGTGGGCGC-3'	Sma I
pTYB11	Up 5'-ATCGTTATTGCTCTTCCAACATGGTGCATGCTACGGCGTGCTCGGAGATC-3'	Sap I
	Down 5'-ATC GTT CTG CAG TCA CCC TGG TGC GGC AAC TGC CGT GGG CGC-3'	Pst I
pTYB12	Up 5'-GATCGATCGAATGCTGTGCATGCTACGGCGTGCTCGGAGATC-3'	Bsm I
	Down 5'-ATCGTTCTGCAGTCACCCTGGTGCGGCAACTGCCGTGGGCGC-3'	Pst I

Disrupted cells (crude extract) and clarified lysate (supernatant) derived from screening optimal over-expression conditions were loaded into a 15 % SDS-PAGE. Proteins were transferred onto a nitrocelluse membrane by Western Blot. Immunodetection was carried out with antibodies against the chitin-binding domain of the fusion protein as a first antibody (New England Biolabs). Secondary antibody, rabbit-IgG linked to alkaline phosphatase

conjugate (Sigma Aldrich) and BCIP/NBT (Sigma Aldrich) was used as a substrate for the latter.

2.2.7.1 Protein purification of ArCP in chitin matrix column.

Once optimal over-expression of the ArCP was determined, protein purification was carried out by affinity chromatography using a chitin column. The chitin-binding domain (CBD) in the intein tag from the vectors allows affinity purification of the fusion protein. A 25 mL chitin matrix column was prepared and equilibrated with 10 times buffer A (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 1 mM EDTA) at 4°C. Optimal over-expression of the ArCP-pTYB1 in *E. coli* ER2566 was found to be at 0.5 mM IPTG and cells growing at 16°C. After induction and growth, cells were harvested, resuspended with buffer A and lysed through two passages using a French Press cell. The extract was clarified by centrifugation at 15, 000 rpm for 30 minutes at 4ºC. The supernatant was collected and loaded onto a chitin bead column, and left for 30 minutes for specific binding of the fusion protein. Then, the column was washed with 10 bed volumes of the column buffer making sure that the clarified extract was not present in the column. The protein was released from the chitin column by induction on-column cleavage by quickly flushing 3 bed volumes of cleavage buffer B (20 mM DTT, 20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and I mM EDTA) and the column was left for further 16 hours. The following day, the protein was eluted with buffer A. Purity of the protein was monited by electrophoresis 15% SDS-PAGE and Coomasie blue staining. Fractions containing the purified protein were pooled and dialysed against storage buffer (10 mM HEPES, 10 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol, pH 8.6), and concentrated using Centricon YM-3 filter unit (Millipore) and stored at -80C ºC until use. Protein concentration was estimated by the BCA assay (Pierce) and by absorbance at 280 nm with the predicted molar extinction coefficient (ε=9121 M^{-1} cm^{-1}).

2.2.7.2 Screening *in vivo* and *in vitro* post-translational modification of the ArCP protein.

A 50 µL stock *E. coli* ER2566 was transformed with *ArCP*-pTYB1 and chemical competent cells were prepared. Large-scale cultures were prepared for protein production. The recombinant protein was purified as in section 2.2.7.1, followed by in-gel trypsin digestion and MALDI-TOF analysis as mentioned in section 2.2.5.1. The intact protein was also analysed by Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR) and Electro Spray-MS (ES-MS).

For *in vitro* post-translational modification, the *ArCP*-pTYB1 and *pptT*-pET28b recombinant proteins were purified and biochemically assayed. The typical biochemical assay for determining the conversion from *apo*-protein to *holo*-protein was carried out as in section 2.2.6.1.

2.2.8 *In vivo* post-translational modification of the carrier proteins of MbtB by a broad substrate phosphopantetheinyl transferase.

The phosphopantetheinyl transferases are classified according to their primary sequence and substrate specificity, and one class exhibits broad substrate specificity that can phosphopantetheinylate diverse carrier proteins from PCPs to ACPs (Gaitatzis *et al*., 2001; Gross *et al*., 2005). This type of PPTases is sometimes named "promiscous PPTase", "relaxed PPTase" or "broad substrate PPTase". In this study, the term "broad substrate PPTAse" will be used. Complete post-translational phosphopantetheinylation of *apo*-MxcF and *apo*-MxcF, nonribosomal peptide synthetases involved in myxochelin biosynthesis, was achieved by co-overexpression of MtaA, a "broad phosphopantetheinyl transferase", from *Stigmatella aurantiaca* DW4/3-1. The protein was also able to modify other carrier proteins, such as the acyl carrier protein (ACP) domain of the *mta* gene cluster for myxothiazol biosynthesis from *S. aurantiaca*, a PCP of unknown function from *Sorangium cellulosum* and the ArCP of the enterobactin synthetase (EntF) from *E. coli* (Gaitatzis *et al*., 2001). Hence, the MtaA represented a potential PPTase to achieve modification of the ArCP and MbtB proteins.

The *mtaA* gene from *S. aurantiaca* has been cloned into pUS vector giving rise to pUSMtaA genetic construct as reported by Gaitatzis *et al*., (2001). The pUS plasmid has the P15A replicon and chloramphenicol (Chl) resistance marker, genetic features that allowed *in vivo* post-translational modification of the ArCP and MbtB proteins by co-overpression of the genetic constructs using *E. coli* as heterologous host. Co-overexpression experiments were carried out as described in section 1.5.1.4., in which *mbtB*-pET23b and ArCP-pTYB1 were transformed in *E. coli* C41 (DE3) and *E. coli* ER 2566, respectively, to prepare chemical competent cells followed by second round of transformation in each set with pSUMtaA. Cultures of each set were initially grown at 37°C until OD_{600}=0.5-0.6 containing Amp (100 μg/mL) and Chl (30 μg/mL) and gene expression was induced with 0.5 mM IPTG. Incubation was continued for further 16 hours at 16°C. Large-scale cultures were prepared to yield and purify each recombinant protein as described above. Determination of post-translational modification was performed by MS analysis as described in section 2.2.5.1.

2.3 Results.

2.3.1 *In vivo* post-translational modification of MbtB by the intrinsic phosphopantetheinyl transferase of *M. tuberculosis*.

E. coli has been successfully used for *in vivo* reconstitution of other carrier proteins from siderophores, phosphopantheinyl transferases (*pptT, acpS*) of aryl-*N*-capped siderophores such as yersiniabactin (Bobrov *et al*., 2002), enterobactin (Gehring *et al*., 1997a) and other molecules produced by NRPSs (Gruenewald *et al*., 2004). The choice of *E. coli* as a heterologous host for expression is due to the following reasons: established cultures, ease of handling, low cost and availability of vectors (Makrides, 1996). Thus, we adopted this system and *E. coli* C41 (DE3) was chosen as the heterologous host to express both *mbtB* and *pptT* simultaneously with the expectation that this strain might modify both ArCP and PCP of MbtB *via* the activity of PptT. Along with the favourable host, the plasmids used in this study, pCDFDuet and pET23b, are compatible vectors which can co-exist in *E. coli* and therefore express the interested genes, *mbtB* and *pptT*.

A requirement for the modification for both carrier proteins, ArCP and PCP, of MbtB is the addition of phosphopantetheinyl (P-pant) arm by PPTtase. Initially, modification of MbtB was attempted by simultaneous induction of recombinant genes from pCDFDuet-*pptT*, encoding the intrinsic PPTase of *M. tuberculosis*, and *mbtB*-pET23b, by heat shock transformation at 42°C in *E. coli* C41 (DE3). To determine if such a modification was achieved, MbtB was purified by affinity chromatography and then analysed by MALDI-MS. The optimal expression conditions for *mbtB* were at 16°C with 1mM of IPTG with 16 hours of incubation, yielding approximately 3 mg/mL. The production of both proteins was difficult to assess by analysis of cell lysates on SDS-PAGE. Although production of a very high molecular protein, presumably MbtB, was apparent under expression analysis.

Identification of a band that could be attributed to recombinant PptT was not possible during expression analysis (data not shown). Previous experiments had suggested that the expression of similar *pptT* construct identified *via* a specific stain for its C-terminal hexa-histidine tag, a property not shared with the current construct in order to limit the possibility of

co-purification, was relatively poor (L.G. Dover, unpublished results, personally communicated) at 16°C, but might be considered significant for its proposed catalytic role here. Regardless, recombinant MbtB was purified from these extracts *via* its His6-tag by Ni^{2+}-affinity chromatography. Analysis by SDS-PAGE revealed that a single protein band was eluted in several fractions that migrated above the 116 kDa lane marker upon 12% SDS-PAGE (Figure 16), consistent with the expected behaviour of the tagged MbtB (151 kDa).

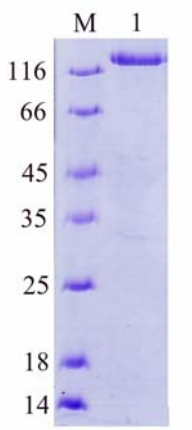


Figure 16. **Coomasie blue stained 12% SDS-PAGE gel of MbtB purified.** M= molecular size standards. Lane 1 shows MbtB purified by Ni^{2+}-affinity chromatography. Protein solution fractions purified were pooled for in-gel trypsin digestion and then analysed by MALDI-MS.

In-gel trypsin digestion of MbtB was carried out and the generated peptides subsequently analysed by MALDI-MS. Two methods for desalting and concentrating the samples were investigated. In the first method, the tips were prepared with 100% ACN, 0.1% TFA, and then the sample eluted using 1% formic acid/ 50% methanol, whereas for the second method tips were hydrated and equilibrated with 50% ACN and 1% acetic acid, respectively, and then the samples eluted using 50% ACN/1% acetic acid. The second method was chosen since it allowed most of the MbtB peptide constituents to be analysed by MALDI-MS.

Virtual trypsin digestion determined the theoretical modification of the peptides in each carrier protein. Since the modification of both carrier proteins of MbtB involves the transfer of the P-pant group which has a mass of 339.32 Da, an increase in mass in the peptides would determine the modification of each carrier protein. Thus, the ArCP peptide of 1903.9984 Da was expected to increase to 2243.3218 Da, whereas the PCP peptide 2914.4693 Da was expectecd to increase to 3253.7927 Da. Peptides generated by in-gel digestion of MbtB were analysed in two different matrices, SA and CCA, which allowed the screening of either small or large peptides. An increase of mass by addition of the (P-pant) group (339.32 Da) from CoA by PPTase was expected in each carrier protein, but neither ArCP nor PCP of MbtB was observed, as analysed by MALDI-MS. Only the peaks corresponding to ArCP or PCP in their *apo*-form were observed (Figure 17).

To screen further for post-translational modification of MbtB, after co-overexpression with *pptT*, in-solution trypsin digestion of MbtB was carried out to identify peptides. After digestion, a sample was taken and analysed by 15% SDS-PAGE. Migration of different size bands on the gel indicated that digestion of MbtB was achieved and the generation of peptides for subsequent labelling with fluorescent dye. Peptides generated by digestion were subsequently labelled with Alexa Fluor-maleimide. Maleimide reacts with thiol groups, and since MbtB possess 10 cysteine residues, it was expected that addition of the fluorescent dye would yield 9 labelled peptides (one peptide has two cysteine residues). Also, by addition of the phosphopantetheine group, which at its end also possesses a thiol group, to modify MbtB in its *holo*-form, two additional peptides were expected to be labelled if the PPTase modified both carrier proteins. Labelled peptides were subsequently analysed by HPLC to separate the interest peptides and elution of Alexa fluor modified peptides was carried out at 493 nm. The HPLC profile showed some peaks that could be labelled, and apparently the maximum number of labelled peptides detected was 8 (data not shown). Most of the peaks overlapped and only two peaks, 7 and 8, were separated at different retention times. Despite this poor resolution, all labelled peptides were purified and analysed by MALDI-MS to determine their nominal mass. However, the determination of the nominal mass of those peptides was not

achieved either because under acidic conditions a spontaneous cleavage of the peptides occurred or during ionisation in MS, desorption of peptides did not occur.

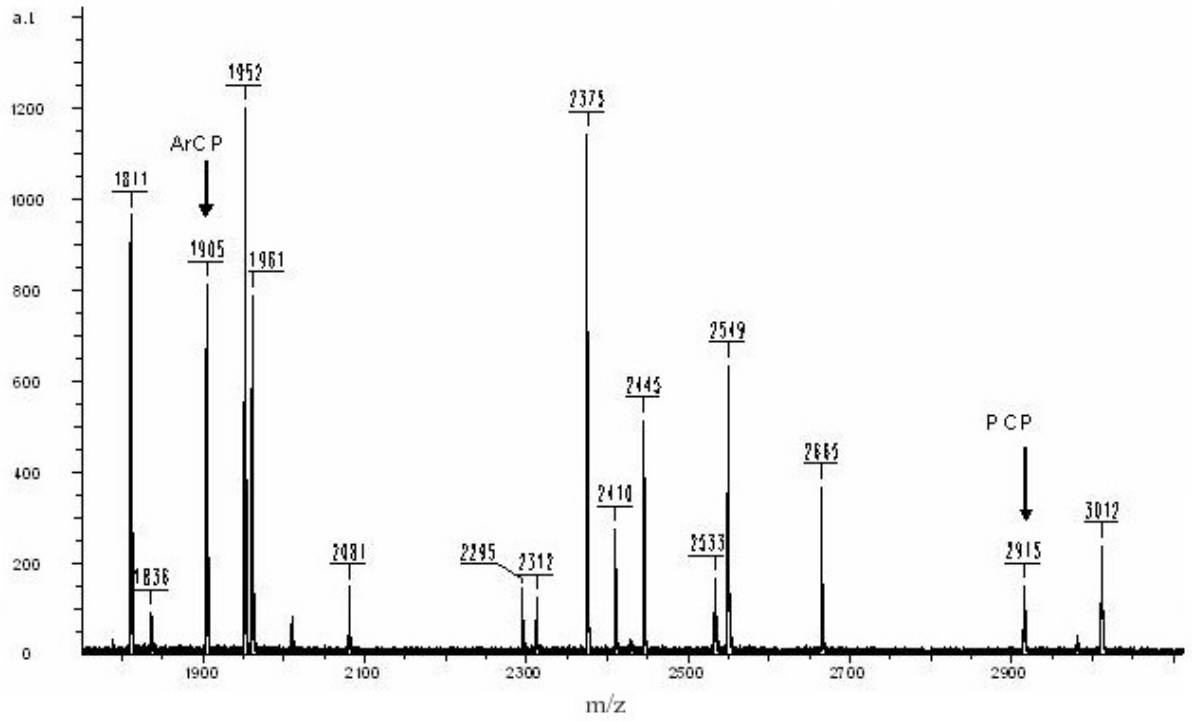


Figure 17. MALDI-MS analysis of peptides from MbtB. After co-overexpression of *mbtb*-pET23b and *pptT*-pCDFDuet-1 in *E. coli* C41(DE3), followed by MbtB purification by Ni^{2+}-affinity chromatography. In-gel trypsin digestion of the MbtB was carried out, and the peptides were then analysed by MALDI-MS to screen for modifications. No modification of both ArCP and PCP of MbtB were observed and only the carrier proteins in their *apo*-form were detected (indicated by arrows).

2.3.2 *In vitro* post-translational modification of MbtB.

Since *in vivo* MbtB post-translational modification by PptT, using *E. coli* as a heterologous host, was not successful, *mbtB* and *pptT* were expressed separately into pET vectors and transformed *E. coli* C41 (DE3) and the modification attempted by *in vitro* assays. The *mbtB* gene had already been cloned into a pET23b-expression vector and expressed in *E. coli* C41

(DE3), followed by Ni^{2+}-affinity chromatography purification. In order to attempt *in vitro* modification, the *pptT* gene was cloned into a pET28b-expression vector with an N-terminal hexa-histidine tag, and subsequently expressed in *E. coli* C41(DE3), achieving good yields of soluble protein. The recombinant PptT was purified by Ni^{2+}-affinity chromatography and it was obtained pure. The purified protein migrated as a single band on 12 % SDS-PAGE with an apparent molecular mass of 30 kDa, although the molecular mass calculated for PptT is 24 kDa (Figure 18). This abnormal mobility of PPTases on SDS-PAGE has been observed previously (Ku *et al*., 1997; Sánchez *et al*., 2001).

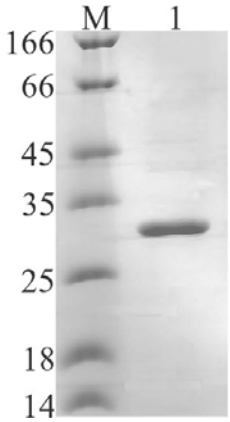


Figure 18. Coomasie blue stained 12% SDS-PAGE of PptT purified. The recombinant protein was purified in a step gradient concentration of imidazole (50-500 mM). The purified protein migrated as single band with an apparent molecular size of 30 KDa (calculated MW for His_6 –tagged PptT is 25.53 KDa) on 12 % SDS-PAGE.

To establish if PPTase could catalyse the transfer of the P-pant group in the conserved serine residue into either *apo*-ArCP or *apo*-PCP of MbtB to their *holo*-forms, both proteins were incubated at 37ºC in a time course assay. Then, for each time point a sample was taken and loaded onto 12% SDS-PAGE, the carrier protein band was excised and in-gel protein

digestion performed by trypsin. Samples were treated to desalt and concentrate with the second method for MS analyses which allowed detection of most MbtB peptides constituents. No modification of either carrier protein was observed when the MbtB peptide constituents were analysed. Only the peptides, which corresponded to either ArCP or PCP in their *apo*-form were observed (data not shown). Therefore, results indicated that this recombinant form of the intrinsic *M. tuberculosis* PPTase did not transfer the phosphopantetheine group from CoA to the serine residues in each carrier protein of MbtB.

2.3.3 Cloning, expression, and post-translational modification of the ArCP.

Since neither *in vivo* nor *in vitro* strategies produced efficient post-translational modification of MbtB by the intrinsic PPTase of *M. tuberculosis*, cloning and expression of the truncated ArCP from MbtB represented an alternative approach. The pTYB expression vectors were used to attempt cloning and expression of the ArCP in *E. coli* ER2566. Hence, the truncated ArCP gene was cloned into pTYB1, pTYB2 and pTYB12.

E. coli was transformed with the ArCP-pTYB1 construct and the cultures (100 mL in LB medium) were grown at 37°C until OD_{600}=0.5-0.6. Cultures were cooled to 16°C and gene expression induced by the addition of different IPTG concentrations (0.1 mM, 0.5 mM and 1 mM). Incubation was continued for a further 16 hours at 16°C with shaking. Cells were disrupted by French Press followed by centrifugation and the clarified (supernatant) and crude extracts analysed by SDS-PAGE. Since the ArCP (MW 9.12 KDa) was cloned in fusion with the Chitin Binding Domain (CDB, 56 KDa) of the pTYB1 expression vector, a fusion (CBD)-recombinant protein ArCP with a MW 65 KDa was expected. A band migrated about 66 KDa in all clarified samples from each IPTG concentration tested, including in the negative control upon 12% SDS-PAGE. Nevertheless, Western Blot and Immunodetection analyses were performed to further screen expression. Analyses of banding patterns suggested that anti-CBD-intein had recognised several molecular species with mass ranging between 66-56 kDa in the clarified samples. The signal intensity was significantly increased in extracts of cells

treated with IPTG consistent with the overproduction of the ArCP-CBD fusion protein (Figure 19A).

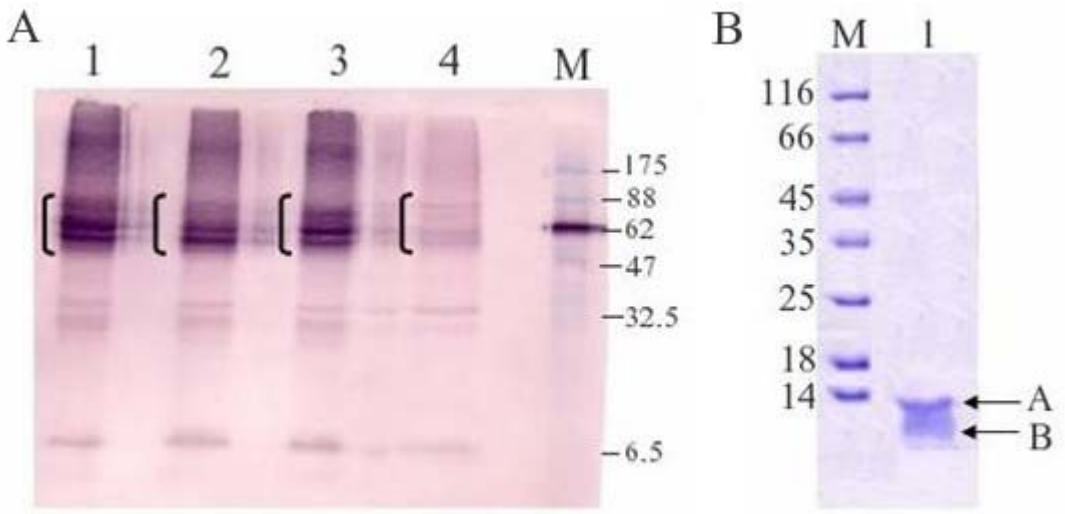


Figure 19. Over-expression and purification of ArCP. A) Western Blot and Immunodetection analysis. Strong signals were observed (indicated between brackets) in samples coming from induced cells with IPTG at: Lane: 1 mM; Lane 2: 0.5 mM; Lane 3: 0.1 mM. In lane 4, the control (no IPTG added) was loaded and no strong signal was observed. Numbers on the right are molecular masses (in kDa) based on broad-range prestained standards (New England Biolabs). B) Coomasie blue stained 15% SDS-PAGE of the ArCP of MbtB. Lanes as following: M: marker; Lane 2: purified protein. The purified protein migrated (pointed with an arrow) below the 14 marker band and this indicated that the interest protein was expressed in *E. coli* ER2566. Due to the presence of another band below the protein, it was assumed that the ArCP was modified *in vivo* by the heterologous host.

Thus, *E. coli* ER 2566-ArCP-pTYB1 was grown in 4 L of TB media and the expression induced with 0.5 mM IPTG at 16ºC overnight. After induction, cells were harvested and treated as before. The clarified supernatant was loaded onto a 25 mL bed of chitin beads and purification performed. Here the chitin-adsorbed fusion protein was treated with 20 mM DTT to release ArCP domain from its intein-linked fusion partner (Figure 19B).

The eluted protein was electrophoresed upon 15% SDS-PAGE and showed the presence of two distinct bands (Figure 19B, pointed by two arrows), suggesting that the ArCP might have been *in vivo* modified by the intrinsic PPTase of *E. coli* ER2566; similar electrophoretic behaviour was observed with AcpM of *M. tuberculosis* that participates in mycolic acid biosynthesis in similar analyses (Kremer *et al*., 2001). To confirm this, FT-ICR and ES-MS analyses were performed for the intact fusion protein and for the two bands, but in both analyses modification of the ArCP was not detected (Figure 20).

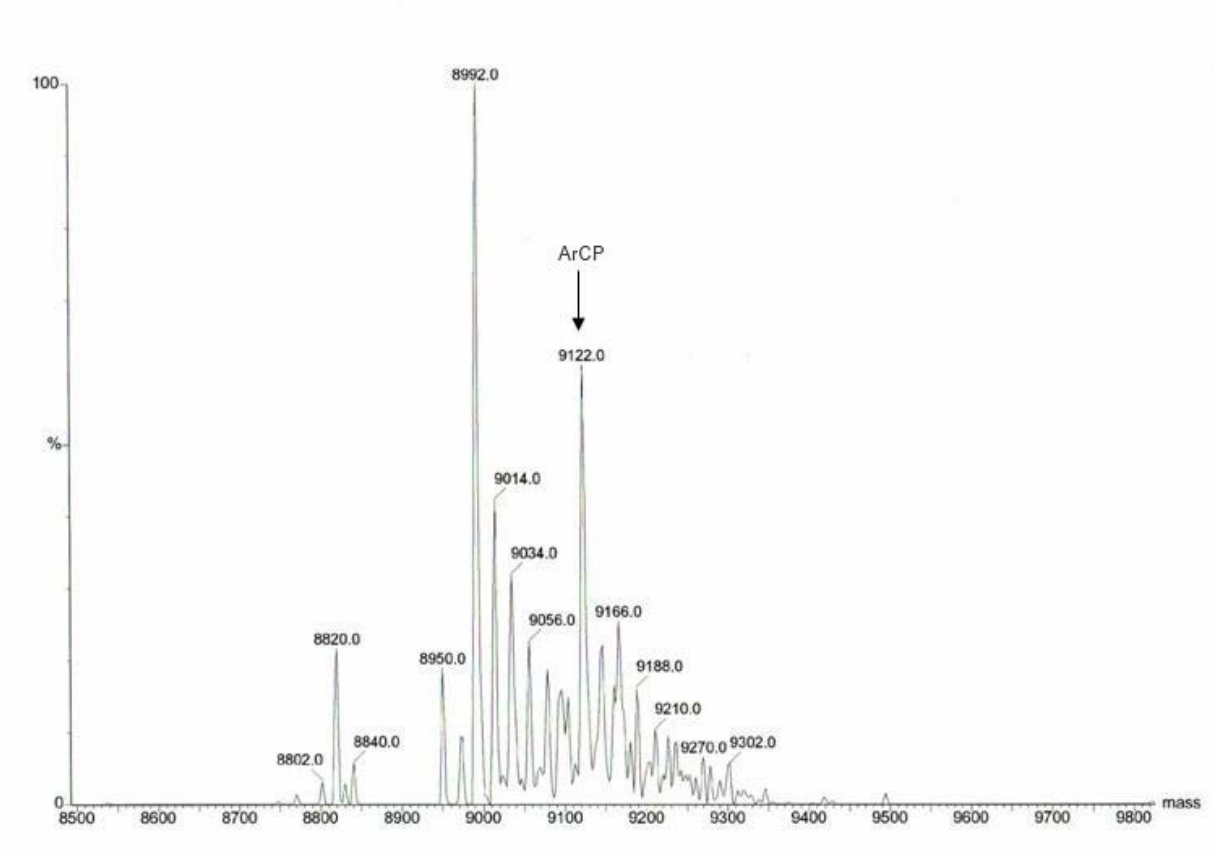


Figure 20. ES-MS analysis of the ArCP. The ArCP was overproduced in *E. coli* ER2566 and purified by chitin bead affinity chromatography. The ArCP was analysed by ES-MS to seek post-translational modification. The molecular weight calculated for ArCP is 9121.57 Da. Addition of the phosphopantetheine group (339.32) should have increased the mass up to 9460.89. However, post-translational modification was not achieved, and only a peptide exhibiting the nominal mass of the unmodified ArCP (indicated with the arrow) was observed.

In another attempt, both the recombinant PptT (decribed earlier) and the ArCP proteins were purified separately and *in vitro* assays were carried out seeking modification of the truncated protein. Here also, MALDI-MS analysis of in-gel trypsin treated samples provided no evidence for the post-translational modification of the carrier protein.

2.3.4 *In vivo* post-translational modification of the ArCP and MbtB by a broad substrate phosphopantetheinyl transferase.

The PPTases are classified into three groups or families based on their sequences, structural similarities and substrate specificities. The first family designated "Sfp-type", denominated after Sfp (surfactin phosphopantetheinyl transferase from *Bacillus subtilis*), include PPTases having ~230 amino acid residues in length and with broad substrate specificity (Lambalot *et al*., 1996). Members of this family often participate in PKSs/NRPSs hybrid systems, such as siderophore biosynthesis, i.e. enterobactin and pyochelin. Since the intrinsic PPTase of *M. tuberculosis* did not carry out the post-translational modification of the carrier proteins, another approach was to examine a broad substrate PPTase. Hence, the MtaA, a PPTase from myxochelin biosynthesis of *Stigmatella aurantiaca* was chosen because of the enzyme's demonstrated ability to carry out phosphopantetheinylation of fatty acid and carrier proteins of NRPSs (Gaitatzis *et al*., 2001). Based on amino acid sequence aligments and conserved motifs, PptT and MtaA appear to be related to Sfp. Therefore, based on these experimental and bioinformatic information, MtaA was viewed as a potential candidate for attempting post-translational modification of the carrier proteins of MbtB and the ArCP domain.

Firstly, *mbtB*-pET23b and ArCP-pTYB1 were transformed into *E. coli* C41 (DE3) and *E. coli* ER2566, respectively, to prepare chemical competent cells. Then, a second round of transformation using the pSUMtaA (genetic construct carrying the *mtaA* gene encoding for the MtaA provided as a gift by Prof. Rolf Müller, Department of Pharmaceutical Biotechnology, Saarland University, Germany), was performed. As negative controls, the *ArCP*-pTYB1 and *mbtB*-pET23b constructs were expressed in their respective heterologous host without pSUMtaA. After co-overexpression of each of the protein sets (ArCP/MbtB) with *mtaA* in

their respective heterologous host, purification, in-gel trypsin digestion and MALDI-MS analysis of the peptides of each protein, both proteins showed mass increases ~340 Da, in the carrier proteins, which corresponds to the mass of the attached phosphopantetheinyl group. Thus, MtaA activated both the ArCP and the MbtB proteins in *E. coli* (Figure 21).

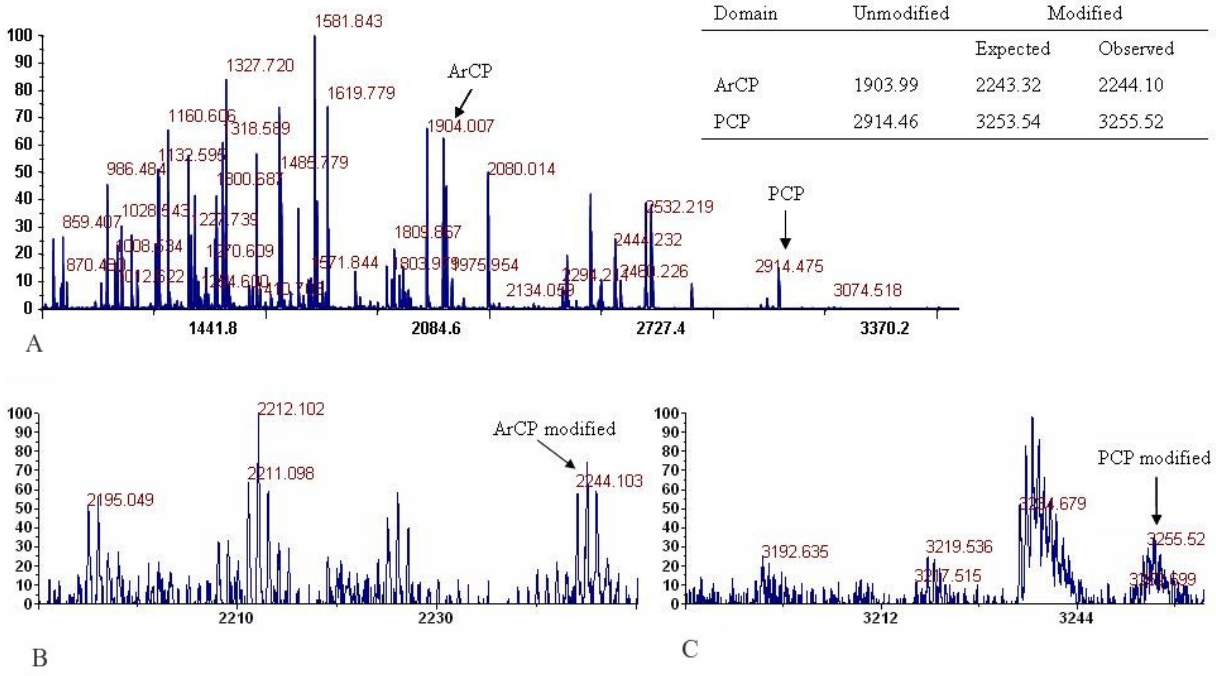


Figure 21. MALDI-MS analysis of the carrier proteins of MbtB. The *mbtB*-pET23b and pSUmtaA genes were co-overexpressed in *E. coli* C41 (DE3). After co-overexpression, purification, in-gel trypsin digestion, and MALDI-MS analysis of the peptide constituents of MbtB, post-translational modification was observed in both carrier proteins. A) As negative control, the *mbtB*-pET23b was overexpressed in *E. coli* C41 (DE3), without the pSUMtaA construct, followed by purification, in-gel trypsin digestion, and MALDI-MS analysis. Only the *apo*-forms of the carrier proteins were observed (indicated by arrows). By co-overexpression of the *mbtB*-pET23b and pSUMtaA both the ArCP (B) and the peptidyl carrier protein (C) were partially phosphopantetheinylated by MtaA.

However, it was clear that complete post-translational modification of MbtB had not been achieved and the efficiency at which the ArCP and PCP domains within MbtB had been modified was not uniform. Approximately 75% of the total ArCP was activated (Figure 21B), judged from the peak area in the MALDI-MS spectra, whereas only 30% of the PCP was modified (Figure 21C). However, when the modification of the isolated ArCP domain was analysed, phosphopantetheinylation appear to be complete (Figure 22).

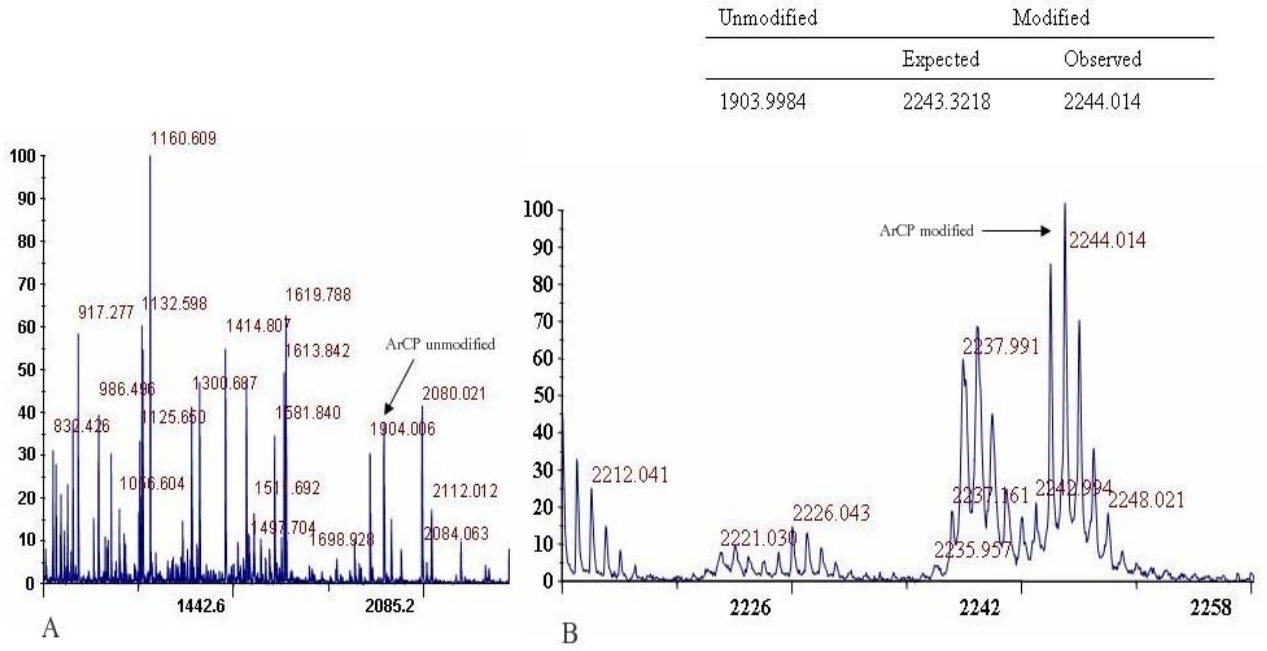


Figure 22. MALDI-MS analysis of the truncated ArCP. The ArCP-pTYB1 was co-overpressed with pSUMtaA in *E. coli* ER2566, followed by purification, in-gel trypsin digestion, and MALDI-MS analysis. A) For negative control ArCP-pTYB1 was overexpressed without pSUMtaA; only the apo-form of the carrier protein was observed. B) Complete *in vivo* post-translational modification of the ArCP by MtaA was observed.

2.4 Discussion.

Numerous antibiotics and other secondary metabolites are produced by complex NRPSs and PKSs megaenzymes. NRPSs and PKSs are organized in modules, and each module contains multiple and different catalytic domains. A simple NRPS module may consist of an adenylation domain (A) for substrate recognition and activation, a carrier protein domain (CP) that accepts the activated substrate and holds its residue as a thioester, and a condensation domain (C) for peptide bond formation. A final domain might be present in this multienzyme complex, the thioesterase domain (TE) that cleaves the growing molecule from the terminal NRPS. Additional enzyme activity can be found by the presence of extra domains such as the epimerisation domain (E) that catalyses racemisation and the cyclisation domain (Cy), catalysing both condensation and intramolecular heterocyclisation of serine, threonine or cysteine (Challis & Naismith, 2004).

A typical PKS module consists minimally of an acyltranferase (AT) to extend unit selection, an acyl carrier protein (ACP) for extender unit loading, and ketoacyl synthase domain (KS) for decarboxylative condensation between the aligned acyl thiosters to elongate the growing polyketide chain (Du *et al*., 2001). NRPSs enzyme activities may be coupled with PKSs to form NRPS/PKS hybrid systems to produce complex molecules, i.e. siderophores, from simple building substrates. NRPSs and PKSs have common organization domains: i) both systems use carrier proteins (PCP in NRPSs and ACP in PKSs) to tether the growing product; ii) both PCP and ACP are post-translationally modified by phosphopantetheinyl transferases; iii) the growing chain is released by a TE domain (Du *et al*., 2001; Schwarzer *et al*., 2003).

There is increasing interest in elucidating the complete structure and function of NRPSs and PKSs enzymes to develop novel biological molecules of pharmacological importance. Furthermore, establishing the biochemical sequence reactions, as well as substrate recognition, during siderophore biosynthesis, would aid in designing new drugs against pathogenic bacteria (Schwarzer *et al*., 2003). However, investigation of these enzymes has been hampered due to their enormous size representing not only a problem for molecular

cloning, but also hindering heterologous expression and purification of the gene products (Keating *et al*., 2000c). In addition, appropriate functions of each module might be coupled with other synthetases as a single complex interaction. All these challenges are represented in cloning and expression the *mbtB* gene of *M. tuberculosis*, a NRPS required for the core structure of mycobactin-carboxymycobactin biosynthesis (De Voss *et al*., 2000; Quadri *et al*., 1998). Furthermore, the high GC content of mycobacteria genomes represents an additional challenge for molecular cloning. Despite these challenges, in this study the full length of *mbtB* was cloned and successfully expressed and purified as a soluble protein in *E. coli* C41 (DE3). The MbtB modular enzyme (~151 KDa) consists of the following five domains: an adenylation domain to activate serine/threonine, two carrier proteins, one for salicylic acid (ArCP) and one for serine/threonine (PCP) covalent tethering, a cyclisation domain (Cy) to condense and cyclise the activated substrates to form the oxazoline ring, and a thioesterase domain (TE) for releasing the growing product.

In order to carry out subsequent biochemical studies, the carrier proteins of MbtB, ArCP and PCP, must be post-translational modified by a dedicated phosphopantetheinyl transferase (PPTase). The PPTase catalyses the transfer of the phosphopantetheinyl moiety from CoA to the hydroxyl group in the side-chain in a conserved serine residue of the carrier protein domains. *M. tuberculosis* possesses only two PPTases: i) Rv2523C, also called *acpS* because the encoded protein displays similarity in activity, amino acid sequence, and substrate specificity, to AcpS, a *E. coli* PPTase (Chalut *et al*., 2006); ii) Rv2794c, which encodes for 227-aa PPTase, named PptT, which is involved in pre-mycobactin biosynthesis (Quadri *et al*., 1998), as well as in numerous PKSs and NRPSs biosynthesis (Chalut *et al*., 2006).

In this study, the first attempt to achieve *in vivo* post-translational modification of the carrier proteins of MbtB was the co-overexpression of the intrinsic *pptT* gene of *M. tuberculosis* in *E. coli* C41 (DE3). Several reasons supported the use of *E. coli* to carry out this study: i) *E. coli* has been successfully used for *in vivo* reconstitution of carrier proteins and PPTases involved in aryl-*N*-capped siderophore production, such as yersiniabactin (Bobrov *et al*., 2002) and enterobactin (Gehring *et al*., 1997a), as well as other molecules

produced by NRPSs (Gruenewald *et al*., 2004); ii) the availability of established cultures, ease of handling, low cost and a range of expression vectors; and iii) co-overexpression of mycobacterial genes in *E. coli* represented a strategy to measure interaction between associated gene products (Mir *et al*., 2006). Hence, the genetically compatible constructs were created: *mbtB*-pET23b and *pptT*-pCDFDuet-1, in which the expression vectors displayed the required genetic features, different replicons and resistance markers, for *in vivo* post-translational modification of MbtB by PptT in *E. coli* C41 (DE3). After co-overexpression, protein purification, in-gel trypisn digestion and MALDI-MS analysis of the peptide constituents of MbtB, phosphopantetheinylation by PptT was not detected. The modification both ArCP and PCP of MbtB was not achieved probably because the intrinsic phoshophopantetheinyl transferase of *M. tuberculosis* was not produced in the concentrations required to carry out its activity under these conditions. Despite the likely copy number of 20-40, production of the protein in *E. coli* C41 (DE3) was probably not achieved at high enough levels. The use of *E. coli* C41 (DE3) as host was due to this strain being modified to avoid high production of proteases and allows good co-expression host for globular and membrane protein over-production (Miroux & Walker, 1996). On the other hand, poor plasmid stability has been observed in this strain, although the target proteins were overexpressed. Expression of some heterologous genes from plasmids may be toxic to *E. coli* C41 (DE3). These plasmids are unstable and may be lost from up to 50% of the population (Dumon-Seignovert *et al*., 2004). Maybe, the *pptT*-pCDFDuet-1 is relatively unstable in *E. coli* C41 (DE3) and therefore the production of PptT was compromised since no modification of MbtB was observed.

Research in PKSs and NRPSs biosynthesis has been difficult due to inability to produce their fully active *holo*-forms in other hosts, because the host PPTases fail to either recognise or act inefficiently on these carrier proteins substrates. Many organisms utilise more than one PPTase-dependent pathway for either primary or secondary metabolism. *E. coli* produces three PPTases, the AcpS required for fatty acid biosynthesis, EntD a PPTase involved in enterobactin production (Lambalot *et al*., 1996; Lambalot & Walsh, 1995), and YhhU (also called o195) rendering function similarity to AcpS under certain cell environment conditions (Flugel *et al*., 2000). It is interesting to note that regardless of similar function of

the AcpS and EntD PPTases, the proteins are able to recognise their respective specific substrates: AcpS recognizing only ACP and EntD only accepting ArCP and PCP of NRPSs (Lambalot *et al*., 1996). It has been shown that EntD does not cross-interact with primary metabolism, since EntD may be only expressed under iron-deficient environments (Lambalot *et al*., 1996). However, partial or fully post-translational modification of the carrier proteins is observed in *E. coli* due to its endogenous PPTases. Obviously, over-expressing ACPs in *E. coli* affords activation of this type of proteins, but levels vary from complete to almost undetectable modification of the proteins (Crosby *et al*., 1995; Jackowski & Rock, 1983; Keating *et al*., 1995; Keating *et al*., 1996; Kremer *et al*., 2001).

The ArCP and PCP domains from vibriobactin biosynthesis of *Vibrio anguillarum* reached 46-56% and 28-70% *holo*-conversion by the *E. coli* EntD. The relative modification of these carrier proteins was attributed to the high similarity with their counterparts in enterobactin biosynthesis (Liu *et al*., 2005). In contrast, attempting to carry out post-translational modification of tyrocidine synthetase 1 (TY1), a NRPS produced by *Bacillus brevis* ATCC 8185, failed when the protein was expressed in *E. coli*. The aminoacylation activity was low as the majority of the enzyme molecules were unmodified (Pfeifer *et al*., 1995). The truncated recombinant PCP from the tyrocidine synthetase A of *B. subtilis* was partially modified in *E. coli*, activity attributed to the EntD enzyme (Stachelhaus *et al*., 1996). Clearly, *E. coli* does not support *in vivo* post-translational modification of NRPSs and PKSs due to its endogenous PPTases. To overcome this problem, co-overexpression of exogenous PPTases in *E. coli* has achieved phosphopantetheinylation of the carrier proteins from diverse systems. The co-overexpression of the *Streptomyces* ACPs from the actinorhodin and griseusin PKSs with *E. coli* AcpS led to high levels (>90%) of *holo*-ACP production (Cox *et al*., 1997).

The PPTase required for the gramicidin biosynthesis from *Bacillis subtilis*, named Gsp (gramicidin sythetase phosphopantetheinyl transferase), was co-overexpressed with the truncated phenylalanine-activating module GrsA protein of gramicidin S synthetase in *E. coli* X90 leading to the isolation of fully modified active *holo*-enzyme (Ku *et al*., 1997). The *sfp*

gene that encodes a broad substrate PPTase from *B. subtilis* was inserted in the chromosome of *E. coli* and subsequently co-overexpressed with the genes encoding two modules, TycA and TycB1, for the biosynthesis of the antibiotic tyrocidine from *B. brevis*. The resulting *E. coli* *sfp*$^+$ strain converted the *apo*-form of the module to their *holo*-form (Gruenewald *et al*., 2004). Also, the *angD* gene that encodes a PPTase for the biosynthesis of anguibactin, a siderophore in *Vibrio anguillarum*, modified ArCP and PCP domains successfully when the gene was expressed in *E. coli entD* mutant AN90 since this strain lacked the PPTase responsible for the NRPS activation for the siderophore enterobactin (Liu *et al*., 2005).

Since *in vivo* post-translational modification of MbtB by PptT did not work, *in vitro* assays were carried out by cloning and expressing the *pptT* and *mbtB*, separately. Thus, the *M. tuberculosis pptT* gene was cloned into another *E. coli* expression vector (pET28b), allowing PptT to be produced as a His-tagged protein and homogenously purified by Ni^{2+}-affinity chromatography. The *mbtB*-pET23b was overproduced in *E. coli* C41(CE3) and purified similar to PptT. To establish if PptT could catalyse the phosphopantetheinylation of MbtB, both proteins were incubated at 37°C in a time course *in vitro* assay. However, after in gel-trypsin digestion and MALDI-MS analysis of MbtB, modification of the carrier domains was not detected. The results obtained in this study differ from that found when both ArCP of MbtB and PCP from MbtE were converted to their *holo*-forms by PptT *in vitro* assays (Quadri *et al*., 1998). The ArCP, PCP and PptT genes were cloned and overproduced as maltose-binding protein fusions (MBP) allowing good production of the recombinant proteins. The PptT activity was assessed by the incorporation of the [H^3]-pantetheine group into the carrier domains. In addition, the quantitative conversion of *apo*-proteins into their *holo*-form was proved by HPLC and MALDI-TOF (Quadri *et al*., 1998). Here, the expression and production of MbtB and PptT was sufficient and the proteins were obtained as soluble enzymes, however *holo*-conversion of the carrier proteins was not achieved probably due to PptT exhibited no catalytic activity.

The initial *in vivo* and *in vitro* approaches were not successful strategies to achieve MbtB modification by PptT, another way to overcome this challenge was to clone the ArCP from MbtB. Hence, the ArCP domain was cloned into pTYB1 as a fusion frame with an intein CBD tag allowing single step purification. Initial expression experiments of ArCP-pTYB1 in *E. coli* ER2566 did not show overproduction of the protein. Western Blot and Immunodetection analysis provided hints of ArCP overexpression, but large amounts of culture (at least 4 L of TB medium) were needed to achieve enough protein yields. Based on 15% SDS-PAGE analysis, the protein showed the presence of two distinct bands, suggesting thus that the ArCP might has been *in vivo* activated by the endogenous *E. coli* ER2566 PPTases. This assumption was supported by an earlier report in which the *M. tuberculosis* AcpM was *in vivo* phosphopantetheinylated by *E. coli*. ES-MS analysis confirmed not only the *holo*-form of AcpM, but also determined the palmitoylated-AcpM form (Kremer *et al*., 2001). Hence, each band derived from ArCP was analysed by ES-MS and FT-ICR MS, but these experiments did not show modification of the truncated protein. Therefore, the presence of these distinct bands of ArCP may be attributed to a limited degradation of the protein during purification process. To seek further modification of the ArCP, *in vitro* assays were attempted to achieve post-translational modification of ArCP. Both proteins (*pptT*-pET28b and ArCP-pTYB1) were overproduced in their respective host and purified separately to carry out *in vitro* assays. However, this approach did not achieve activation of the ArCP either.

MbtB represents a potential enzyme for not only understanding mycobactin biosynthesis, but also the protein could be the basis to exploit its structure for rational design of new drugs to combat TB. Thus, a further attempt embarked on the use of broad substrate phosphopantetheinyl transferases that could allow modification of the MbtB, as well as the truncated ArCP. The phosphopantetheinyl transferases form a superfamily of enzymes, which are classified into three groups based on their sequences, structural similarities, and susbtrate specifities (Lambalot *et al*., 1996). The first group is exemplified by AcpS of *E. coli*, which consist of PPTases about 120 amino acids in length, function as homotrimers, and modify only ACP of FAS and PKSs (Lambalot *et al*., 1996). Members of the second group, typified by the Sfp of *B. subtilis*, are usually at least 240 residues long, function as monomers, and

have very broad substrate specifities. These PPTases are mainly found in NRPSs and PKSs as well as in siderophore biosynthesis (Reuter *et al*., 1999). The PPTases of the third group act as integrated domains on their cognate ACP of type I FAS in *Saccharomyces cerevisae* (Fichtlscherer *et al*., 2000). The remarkable broad substrate of the second PPTase group provide another alterative to accomplish post-translational modification of the carrier proteins in MbtB. Hence, the MtaA was chosen due to: i) the similarity of MtaA in activity and substrate recognition compared to that of Sfp-prototype and PptT (Silakowski *et al*., 1999); ii) the ability of the protein to modify *in vivo* diverse carrier proteins using *E. coli* as heterologous hosts; iii) the features of the pSUMtaA genetic construct, different replicon and resistance marker (Gaitatzis *et al*., 2001). These features pointed to MtaA as a valuable tool to carry out co-overexpression along with *mbtB*-pET23b and ArCP-pTYB1 in *E. coli*. Therefore, co-overexpression was performed for each set of carrier proteins with and without MtaA. After analysis, both carrier proteins were phosphopantetheinylated to some extent. Co-overexpression of *mbtB*-pET23b and pSUMtaA in *E. coli* C41(DE3) led to activation of the ArCP domain to 75%, whereas only 30% of the PCP domain was modified. There was no modification, when only the carrier proteins were overproduced in *E. coli*, indicating that phosphopantetheinylation observed was entirely dependent upon MtaA and was not mediated by the endogenous *E. coli* PPTases.

The proportional modification of the carrier proteins may be attributed either to a stress response (metabolic burden) of *E. coli* or substrate preference by MtaA towards ArCP. Metabolic burden is defined as the amount of resources (raw material and energy) that are withdrawn from the host metabolism for maintenance and expression of foreign DNA. As result of this metabolic burden due to high synthesis of protein, amino acid starvation occurs as well as other resources (Bentley *et al*., 1990). It has been suggested that the intracellular concentrations of the CoA and Mg^{2+}, the required substrate and co-factor for PPTase activity, tend to be reduced during cellular growth and thus become a limiting factor for complete activation of the carrier proteins. Expansion of the intracellular CoA pools in cells overproducing ACP (through pantothenate supplementation together with a mutation giving a feedback-insensitive pantothenate kinase) markedly increased the conversion of *apo*-ACP to

its *holo*-form (Keating *et al*., 1995). Furthermore, the CoA pool limitations decrease translational efficiency of certain amino acids, mainly glutamate and other derived amino acids from tricarboxylic acid, which in turn affects synthesis of proteins (Keating *et al*., 1996). Taking into account the length of the overproduced recombinant proteins (MbtB:1414 and MtaA:277), the incomplete activation of the overproduced recombinant protein by MtaA may be due to low concentrations of CoA, Mg^{2+} and amino acids in the media during growth of the heterologous host. Interestingly, co-overexpression of ArCP-pTYB1 and pSUMtaA in *E. coli* ER2566 achieved full phosphopantetheinylation of the truncated aryl carrier protein (89 amino acids in length), supporting the above hypothesis that such metabolic burdens may be of significance in this process; this assumption must be further tested by other means. It is interesting to note that other studies have found partial modification of carrier proteins when co-overpression was attempted. Co-overexpression of PspT, a PPTase from *Pseudomonas syringae*, and Cfa1 (ACP), in *E. coli* DH10B resulted in only 25% conversion of Cfa1 to its *holo*-form (Seidle *et al*., 2006). There was only 90% conversion of *Streptomyces apo*-ACP to *holo*-form when co-overexpressed along with the *acpS* gene in *E. coli* K38 (Cox *et al*., 1997).

Although, the broad substrate PPTases are well known to modify diverse carrier proteins, the means by which they recognise their substrates are not fully understood. Most organisms employ more than one phosphopantetheine-dependent pathway also contain more than one PPTases, but *Cyanobacteria* and *Pseudomonas* seem to have only one PPTase suggesting their essentiality to carry out the modification of both carrier proteins of primary and secondary metabolism (Copp & Neilan, 2006; Finking *et al*., 2002; Seidle *et al*., 2006). In bacteria that lack a PPTase of primary metabolism, the Sfp-type seems to have been evolutionarily selected for high efficiency with CPs of primary metabolism, although the enzymes maintain broad substrate tolerance (Finking *et al*., 2002; Keszenman-Pereyra *et al*., 2003). Moreover, genes encoding for NRPSs and PKSs are often clustered in one region of the bacterial chromosome. Then, if every NRSP and PKS has its own dedicated PPTase, one should expect to find a PPTase gene within each cluster. Indeed, some genes encoding for PPTases are found clustered, for instance enterobactin in *E. coli* (Lambalot *et al*., 1996), surfactin in *B. subtilis* (Nakano *et al*.,1992) and gramicidin in *B. brevis* (Borchert *et al*., 1994).

However, there are exceptions in which the PPTases genes are not clustered within the NRPSs and PKSs, such as the mycobactin-carboxymycobactin biosynthesis in some mycobacteria species (Quadri *et al*., 1998). In addition, the structure, physical and biochemical properties of ACPs and PCPs are different, features that may account into PPTase recognition (Mofid *et al*., 2002). All these observations raise the question how the PPTases interact with their corresponding carrier proteins.

The answer may lie in the structures of both PPTases and the carrier proteins. Independent of substrate specificity, all biochemically characterised PPTases have three conserved motifs (Table 10). Additionally, the resolved crystal structures of AcpS and Sfp revealed a striking similar folding (Chirgadze *et al*., 2000; Parris *et al*., 2000). However, these structures revealed first that all AcpS-type PPTases are enzymatically active homotrimers (Chirgadze *et al*., 2000; Parris *et al*., 2000) whereas Sfp-type is a pseudohomodimer composed of two halves that resemble the AcpS monomer (Reuter *et al*., 1999), suggesting that Sfp arose from a gene duplication and further accumulation mutations of *acpS*. The Sfp-type CP recognition site is a flexible loop, whereas AcpS uses a rigid helix for the interaction with ACP, which may contribute to substrate recognition among CP domains (Parris *et al*., 2000).

Table 10. Conserved motifs in PPTases. Independent of their recognition substrate, three conserved motifs are present in all PPTases. *h refers to a hydrophobic residue in motif 3.

Motif	Conserved residues
P1	PXWPXGX2GS(M/L)THCXGY
P2	(V/I)G(V/I)D
P3	(F/W(S/C/T)XKE(A/S)hhK*

On the other hand, based on the crystal structures of *E. coli* ACP and *B. brevis* TycC3-PCP, the construction of a hybrid PCP/ACP, and mutational analysis of ACPS and PCPs revealed that the carrier proteins may also play an important role in PPTase recognition. The PCP and ArCP domains in NRPSs and the ACPs in FASs and PKSs share a conserved sequence motif (GX(D/H)S(L/I)(D/K) containing an invariable serine residue. The tridimensional structure of ACP and PCP show no significant differences in the overall folding pattern, having both CPs a characteristic four-helix bundle folding (Weber *et al*., 2000). Further analysis revealed some differences between ACPs and PCPs, being the difference in the electrostatic surface potential, especially around the invariant serine residue and adjacent helix 2 (Mofid *et al*., 2002). Mutational analysis in PCPs and ACPSs, determined that a single point mutation in PCP (residue 47, numbering based on the crystal structure of TyC3-PCP) domains allowed to be recognised *in vivo* and *in vitro* by AcpS (Finking *et al*., 2004). However, since the NRPSs and PKSs are organised in modules which might act either *in cis* or *in trans*, it cannot be ruled out that conformational change of the proteins contributes to the recognition of substrates as well (Marshall *et al*., 2002).

2.5 Further work.

Despite the partial modification of MbtB carrier proteins, optimal conditions during growth must be examined to achieve higher phosphopantetheinylation. Biochemical characterisation of the MbtB domains should then be attempted, particularly the communication between the A domain, Cy domain and PCP domain for substrate recognition (serine/threonine) and formation of the oxazoline ring following experimental methods reported elsewhere (Keating *et al*., 2000b). Mutation of the active sites of ArCP and TE domains would be needed to address some of these experiments. Hence, all the above experiments will allow further testing of different drugs obtained either *in house* or *in silico* to block early mycobactin-carboxymycobactin biosynthesis, although the first step can be already assessed (Chapter 3). The need for rational design of drugs to combat TB, prioritises the structural characterisation of MbtB and the modification of the protein achieved here offers the possibility that mutants can be constructed that accumulate thioster-bound precursors and reveal useful data.

3. Identification of potential inhibitors of mycobactin biosynthesis

3.1 The old anti-TB drugs and their drawbacks.

The World Health Organisation has recently reported that TB is still a menace for the human kind. Despite current chemotherapy, vaccination and the implementation of the DOTS program, the incidence of TB has not decreased. Contributing factors are the emergence of *M. tuberculosis* strains resistant to both first and second line drugs, co-infection with HIV, and related social and economical problems in high incidence countries (WHO, 2008).

The current TB drugs were developed between the 1940 and 1960s by screening a multitude of synthetic compounds, as well as natural products derived from other bacteria. However, following this period, no effective antibiotics have been developed to treat TB, except for the quinolones that were developed in the 1980s (Zhang, 2005). Because no biochemical tools were available and there was a general lack of understanding of *M. tuberculosis* biology, the target of these drugs was not identified. In fact, soon after STR was introduced for TB treatment, it became apparent that the bacillus could acquire resistance. The subsequent discovery of PAS and its combination with STR for TB treatment either delayed or reduced STR resistant. That observation suggested that TB could not be effectively treated with a single drug from those available.

The discovery of INH changed TB chemotherapy completely; the combination of this highly active and inexpensive drug with PAS and STR led to improved efficacy. Through, various experimental and clinical studies, modern TB chemotherapy was developed ultimately to the DOTS program (Saltini, 2006). The chemotherapeutic aspect of this broad public health initiative comprises a six to eight month regimen with four different drugs for treating patients infected by drug-susceptible organisms. For new TB cases, the regimen consists of an initial two-month bactericidal phase of treatment including four drugs (INH, RIF, PZA, and EMB), followed by a continuation phase of treatment with INH and RIF for four months (WHO, 2003). The regimens can be more complicated and extended with patients suffering co-infection with HIV-AIDS or infected by MDR-TB and therefore DOTS-Plus is implemented (WHO, 2003).

Although TB can be cured with the current treatment, complications may arise from the necessary duration, complexity and associated secondary effects of TB treatment which lead to poor patient compliance. Compared with treatment of other bacterial infections, such as *Helicobacter pylori* and pneumococcal infections, taking usually two or three weeks, TB chemotherapy requires at least six months. This requirement for such an extended treatment time is related to the disease pathology, biology of the bacillus, and immunology of the host. As aerosol particles, TB infection begins with the inhlation of *M. tuberculosis*. Afterwards, the bacilli are engulfed by alveolar macrophages and at this stage the bacterium either survives to continue infection or is contained by the activation of more macrophages and cellular immunity at the site of infection leading to the formation of a tubercle or granuloma (Russell, 2007). *M. tuberculosis* survives and multiplies in different environmental niches. Subpopulations of the bacillus are mainly located in necrotic lesions, but a significant portion of bacteria are associated with macrophages located in the periphery of the lesion (Russell, 2007). Moreover, in some cases the granuloma becomes quite large and the caseous material liquifies into the airway in which a large number of bacilli is present, representing a major problem for chemotherapy (Woolwine & Bishaie, 2006). Individuals with a strong immune system develop granulomas in which viable bacilli may be present and represents latent TB infection (LTBI). In this physiological state, *M. tuberculosis* becomes "dormant or latent" and no clinical symptoms and signs characteristic of TB infection are shown (Cosma *et al*., 2003). During development of TB infection, all of these subsets maybe present within *M. tuberculosis* populations and their metabolic heterogeneity hinders TB drug therapy. According to their metabolic activity, these sub-populations can be divided into four subsets. (i) Actively metabolising and rapidly growing, (ii) semidormant in an acidic intracellular environment, (iii) semidormant in a non-acidic intracellular environment and (iv) dormant (Mitchison, 1985). Certain drugs are more effective against some of these subpopulations INH, RIF, STR and EMB exhibit rapid bactericidal action against actively metabolising organisms, whereas pyrazinamide (PZA) appears to be more effective against semidormant bacilli in an acidic/hypoxic intracellular environments and RIF is effective against semidormant bacilli in non-acidic environments. Thus, current TB drugs are only active against growing bacilli, but there is no drug to kill "dormant bacteria"(Zhang, 2005). On the

other hand, the host immune system may not effectively eliminate residual tubercle bacilli not killed by TB chemotherapy.

Intrinsic and acquired drug resistance are observed in *M. tuberculosis*. Intrinsic resistance is attributed to: (i) the formidable permeability barrier afforded by the lipid-rich cell wall; (ii) mechanisms that activate genes for self-defending secondary metabolites or the production of enzymes that degrade or modify the drugs rendering them useless; and (iii) spontaneous chromosomal mutations of the drug targets (David, 1970; Leonard, 2006; Morris *et al*., 2005; Nguyen & Thompson, 2006). The latter mechanism is considered the most significant driving force for adaptation and survival of microorganisms in new environments, rendering benefical mutations and fitness factors that explain antibiotic resistance and virulence in bacteria (Perfeito *et al*., 2007; Woodford & Ellington, 2007). In fact, mutations in the genome of *M. tuberculosis*, that can confer resistance to anti-TB drugs, occur spontaneously with an estimated frecuency (*per* bacterium *per* generation) of 3.5×10^{-6} for INH, 3.1×10^{-8} for RIF, 3.8×10^{-8} for STR and 0.5×10^{-4} for EMB (David, 1970). In clinical terms, acquired resistant mutants are selected as a result of ineffective treatment due to noncompliance. This selection favours the evolution of *M. tuberculosis* by acquiring sequential mutations in the drug target, which provides resistance and has promoted the emergence of MDR- and XDR-TB (CDC, 2006; Crofton *et al*., 1997). During infection, antibiotics kill most of the growing bacilli, but some may become phenotypically resistant to the drugs, i.e. “persisters” (Zhang, 2005). This subpopulation may become actively growing when antibiotics are removed or present in low concentrations (Zhang, 2007). This phenomenon of reverted-sub-populations has effects in the selection of competitive fitness of *M. tuberculosis* resistant to drugs with a high probability to spread and become prevalent in human populations (Gagneux *et al*., 2006). Nowadays, *M. tuberculosis* strains resistant to almost all the front line TB drugs have emerged, demonstrating the high adaptability of the bacterium (Table 11).

Table 11. **Mechanism of current anti-TB drugs action, genes associated with resistance and adverse effects** (Hopewell, 2006; Sacchettini *et al*., 2008; Zhang, 2007).

Antibiotic	Mechanism and Target	Gene mutations associated with resistance	Toxicity
Isoniazid	Inhibits mycolic acid synthesis; primary targets InhA and secondary targets as KasA and DfrA	*katG*, *inhA*, and others	Hepatoxicity and peripheral neuropathy
Rifampicin	Inhibits transcription; RNA polymerase β-subunit	*ropB*	Display a range of adverse effects: rashes, hepatitis, gastrointestinal upset
Ethambutol	Inhibits arabinogalactan synthesis, EmbB	*embB*	Inflammation of the optic nerve
Pyrazinamide	No specific target has been found; probably inhibits FAS-I or alters membrane proteins	*pncA* (required for drug activation)	Hepatitis, hyperuricaemia (high levels of uric acid in blood)
Streptomycin	Inhibits protein synthesis; 30S ribosomal subunit.	*rpsL*, *rrS*	Ototoxicity (damage of the ear)
p-Aminosalicylic acid	Folate or mycobactin biosynthesis.	*thyA* (folate biosynthesis)	Gastrointestinal upset
Kanamycin	Inhibitis proteins biosynthesis	*rrS*	Hearing loss, Renal malfunction
Fluroquinolones	Inhibits DNA gyrase	*gyrA*	Nausea, vomiting, dizziness
Ethionamide	Inhibits mycolic acid biosynthesis: InhA	*inhA*, *ethA*, *ethR*	Gastrointestinal upset

The phenomenon of latent TB infection adds another complication for TB control. The WHO has estimated that one-third of the world's population is latently infected with TB. In 2000, there were an estimated 8-9 million TB new cases and this scenario has not changed and around 9.2 million new TB cases were reported in 2008 (WHO, 2008). This may be a reflection of latent TB and co-infection with HIV might trigger the emergence of new TB cases, specifically in those countries in which TB-HIV synergy has high incidence rates (WHO, 2008).

Another problem of TB chemotherapy is the adverse effects of the drugs (Table 11). The long last-duration of TB chemotherapy with INH may cause hepatoxicity and peripherial neuropathy (damage to nerves of the peripherial nervous system). Rashes, hepatitis, gastrointestinal upset, are adverse effects due to daily use of RIF (Hopewell, 2006). The emergence of MDR-TB requires the use of second-line drugs which are more expensive and have more side effects (WHO, 2008). Furthermore, there is evidence of drug-drug interactions between some of the antiretroviral agents for HIV treatment and key antitubercular drugs, in particular RIF (Ginsberg & Spigelman, 2007). RIF is a potent inducer of the hepatic cytochrome P450 isoenzyme CYP3A4. Protease inhibitors and non-nucleoside reverse transcriptase inhibitors are metabolised by this enzyme and it has been documented that co-administration results in reduced levels of the antiretroviral drugs (Pozniak *et al*., 1999).

3.2 The new generation of anti-TB drugs.

All the above observations have prompted a search for new drug targets and novel compounds to alleviate *M. tuberculosis* infection. Thus, the new goals for improving TB chemotherapy are: (i) shortening and simplifying the treatment of active-TB; (ii) treating MDR and XDR-TB; (iii) simultaneously treating TB and HIV-AIDS; and (iv) shorting the treatment for LTBI (Ginsberg & Spigelman, 2007; Sacchettini *et al*., 2008). A new era of TB drug discovery has emerged and there is an increasing interest from both private and public sectors to develop new drugs. The Global Alliance for TB Drug Development is coordinating all efforts at all stages of the TB drug development pipeline (Dover *et al*., 2008; TB-Alliance, 2007).

However, new drugs must meet certain characteristics to improve TB treatment. (i) The new drug target should be essential for bacterial growth, metabolism, viability and persistence (genes expressed and essential during the time that treatment occurs); (ii) the targets must be unique to mycobacteria, which are unrepresented in the human genome and absent in other bacteria (gut flora); (iii) the new compounds should be "druggable", that is they should have a range of biochemical and physical properties (drug-like properties); and (iv) the new compounds must modulate their action towards their targets. In order to facilitate these goals, development of appropriate *in vivo* and *in vitro* models to study "persisters" and "dormant" populations of *M. tuberculosis* are required (Balganesh *et al*., 2004; Ginsberg & Spigelman, 2007; Williams & Duncan, 2007).

Many promising new drug targets and active compounds have been identified in recent years. After several decades in which little TB research and development was carried out, a list of new compounds are being tested for their potential effectiveness in the treatment of TB. Some of these compounds are an improvement of the existing TB drugs, such as rifapentine (RPT) a long-lasting compound derived from rifamycin, the fluoroquinolone family, macrolides, oxazolidinones and nitroimidazoles. Representative of novel compounds are the pyrroles, pleuromutilins, and diarylquinolines (Spigelman, 2007; Zhang, 2007). Despite these advances, only a few drugs are under clinical trials (Table 12) and, therefore, new drug targets and novel compounds are urgently needed.

3.3 Tools and basis for the identification of new drug targets.

Although, there are promising new candidates in the drug pipeline and new targets, the main aim is to improve TB chemotherapy by shortening the regimen and preventing selection of resistant *M. tuberculosis* strains (Ginsberg & Spigelman, 2007). Ideally, new anti-TB drugs should have certain characteristics and it is essential to know the precise targets (Balganesh *et al*., 2004; Williams & Duncan, 2007).

Table 12. Potential anti-TB drug candidates under clinical tests (Ginsberg & Spigelman, 2007; Sacchettini *et al*., 2008; Spigelman, 2007; TB-Alliance, 2007; Zhang, 2007).

Drug	Target	Features, drawbacks, and clinical trial stage
Moxifloxacin	DNA gyrase	It could replace INH or EMB. Compatibility with HIV therapy. Phase IIIB.
Gatifloxacin	DNA gyrase	It could replace INH or EMB. Compatibility with HIV therapy. A particular concern is the reported increased risk of dysglycemia (abnormal blood sugar metabolism). Phase IIIB.
Nitroimidazopyran (PA-824)	Prodrug activated by F420-dependent glucose-6-phosphate dehydrogenase. Probably inhibits cell wall biosynthesis	No specific mode of action identified yet. However, exhibits activity against both replicating and non-growing *M. tuberculosis*. Phase I
Nitrodihydroimidazo-oxazole (OPC-67683)	Inhibits mycolic acid biosynthesis	No specific mode of action identified yet. Extremely potent, *in vitro* and *in vivo* activity. It could be used in combination with HIV therapy. Phase I.
Diarylquinoline (R207910, TMC207, or J compound)	Fo subunit of ATP synthase (*atpE*)	Initial tests indicate that there are no toxicity effects. Phase II.
Diamine analog (SQ-109)	Inhibition of cell wall biosynthesis.	No specific target identified yet. Shares structural similarity to EMB, but superior *in vitro* and *in vivo* activity. Phase I.
Pyrrole (LL-3858)	No information available	Bactericidal activity similar to INH. No public information available about mode of action. Phase I.
Metronidazole	Damage to DNA by reactive radicals	Active against dormant *M. tuberculosis*. Phase II.
Rifapentine	Inhibition of RNA synthesis	Superior activity compared to RIF. Long life in serum. Phase IIIB

The availability of the genome sequences of several strains of *M. tuberculosis* as well as other mycobacteria and members of the Corynebacterineae has afforded an opportunity to further investigate the biology of the organism, informing the selection of a range of potential drug targets to be screened (Cole *et al*., 1998). Furthermore, the advancement of drug research technology, as well as increasing genetic and biochemical tools in mycobacterial research have enhanced and expedited the identification of new drug targets in TB (Balganesh *et al*., 2004). Modern drug discovery strategies exploit two main approaches, (i) whole-cell target and (ii) target-based screening. The whole-cell strategy involves screening for potent inhibition of bacterial growth or killing activity of potential drugs. The fact that the compounds have activity implies that these drugs can penetrate the rigid *M. tuberculosis* cell wall. However, as this is not target based, there is a considerable risk of finding compounds that have generalised toxicity and the lack of information on the target will hinder the optimisation process (Balganesh *et al*., 2004; Sacchettini *et al*., 2008; Williams & Duncan, 2007). In contrast, target-based screening or enzyme-inhibition permits high-throughput automated screening of a large number of compounds against a purified protein. Nevertheless, the "hit" may have high *in vitro* activity but poor whole-cell potency (penetration) due to the *M. tuberculosis* cell wall (Balganesh *et al*., 2004; Williams & Duncan, 2007), efflux pump mechanisms and antibiotic degradation/modifiying enzymes (Nguyen & Thompson, 2006). The availability of X-ray crystal structures of interesting proteins provides the opportunity to carry out virtual screening (*in silico*). *In silico* screening, using computational methods, can be complementary to a biochemical high-throughput screen due to the potential for screening a larger chemical space quickly (drug libraries) and inexpensively. Virtual screening can be used in two ways, (i) to identify compounds that are consistent with a pharmacophore (the chemical functional group or groups possessed by a molecule that enables its biological activity) model irrespective of the identity of the structure of the pertinent protein target or targets and (ii) to develop inhibitors of a protein (or related protein) based on its known three-dimensional structure (Arcus *et al*., 2006; Balganesh *et al*., 2004; Williams & Duncan, 2007). Undoubtely, combining these drug-discovery methods increases the possibility to find potential novel compounds that could be used for further investigation.

3.4 Mycobactin biosynthesis: a potential new drug target.

Recently, products of several *M. tuberculosis* genes have been identified as potential drug targets. These mainly participate in cellular metabolism, cell wall biosynthesis, signal transduction, transcription, translation, persistence and virulence (Hasan *et al*., 2006; Vohra *et al*., 2006). Snow (1970) proposed that enzymes participating in mycobactin biosynthesis are potential drug targets since mammalian cells do not produce siderophores and thus adverse effects might be avoided. Hence, salicylic acid biosynthesis itself from chorismate, the salicylate adenylation activity of MbtA, the formation of oxazoline/thiazoline rings, the formation of *N*-hydroxylysine, the introduction of acetyl groups into the central lysine, and the phosphopantetheinylation mediated by the mycobacterial PPTases, PptT and AcpS, are considered potential drug targets (Chalut *et al*., 2006; Krithika *et al*., 2006; Ratledge, 2004). Moreover, the idea of using mycobactins as a "Trojan horse" is also attractive. Since *M. tuberculosis* possesess a complex cell wall that prevents drug permeability, the use of mycobactins and its analogues by coupling a known bacterial growth inhibitor may overcome permeability issues (Vergne *et al*., 2000). However, this Trojan horse concept has the inherent danger of allowing the bacteria to become resistant to the agent more easily (Ratledge, 2004).

What evidence exists to consider mycobactin biosynthesis as a potential drug target? Iron is an important element for diverse cell process in eukaryota and prokaryota, and its role in virulence of pathogenic bacteria has been clearly demonstrated (Andrews *et al*., 2003; Miethke & Marahiel, 2007). *M. tuberculosis* is an intracellular pathogen that resides within the macrophage phagosome and prevents-lysosome fusion. The phagosome is considered to have an acidic pH and *M. tuberculosis* perceives it as a low-iron environment, thus iron-acquisition becomes important for the survival of the bacilli (Cosma *et al*., 2003). It has been demonstrated that intraphagosomal *M. tuberculosis* can acquire iron from lactoferrin, ferritin and citrate (Olakanmi *et al*., 2004). The cell-bound mycobactin has not been isolated *in vivo* and this detracts its attractiveness as a potential drug target. However, this lack of detection *in vivo* should not be taken as absolute absence but may indicate that a physiologically relevant concentration of mycobactins is beyond the limits of detection of the analytical methods applied. The impressive yields of mycobactins seen in iron-limited batch culture *in vitro* are

almost certainly an exaggerated response to an environment with an absolute limitation on iron rather than the *in vivo* situation characterised by an abundance of sequestered iron. This absence may be due to many factors. The pH of the phagocytic vacuole lies somewhere between 4.5 and 6.0, allowing the dissociation of iron from host iron carrier proteins generating a state of iron sufficiency, which may ultimately eliminate the need for mycobactins. The relationship between the stage of an infection and iron sufficiency/deficiency is unknown and the lack of any detectable mycobactins does not necessarily mean they were never involved in iron transport. The mycobactin may be produced at an early stage of infection and then degraded when a state of iron sufficiency is reached (Lambrecht & Collins, 1993). This scenario may be different during LTBI, in which siderophore biosynthesis could be re-activated for iron-uptake. However, recent studies provide genetic and biochemical data supporting the importance of mycobactin biosynthesis, as well as iron homeostasis during *M. tuberculosis* infection (Rodriguez, 2006). *M. tuberculosis* is able to acquire iron from transferrin and lactoferrin by secreting carboxymycobactins when the bacilli are grown in medium (Farhana *et al*., 2008; Gobin & Horwitz, 1996) and the production of the siderophores is tightly regulated by IdeR (Rodriguez, 2006) and required for growth in macrophages (De Voss *et al*., 2000). Furthermore, transcription analysis during *M. tuberculosis* infection has demonstrated the up-regulation of mycobactin biosynthesis as a survival response in macrophages (Schnappinger *et al*., 2003). Iron limitation induces transcription of at least 35 *M. tuberculosis* genes, including *mbtA-J*, but represses *bfrA*, which encodes an iron-storing bacterioferritin (Schnappinger *et al*., 2003). In addition, during immune control of *M. tuberculosis* replication, *mbtB* mRNA levels increase and *bfrB* mRNA levels decrease several weeks after infection in mice (Dubnau *et al*., 2005; Timm *et al*., 2003). The fact that the expression of *mbtA-J* genes are induced under iron-deficient environments during growth in culture medium and in macrophages strongly suggests that siderophores are important for survival and therefore represent potential new drug targets.

The microbial activity of PAS was first reported in 1946 (Lehmann, 1946). PAS is highly effective against *M. tuberculosis*, but its use was discontinued due to adverse effects. However, the emergence of MDR-TB has indicated PAS as an alternative theraupetic option, but it is rarely used in the regimens for the treatment of TB. The mode of action of PAS is still unclear, but it has been suggested to inhibit mycobactin biosynthesis by interfering with the salicylate adenylation (Brown & Ratledge, 1975; Ratledge & Dover, 2000). On the other hand, PAS may also target thymidylate synthase (*thyA*), an enzyme involved in folate biosynthesis (Rengarajan *et al*., 2004). Recently, the inhibition of aryl-*N*-capped siderophore biosynthesis in pathogenic bacteria such as *Y. pestis*, *M. tuberculosis* and *P. aeruginosa* has been demonstrated (Ferreras *et al*., 2005; Miethke *et al*., 2006a; Somu *et al*., 2006a). The first step during aryl-*N*-capped siderophore biosynthesis is the activation of either salicylic acid or DHB depending on the enzymatic activity of the microorganism by the adenylation domain. In this reaction the α-phosphate of ATP is charged by the substrate to generate a tightly bound acyl-adenylate, salicyl-AMP for instance, with subsequent release of pyrophosphate. This biosynthetic step resembles a similar reaction mechanism in all members of the adenylate enzyme superfamily and the functionally related aminoacyl tRNA synthetases (Schimmel *et al*., 1998). In the next reaction, the adenylation domain binds the phosphopantetheine arm of the thiolation domain, ArCP domain, and transfers the aryl-adenylate onto the nucleophilic sulphur atom of this cofactor moiety to provide salicyl-bound-ArCP (Figure 23)(Quadri *et al*., 1998).

In this study, analogues of salicylic acid derived from *in house* synthesis and *in silico* screening have been tested to inhibit early steps in mycobactin biosynthesis. Activity of the drugs is being assessed by a combination of target-based enzyme and whole-cell methods.

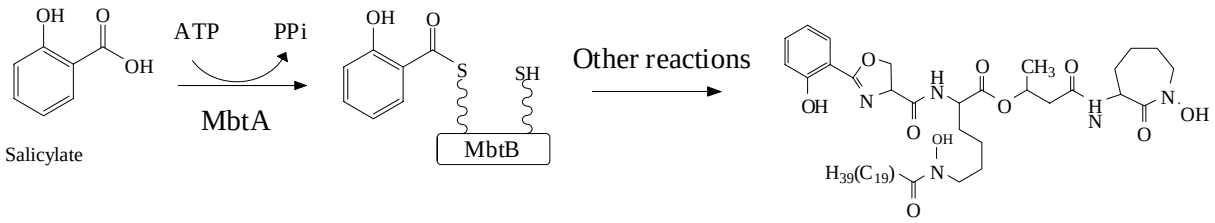


Figure 23. **Enzymatic activity catalysed by MbtA.** The adenylation domain activates salicylic acid by ATP reaction dependent to form acyl-adenylate (salicyl-AMP), and then the enzyme transfers the substrate to the phosphopantethein arm of the ArCP domain. Analogues of salicylic acid should block the salicyl-AMP formation as first step as well as the salicyl-ArCP domain intermediate, which eventually stops mycobactin biosynthesis.

3.5 Material and methods.

3.5.1 *In silico* approach to seek potential inhibitors of MbtA.

There is no crystal structure solved for MbtA, however the availability of the co-crystal structure of DhbE with its substrate from *B. subtilis* has provided the correct core motifs for the deduction of substrate specifity between amino and aryl acid-activating domains and for discriminating between DHB (2,3-dihydroxybenzozate) and SAL (salicylic acid) activating enzymes (May *et al*., 2002). Therefore, an MbtA homology model was constructed using the structure of the DhbE-adenylate ligand (PDB code 1md9) in the CPHmodels 2.0 server (Lund *et al*., 2002). Then, the program Insight II (Accelrys Inc) was used to dock potential inhibitors in this structure by searching a 3-D compound database (Maybridge Library) to identify "hits" that satisfy chemical and geometrical requirements of the model.

3.5.2 *In house* chemical synthesis of analogues of salicylic acid.

Based on the PAS structure, 13 analogues were chemically synthesized (a collaboration with Prof. Colin Ratledge, University of Hull, UK). Due to intellectual property rights, structures and methods for preparation of these compounds are not described here. To validate their target and specifity inhibition towards MbtA, the hits and analogues of SAL compounds will be assessed by *in vitro* assay as described in 3.5.4. In addition, *in vivo* activity of the compounds was investigated against *M. bovis* BCG as described in 3.5.5.

3.5.3 Molecular cloning and expression of *M. tuberculosis mbtA* in *E. coli* and *M. smegmatis* mc^{2}155.

The adenylation enzyme (*mbtA*) was amplified from *M. tuberculosis* H37Rv genomic DNA. The cloning of *mbtA* was attempted in five different vectors: pTYB1 (New England Biolabs), pMAL-C2X (New England Biolabs), pMV261, pSD26 and pVV16. Amplification was performed with different polymerases KOD DNA (Novagen), *Pfu* Platinum (Promega), Accusure (Bioline), Deep Vent^{TM} and Phusion High-Fidelity (New England Biolabs). Primers used for each vector are listed in Table 13. However, PCR products were obtained only with

Pfu Platinum DNA and Phusion High-Fidelity polymerases. Vectors and amplicons were digested with their appropriate restriction endonucleases (New England Biolabs), followed by ligation with T4 DNA ligase (New England Biolabs) at 16°C for 16 hours. Each ligation mix was used to transform *E. coli* TOP 10 (Novagen). Selection of *mbtA*-pMAL-C2X and *mbtA*-pTYB1 were performed on LB agar plates containing ampicillin (100 μg/mL), whereas for *mbtA*-pMV261, kanamycin (50 μg/mL) was added to the media. For *mbtA*-pSD26 and *mbtA*-pVV16, hygromycin (200 μg/mL) was added to LB agar. Screening was performed by double digestion followed by electrophoresis analysis on agarose gel and staining using EtBr. Integrity of genetic constructs was verified by nucleotide sequencing.

Table 13. Primers used to clone *mbtA*. Restrictions sites are underlined and shown. [a]The plasmid was digested with HindIII/MscI to ligate the amplicon.

Vector	Primers	Restriction sites underlined
pTYB1	Up5'-GATCGATCCATATGCCACCGAAGGCGGCAGATGGC-3'	NdeI
	Down5'-GATCGATCTGCTCTTCCGCATCAATGGCAGCGCTGGGTCGT-3'	SapI
pMAL-C2X	Up5'-GATCGATCGGAAGGATTTCAATGCCACCGAAGGCGGCAGA-3'	XmnI
	Down5'-GATCGATCAAGCTTTTAATGGCAGCGCTGGGTCGTC-3'	HindIII
pMV261[a]	Up5'-CCCCACCGAAGGCGGCAGATGGCCGCCG-3'	HindIII
	Down 5'-GATCGATCAAGCTTTCAATGGCAGCGCTGGGTCG-3'	HindIII
pSD26	Up5'-GATCGATCGGATCCATGCCACCGAAGGCGGCAGAT-3'	BamHI
	Down5'-GATCGATCGATATCATGGCAGCGCTGGGTC GT-3'	EcoRV
pVV16	Up5'-GATCGATCCATATGCCACCGAAGGCGGCAGAT-3'	NdeI
	Down5'-GATCGATCAAGCTTATGGCAGCGCTGGGTCGT-3'	HindIII

The *mbtA*-pMAL-C2X and *mbtA*-pTYB1 constructs were transformed by heat shock at 42°C into *E. coli* K12 and *E. coli* ER2566, respectively. Gene expression analysis was determined by testing different temperatures, incubation times and IPTG concentrations as described in Chapter 2, section 2.5.1.2. To seek expression of *mbtA*-pMV261, *mbtA*-pSD26

and *mbtA*-pVV16, stocks of 50 μL electrocompetent *M. smegmatis* mc^2155 cells were transformed by electroporation. Briefly, aliquots were transferred into an ice-cold cuvette (1 mm, Cell projects) and 1 μL of each construct added, followed by incubation on ice for 20 minutes. The cuvettes were then transferred into an electroporator (Eppendorf Electroporator 2510, settings: 1800 mV, 1000 Ohms, 25 μF), with subsequent incubation on ice for 20 minutes. Afterwards, 1 mL of LB broth was added to the cuvettes and incubated at 37°C for 4 hours without shaking. The electrotransformed cells were selected on LB agar with the appropriate antibiotic [for *mbtA*-pMV261 (Kan 25 μg/mL), *mbtA*-pSD26/pVV16 (Hyg 50 μg/mL)] further incubated at 37°C until single colonies appeared on plates.

Cells from a single colony on a LB agar plate were used to inoculate 5 mL of LB broth supplemented with a suitable antibiotic and incubated at 37°C with shaking (180 rpm) until culture was turbid. This starting culture was used to inoculate 1 L of fresh LB broth supplemented with the antibiotic and 0.05% Tween 80, and the culture incubated at 37°C with shaking (180 rpm) until an OD_{600}=0.5-0.6 was obtained. To induce expression for *mbtA*-pSD26, 0.2 % acetamide was added to cultures and the cells allowed growing for an additional 4 hours. Cells were harvested by centrifugation at 5, 000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.9 and 500 mM NaCl) containing DNAase and serine protease inhibitors (PMSF and benzamidine at 1 mM final concentration) and disrupted by sonication (Soniprep 150, settings: Amplitude microns 1,100, 7 cylces, 30-s burst/45-s of cooling). The extract was clarified by centrifugation at 15, 000 rpm for 30 minutes at 4°C. The supernatant was collected and loaded into Ni^{2+}-charged and equilibrated His-Trap column (5 mL) following the manufacturers' instructions (Amersham). The clarified extract was applied to the column and non-binding material collected for analysis. The column was then washed with 50 mL of binding buffer A (50 mM Tris-HCl, pH 7.9, 500 mM NaCl) and the protein eluted with a step-wise gradient of 10-500 mM of increasing imidazole concentration in buffer A. The purity of the protein was monitored by 12% SDS-PAGE and Coomasie blue staining. Purified protein fractions were pooled and dialysed against buffer B (10 mM Tris-HCl, pH 7.9, 1 mM EDTA and 5% glycerol), concentrated using a Centricon

YM-10 filter unit (Millipore) and stored at -80°C until use. The protein concentration was estimated using the BCA protein assay (Pierce) and by absorbance at 280 nm with the predicted molar extinction coefficient for each recombinant protein.

3.5.4 Salicylation and arylation assays.

The assay was carried out as that reported by Quadri *et al*. (1998) with slight modifications. Incorporation of [^{14}C]-salicylic acid (50 mCi/mmol, purchased from American Radiolabeled Chemicals Inc.) by MbtA into *holo*-carrier proteins of MbtB was be quantified in 100 μL reactions containing 10 mM ATP, 25 mM DTT, 25 mM $MgCl_2$, 75 mM Tris-HCl, pH 7.5. Enzyme concentrations and [^{14}C]-salicylic acid were varied to obtain the kinetic data. Reactions were incubated at 37°C for up to 15 hours. After incubation, reactions were quenched with 900 μL of 10% ice-cold trichloroacetic acid (TCA) and 50 μL of bovine serum albumin (BSA, 2 mg/mL) as a carrier. Precipitation was completed by incubation on ice for 10 minutes, followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The protein pellet was washed with 900 μL of 10% TCA before dissolving in 20 μL of 2% SDS containing 20 mM aqueous NaOH. The amount of radioactivity (cpm) incorporated was quantified by re-dissolving the proteins with 5 mL of scintillation cocktail (EcoScintA).

3.5.5 Determination of the *in vivo* effect of inhibitors against *M. bovis* BCG.

Compounds were evaluated for whole-cell activity against *M. bovis* BCG in iron-sufficient medium. Cells were initially grown in Middlebrook 7H9 media supplemented with OADC and 0.05 % Tween 80 (v/v) until OD_{600} ~0.2. Low-cell density allows the potency of the inhibitors and avoids their inactivation by inoculum size. Hence, the starting culture was used to determine the minimum inhibitory concentrations (MIC_{99}) that inhibited >99% cell growth in 96-well microplates using Proskauer and Beck medium (PB) (Allen, 1998). Prior PB medium preparation, glassware was first deferrated as follows. Glassware were filled with alcoholic KOH (2%) and left overnight. Then, glassware was washed with deionised water

and filled with 8 M HNO_3 overnight. Glassware was again washed with deionised water and autoclaved at 127ºC for 15 minutes prior to use. Afterwards, PB medium was prepared in the deferrated glassware.

3.5.5.1 Preparation of 96-well microplates.

The method reported by Franzblau *et al*. (1998) was followed to prepare the 96-well microplates (Corning Incorporated Costar 96-well microplates). Two hundred microliters of deionised water were added to all outer well perimeters to minimise evaporation of the medium during the experiment. The wells from B to G received 100 μL of PB media along with 0.05% Tween 80 as shown in Figure 24. Column 10 was used as a positive control, with the bacteria in the media, whereas column 11 was used as a negative control, with media only. Additionally, 100 μL of media was added to columns B2, D2 and F2 for diluting the stock drug solutions.

3.5.5.2 Preparation of drug stock solutions.

One mg of each compound was resuspended in 100 μL of DMSO giving a 10 mg/mL stock solution. Serial 1:2 dilutions were continued from the initial high concentration. From the initial *M. bovis* BCG growth culture, suspensions of 1:25 dilution were prepared in PB media and 0.05 % Tween 80. From this dilution, 100 μL were added to the wells in rows B to G in column 2 to 10, yielding a final volume of 200 μL per well. Plates were sealed with parafilm and incubated at 37°C in a static incubator for 6-8 days. Isoniazid, chloramphenicol, rifampicin, and ethambutol were included as positive controls for comparison.

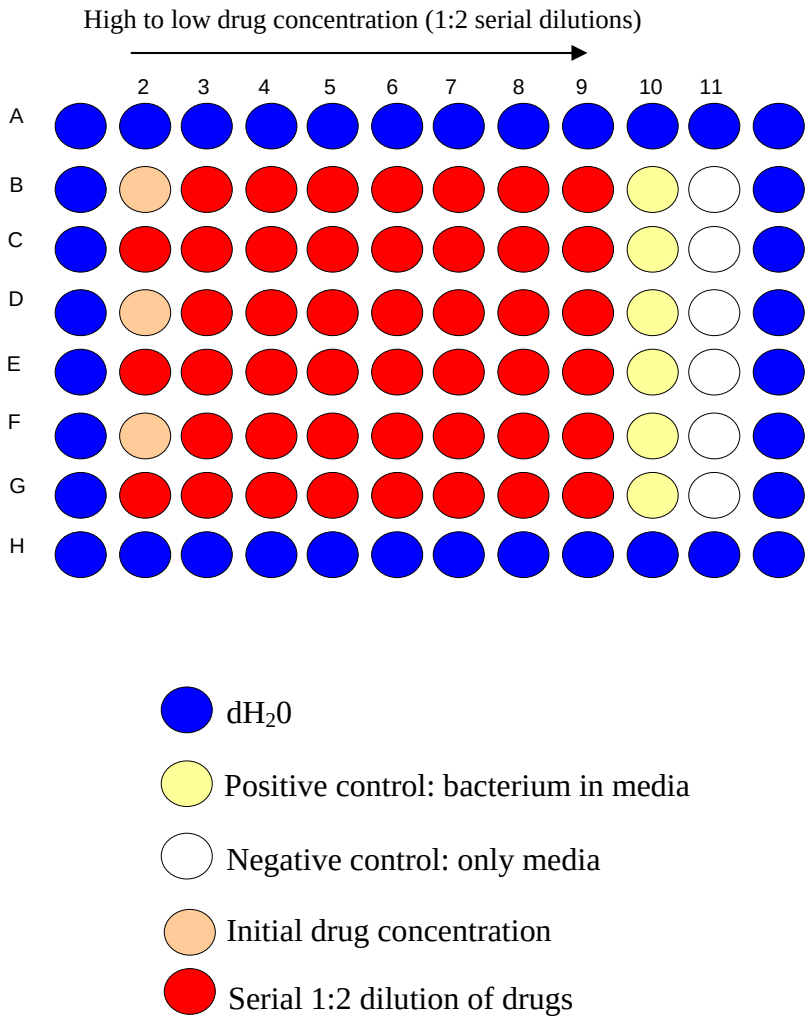


Figure 24. Schematic diagram depicting the order of solutions, medium and drug solutions, added into 96-well microplates.

3.6 Results.

The results shown here are preliminary and, therefore, they are not intended to provide conclusions derived from initial experiments.

3.6.1 *In silico* screening for potential inhibitors of MbtA.

The availability of the DhbE crystal structure from *B. subtilis* provided the correct core motifs for the deduction of substrate specifity between amino and aryl acid-activating domains and for discriminating between DHB and SAL activating enzymes (May *et al*., 2002). Hence, a homology model of MbtA was constructed based on DhbE-DHB (PDB 1md9) to determine the putative binding pocket valid. Analysis of the primary sequence between the DhbE and MbtA aryl acid-activating domains showed 42.1 % identity (Figure 25). Although, below the threshold (70 %) for predicting good homolog models, the MbtA structure taking DhbE as a template could be predicted. This model structure was very similar to the structure 1md9 with a RMSD= 0.69 Å. The co-crystallisation of DhbE with ATP and DHB determined the core motif for recognising the substrate specifity in aryl acid-activating adenylation domains. In agreement with this, amino acid residues Asn235, Ser240, and Val377 of DhbE and the corresponding residue Cys263 of MbtA seem to be involved in key interactions with the DHB and SAL aryl substrates. The conserved Asn235 and Ser240 residues of DhbE bind to the 2 and 3 hydroxyl groups of DHB, respectively. However, the Ser40 residue is replaced by Cys residue in SAL-activating domains as in MbtA and YbtE (Figure 26) (May *et al*., 2002).

The individual molecules of the Maybridge screening collection were as virtual ligands using the autodock facility of Insight II (Accelrys Inc.) for the modeled MbtA structure. Six compounds from this library from over 56,000 molecules were predicted to be potential inhibitors of MbtA on the basis of their predicted dissociation constant (*Ki*), which in all cases were $<7.40 \times 10^{-17}$ M (HTS07619) at 25°C (Figure 27). Other hits from the library were predicted with *Ki* values at least six order of magnitude greater and were not considered for testing. In the simulations, these molecules all bound to a region near the entrance to the active site with most (except HTS10034 and SPB01127) penetrating into the site (Figure 28).

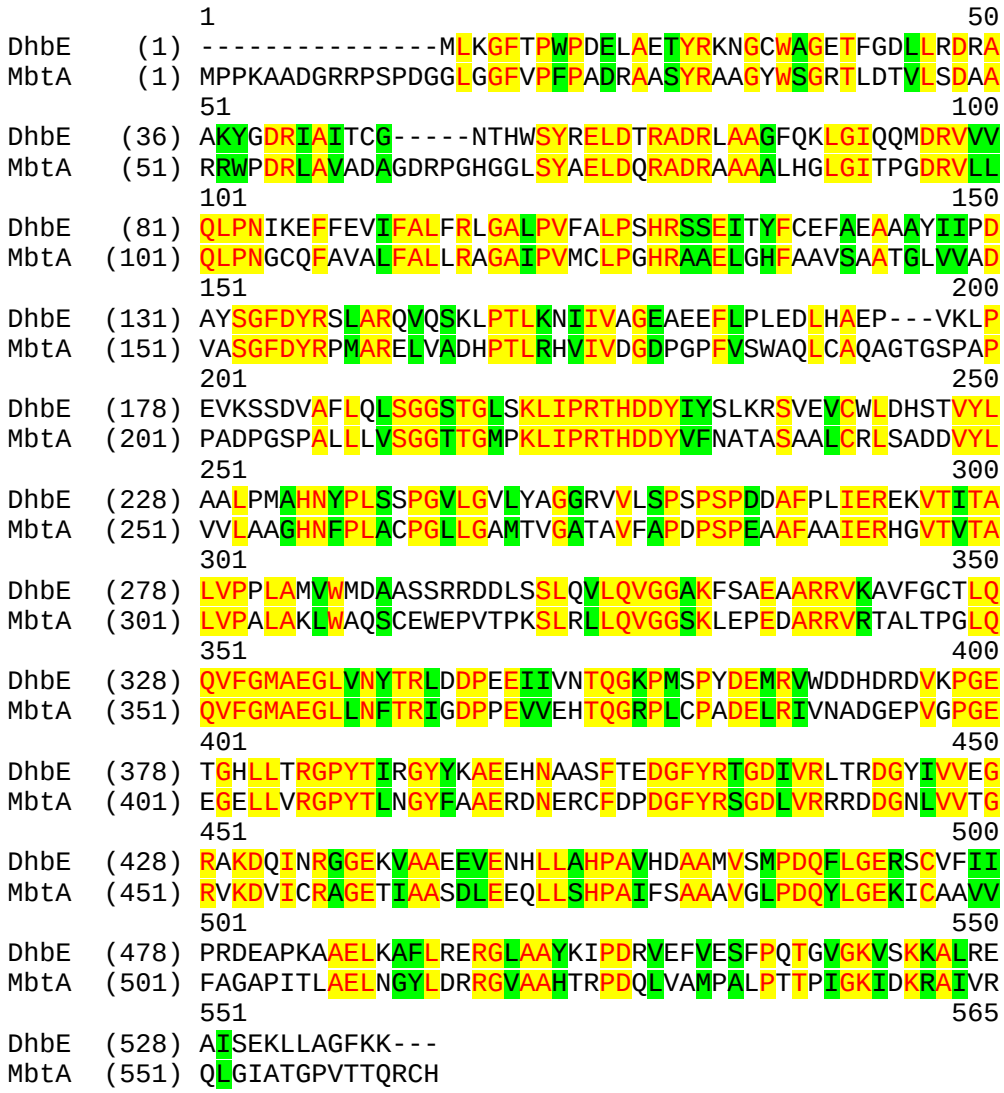
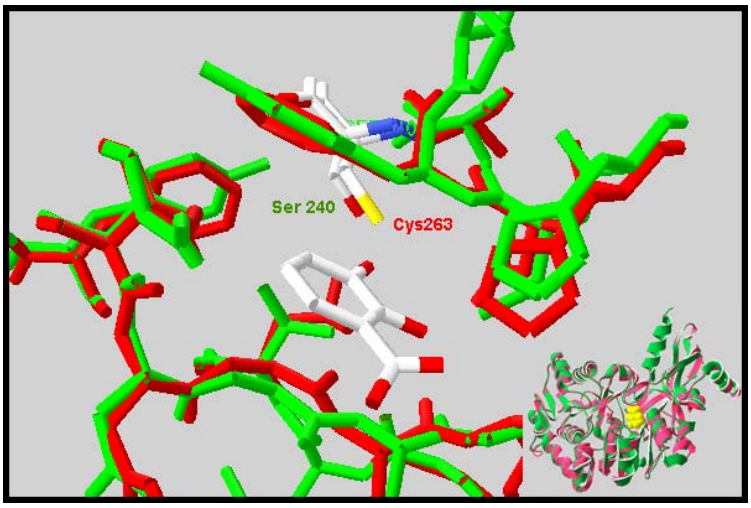
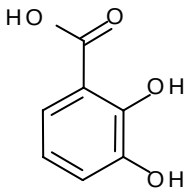
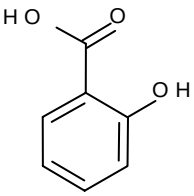


Figure 25. Alignment of the primary structures of *Bacillus subtilis* DhbE and *M. tuberculosis* MbtA. Sequences were aligned using the ClustalW algorithm within the AlignX component of the Vector NTi suite of programs (Invitrogen). The algorithm predicted amino acid identity at 42.1% and consensus at 57.5%.





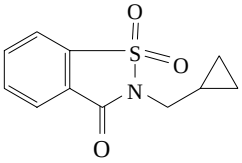
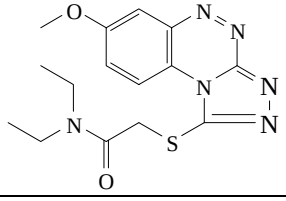
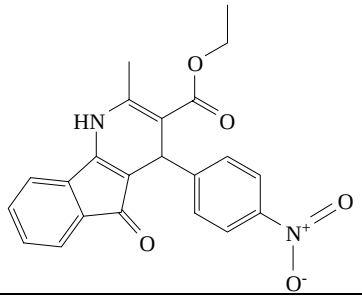
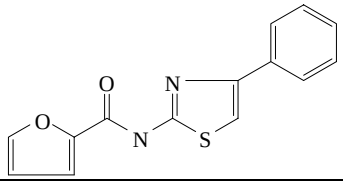
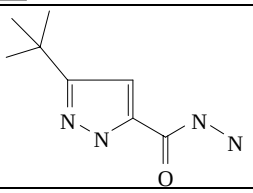
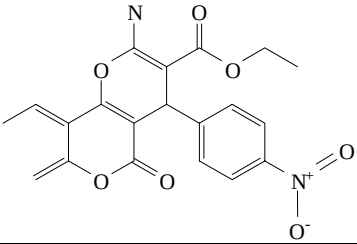
DHB



Salicylate

Figure 26. Homology model of MbtA. The MbtA model (red) was made using CPH models 2.0 Server (http://www.cbs.dtu.dk/services/CPHmodels/) and DhbE (1md8 pdb, in green) from *Bacillus subtilis* as template. Superimposition of model on template is shown in lower right hand corner (RMSD = 0.69Å). The main figure shows the positions of DhbE-bound 2,3-dihydroxybenzoate (DHB) and important residues for substrate specificity; in this class of enzyme Ser (DhbE) selects for DHB while Cys (MbtA) selects for 2-hydroxybenzoate (salicylate).

The predicted binding of these compounds at this site in combination with such low dissociation constants indicated that *in vitro* analyses of MbtA inhibition was appropriate. The compounds in the Maybridge library generally obey the Lipinski's "rule of five" that provide an estimation of drug solubility and permeability, important parameters for initial drug discovery and drug development (Lipinski *et al.*, 2001). These hits along with 13 compounds synthesised elsewhere resembling the PAS structure, were initially evaluated for whole-cell activity against *M. bovis* BCG as described below.

Compound	Exp num	Predicted *Ki*	Structure
2-(cyclopropylmethyl)-1H-1,2-benzisothiazole-1,1,3(2H)-trione (HTS10034)	1	$9.66x10^{-25}M$	
N1,N1-diethyl-2-[(7-methoxybenzo[e][1,2,4]triazolo[3,4-c][1,2,4]triazin-1-yl)thio]acetamide (BTB11368)	4	$6.61x10^{-21}M$	
ethyl 2-methyl-4-(4-nitrophenyl)-5-oxo-4,5-dihydro-1H-indeno[1,2-b]pyridine-3-carboxylate (JFD00435)	2	$2.5x10^{-18}M$	
N-(4-phenyl-1,3-thiazol-2-yl)-2-furamide (NH00363)	3	$4.08x10^{-18}M$	
3-(tert-butyl)-1H-pyrazole-5-carbohydrazide (SPB01227)	5	$6.03x10^{-17}M$	
ethyl 2-amino-4-(4-nitrophenyl)-5-oxo-4H,5H-pyrano[3,2-c]chromene-3-carboxylate (HTS07619)	6	$1X10^{-17}M$	

Exp num= number of compound assigned for whole-cell assay experiments (section 3.6.2, Table 14).

Figure 27. Potential inhibitors of MbtA. Based on the co-crystal structure of DhbE and DHB, an MbtA homology model was constructed. The model was used to dock potential inhibitors by searching a 3-D compound database (Maybridge Library) to identify "hits" that satisfy chemical and geometrical requirements. Six compounds were predicted to be potential inhibitors and their name, dissociation constant (*Ki*) and structure are shown below. Code within parenthesis indicates catalog number.

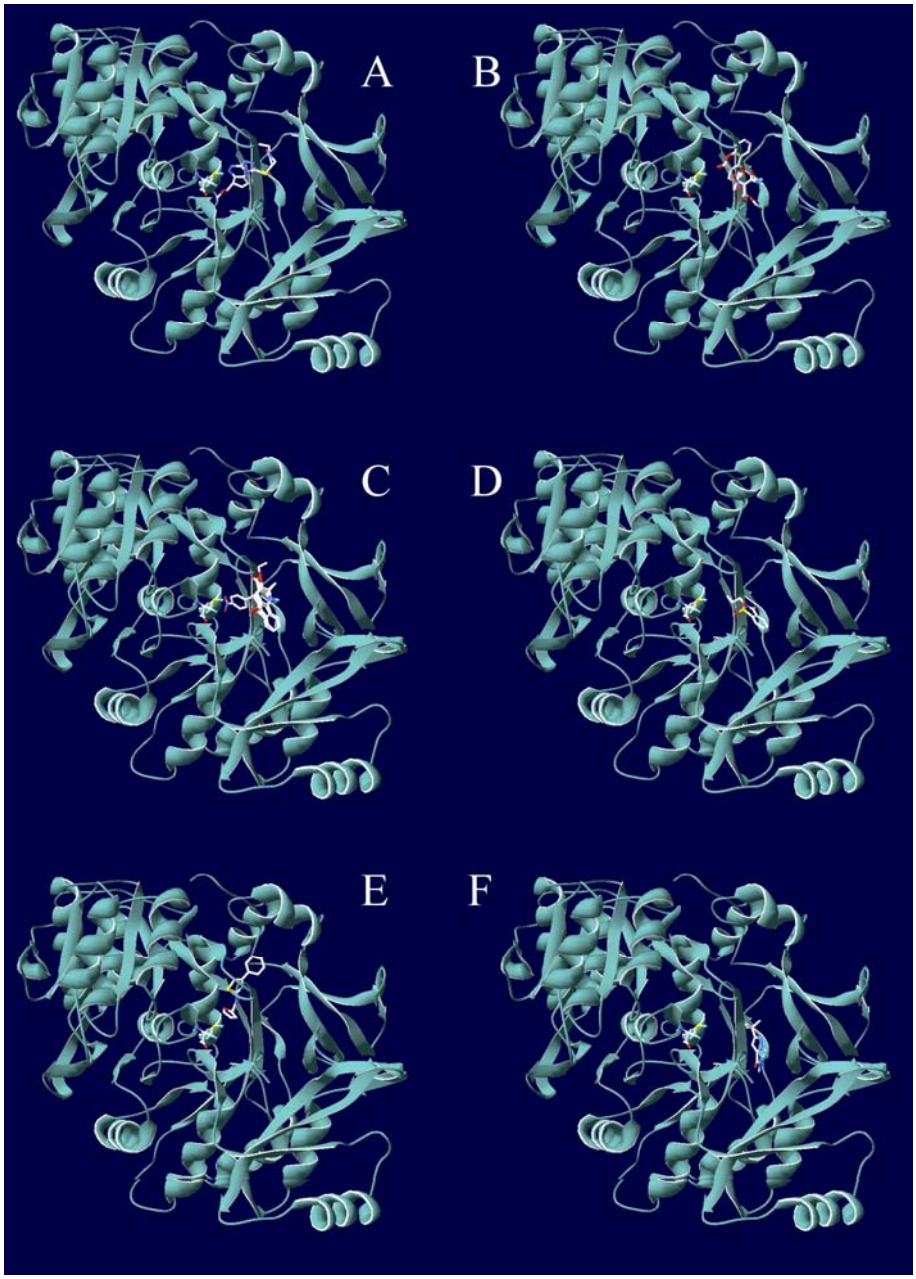


Figure 28. Predicted binding sites for virtual screening hits. The molecules identified from the Maybridge library as potential inhibitors of MbtA, by virtue of the predicted *Ki* in autodock, are shown in relation to the homology model produced as part of this study. For orientation purposes, the side chain atoms of active site residue Cys263 which interact with the 3-position of the salicylate ring are shown. Panel A, BTB11368; Panel B, HTS07619; Panel C, JFD00435; Panel D, HTS10034; Panel E, NH00363; Panel F, SPB01127.

3.6.2 *In vivo* activity of inhibitors on *M. bovis* BCG.

Initial attempts to determine accurate MIC by different methods were hindered due to the drug behaviour in the media. Firstly, the Alamar Blue Assay (MABA) was tested, since this assay determines metabolically active cells by measuring their capacity to convert rezarusin to a fluorescent resorufin. However, the reagents did not react uniformly, even in the controls, a result that may indicate an incompatibility with the PB medium used here. Optical density was not used as an indicator of growth inhibition because the drug solutions showed some turbidity that could bias readings. Therefore, the classical microdilution minimum inhibitory concentrations (MICs) method provided the best mode to determine *in vivo* activity of the compounds. Hence, all potential inhibitors were evaluated against *M. bovis* BCG under iron-sufficient conditions in 96-well microplates.

The MIC at which growth of *M. bovis* BCG was completely inhibited were recorded when bacteria reached good macroscopic growth in control wells, generally after 6 days of incubation (Table 14). None of these compounds exhibited *in vivo* activity that was comparable to that of current anti-TB drugs. The lowest MIC observed shared by seven of the compounds was 32 μg/mL, whereas six other antibiotics did not show growth inhibition at all. Although, all compounds were selected to target mycobactin biosynthesis, particularly salicylate adenylation, unexpectedly, compounds 2,3, and 4 derived from the virtual screening and those chemically prepared 8,9,10 and 11 had similar *in vivo* activity despite their unrelated chemical structure. These preliminary data shows the activity of compounds under iron-sufficient conditions, and to validate their target it will be more interesting to evaluate the inhibitors in enzyme assays and both iron-deficient environments and over-expressing the *mbtA* in *M. bovis* BCG. At this point, there are only seven compounds providing interesting activity in whole-cell assays.

Table 14. ***In vivo*** **activity of compounds against** ***M. bovis*** **BCG.** MICs were determined after six days of incubation at 37°C. Isoniazid (ISO), Rifampicin (RIF), Chloramphenicol (CHL) and Ethambutol (EMB) were included in the experiments as positive controls. NI= no growth inhibition. [a]Compounds derived from *in silico* screening. [b]Compounds analogues to PAS. Experiments were performed in triplicate.

Compound	MIC_{99} (μg/mL)	Compound	MIC_{99} (μg/mL)
1[a]	128	13[b]	NI
2[a]	32	14[b]	64
3[a]	32	15[b]	128
4[a]	32	16[b]	NI
5[a]	NI	17[b]	64
6[a]	NI	18[b]	NI
7[b]	64	19[b]	NI
8[b]	32	ISO	4
9[b]	32	RIF	0.5
10[b]	32	CHL	8
11[b]	32	ETH	4
12[b]	128		

3.6.3 Cloning and expression analysis of MbtA in *E. coli* and *M. smegmatis* mc^{2}155.

The *mbtA* gene was cloned in five expression vectors, however, only three constructs provided gene expression; in one case, the MbtA was insoluble but two provided soluble recombinant protein. Firstly, expression of *mbtA*-pTYB1 in *E. coli* ER2566 was attempted. Since, MbtA (MW 59.28 KDa) was cloned in fusion with the Chitin Binding Domain (CDB, 56 KDa) of the pTYB1 expression vector, a fusion (CBD)-recombinant protein (MbtA) with a MW ~115 KDa was expected to be detected upon analysis by 12% SDS-PAGE. However, no band was observed with the expected mobility in any of the clarified samples in each condition tested (IPTG concentration, time and temperature incubation). In further attempts to seek expression of *mbtA*-pTYB1, Western Blot and Immunodetection analyses were performed. Consistent with the previous analyses no evidence for *mbtA* expression in *E. coli* ER2566 was found. Then, over-expression of *mbtA*-pMAL-C2X was analysed using *E. coli* K12. The *mbtA* gene

was cloned as a fusion with the Maltose Binding Protein (MBP, MW ~42.48 KDa) in the pMAL-C2X vector. Hence, a recombinant fusion protein MBP-MbtA with MW ~102 KDa was expected to be detected on 12% SDS-PAGE. The fusion recombinant protein was expressed in all investigated conditions (IPTG concentration, time and temperature incubation), although higher expression was observed when *E. coli* K12-*mbtA*-pMAL-C2X was incubated at 16°C for 16 hours (Figure 29). However, analysis of the supernatant after centrifugation at 15, 000 rpm showed that the recombinant protein was not present suggesting that the fusion was in an insoluble form. Solubilization experiments were not attempted since this does not always lead to preparation of an active protein. Instead, the cloning of *mbtA* was further attempted into *E. coli*-mycobacteria shuttle vectors, pMV261, pSD26 and pVV16.

M. smegmatis mc^2155 was electrotransformed using *mbtA*-pMV261 and expression analysis was carried out. However, in the conditions investigated, expression of *mbtA* was not observed (data not shown). Then, each of the *mbtA*-pSD26 and *mbtA*-pVV16 constructs were also used to electrotransform *M. smegmatis* mc^2155 to seek protein expression. Both constructs feature a C-terminal hexa-histidine tag. The *mbtA*-pSD26 was induced using 0.2% acetamide, whereas *mbtA*-pVV16 expression was assured by the relatively *hsp60* strong promoter. Transformed *M. smegmatis* mc^2155 was grown at 37°C. Recombinant MbtA was purified by Ni^{2+}-affinity chromatography. The purified protein migrated as a single band on 12% SDS-PAGE above the expected molecular mass calculated ~60.90 KDa (MbtA: 59.28 kDa plus amino acids of the acetamidase promoter and six histidine residues) (Figure 30). Identity of the protein was verified by MALDI-MS analysis. The protein yield from each plasmid was approximately similar (0.17 mg/L). Hence, the adenylation domain, MbtA, was cloned and expressed in *M. smegmatis* mc^2155 allowing the activity of potential the inhibitors to be tested *in vitro*.

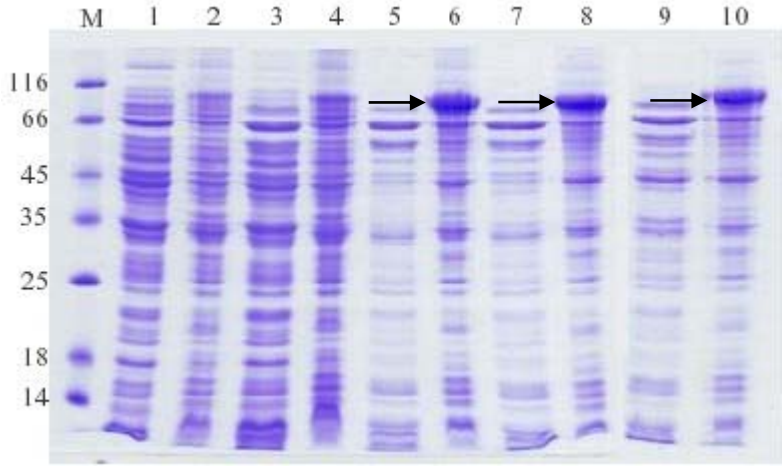


Figure 29. **Expression analysis of *mbtA*-pMAL-C2X in *E. coli* K12.** The *mbtA* was cloned as fusion with the maltose binding protein (MBP) in pMAL-C2X. Analysis of the crude lysate and supernatant extract, a band in lanes 6, 8 and 10 (crude lysates) with the expected MW ~102 KDa of the fusion recombinant protein migrated on 12% SDS-PAGE, indicating that the fusion recombinant protein was expressed. However, the protein was in insoluble form. M= standard molecular protein marker; Lanes 1 and 2: extracts from *E. coli*-pMAL-C2X (1: supernatant, 2: crude lysate); Lanes 3 and 4: extracts from *E. coli-mbtA*-pMAL-C2X without IPTG (3: supernatant; 4: crude lysate); Lanes 5 and 6: *E. coli-mbtA*-pMAL-C2X plus 0.1 mM IPTG (5: supernatant; 6: crude lysate); Lanes 7 and 8: *E. coli-mbtA*-pMAL-C2X plus 0.5 mM IPTG (7: supernatant; 8: crude lysate); Lanes 9 and 10: *E. coli-mbtA*-pMAL-C2X plus 1 mM IPTG (9: supernatant; 10: crude lysate). Here, it is only shown expression analysis carried out at 16°C for 16 hours; the fusion recombinant protein was also expressed at 37°C in all investigated conditions, but lower expression was observed as compared with the above conditions.

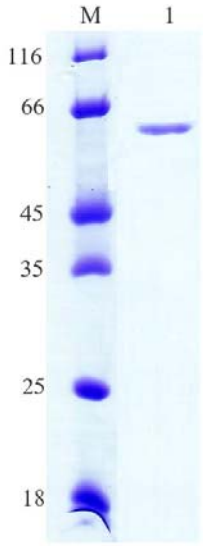


Figure 30. Coomassie blue stained 12% SDS-PAGE of MbtA purified. The recombinant protein was purified using Ni^{2+}-affinity chromatography in a step gradient concentration of imidazole (10-500 mM). The purified protein migrated as a single band consistent with the expected molecular mass calculated ~60.90 kDa. M= standard molecular protein marker. Lane 1: MbtA purified. Here the product of *mbtA*-pVV16 is illustrated; a similar result was obtained from *mbtA*-pSD26.

3.6.4 Salicylate adenylation assays.

This part of the project was significantly delayed by the difficulties encountered in producing phosphopantetheinylated acceptors. However, preliminary results have shown that the recombinant MbtA has enzymatic activity. During initial screening for MbtA activity, a set of different reactions were performed in which the presence and absence of MbtA and SAL were investigated. In presence of MbtA and SAL, higher counts *per* minute (CPM) were observed compared to reactions in which MbtA and substrate were absent, indicating that MbtA exhibited enzymatic activity (Figure 31A). Then, the concentration of SAL was held, whereas MbtA was varied to determine the initial rate (Figure 31B). Although, these preliminary data provide hints of MbtA activity, its catalytic function will be further investigated to establish the true kinetics of the enzyme.

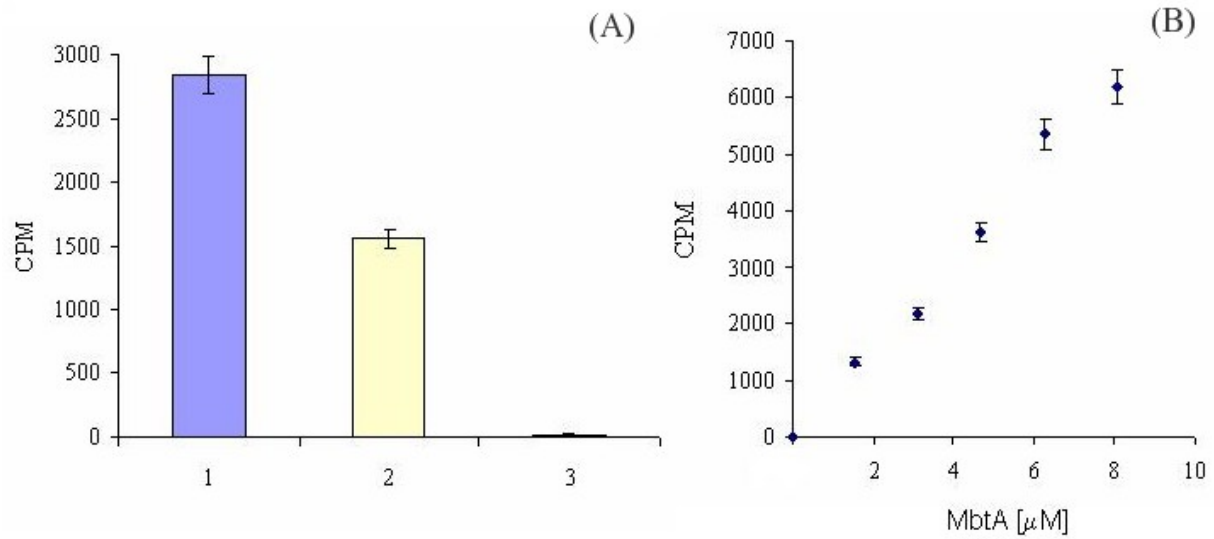


Figure 31. Enzymatic activity of MbtA. A) The assay was carried out (1) in presence of the enzyme [~8 μM] and substrate [~60 μM], (2) in absence of enzyme, (3) in absence of enzyme and substrate (3). B) Concentration of MbtA was varied to determine initial rate reactions. Experiments represent the mean of two repeats.

3.7 Discussion.

The fact that TB is responsible for 2 millions deaths each year and that a third of the world population may be latently infected with *M. tuberculosis*, twinned with the emergence of MDR and XDR-TB, has urgently prompted the search for novel drugs against TB (WHO, 2008). Hence, discovery and characterisation of new enzymes involved in *M. tuberculosis* biosynthetic pathways is important for the designation of novel targets and therefore development of new drugs to combat TB.

The new targets should fulfil certain characteristics to be considered in TB treatment. The targets must be essential for bacterial growth, metabolism, viability, persistence and must be unique to mycobacteria. Thus, mycobactin biosynthesis represents a good target, since the enzymes involved in the biosynthesis of these siderophores have no homology in mammals. The disruption of *mbtB* also impaired growth of *M. tuberculosis* H37Rv in macrophages (De Voss *et al*., 2000). The understanding of the mechanisms involved in the enzyme catalysed reactions toward mycobactin biosynthesis has provided opportunities to design new inhibitors that can block siderophore biosynthesis and, hence, bacterial growth and virulence during infection. In the early stages of mycobactin biosynthesis, the salicylate adenylating enzyme (MbtA) catalyses the activation of SAL and transfers it to the ArCP domain of MbtB to form a salicyl-ArCP domain thioester intermediate through a two step-reaction. The first step, in which ATP and SAL are bound, is followed by the nucleophilic attack of the substrate carboxylate on the α-phosphate of ATP to generate a tightly bound aryl adenylate (salicyl-AMP) intermediate and the release of pyrophosphate. In the second, MbtA binds the phosphopantetheine cofactor of the ArCP domain and transfers the aryl adenylate to the nucleophilic sulphur atom of this co-factor to provide salicyl-bound-MbtB (Quadri *et al*., 1998). It has been reported that the adenylate-forming enzymes bind their cognate acyl-AMP intermediates 2-3 orders of magnitude more tightly than their carboxylic acid and ATP substrates (Schimmel *et al*., 1998). Thus, analogues based on the aryl-adenylate could serve as mechanism-based inhibitors of adenylate-forming enzymes, such as MbtA.

Since there is no crystal structure for MbtA, a homology model, based on the co-crystal structure of DhbE and its substrate, was constructed to screen for potential inhibitors in a drug library. Six compounds were selected that were predicted to bind to the active site of MbtA, and based on their low dissociation contants those compounds promised to be potential inhibitors of the adenylation enzyme. Additionally, 13 other compounds were designed as analogues of PAS were tested to investigate their potential as anti-TB drugs. Whole-cell assays against *M. bovis* BCG under iron-sufficient conditions were performed to determine MIC_S. Only seven compounds exhibited significant growth inhibiton, and unexpectedly, these compounds had similar MIC values despite their unrelated chemical structure. The compounds derived from virtual screening are structurally diverse and were selected based on the binding site of MbtA, whereas other compounds are analogues of PAS. It is important to highlight that the condition in which the antibiotics were tested does not reflect their activity to a specific target, in this case MbtA. These preliminary data serve to highlight several lead compounds that might target iron uptake *via* their inhibition of the early stages of mycobactin biosynthesis. The reproducible phosphopantetheinylation of MbtB and ArCP now allow the detailed study of the mode of action of these lead compounds *in vitro*. On the other hand, analogues of aryl adenylate have been investigated to block mycobactin biosynthesis of *M. tuberculosis*. Although, these compounds inhibited mycobactin production in iron-deficient medium, the compounds were also able to inhibit growth of bacteria under iron-supplemented media, suggesting that the analogues may act on other targets by other mechanisms (Ferreras *et al*., 2005; Qiao *et al*., 2007; Somu *et al*., 2006a; Somu *et al*., 2006b). Based on the salicyl adenylate scaffold, four structural domains (aryl, linker, glycosyl and base) (Figure 32) have been exploited to design inhibitors towards activity of MbtA (Somu *et al*., 2006a) as well as other adenylating enzymes from other bacteria. The first inhibitor, salicyl-AMS (SAL-AMS), in which the phosphate group of the linker was replaced by an uncharged sulfamate, was found to be a potent inhibitor of MbtA and was also shown to inhibit siderophore biosynthesis of *M. tuberculosis* and *Yersinia pestis* under iron-limiting conditions (Ferreras *et al*., 2005). Furthermore, SAL-AMS was tested in iron-suficient conditions, in which siderophore biosynthesis is not required, and was found to inhibit *M. tuberculosis* growth but not *Y. pestis*, although it was required to increase the inhibitor concentration up to 18-fold. This result

suggests that SAL-AMS may also inhibit another target other than mycobactin biosynthesis (Ferreras *et al*., 2005).

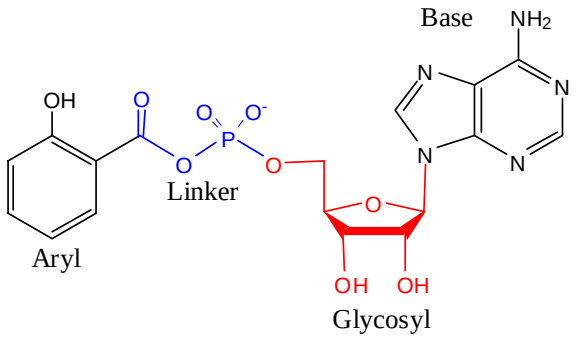


Figure 32. Salicyl adenylate scaffold. Four structural domains have been exploited to design novel inhibitors towards activity of adenylation enzymes [Adapted from Somu *et al*., (2006a)].

In another study, the substitution of the sulfamate oxygen in the linker domain with nitrogen afforded a new inhibitor, salicyl-sulfamide. This inhibitor was directly tested against whole-cell *M. tuberculosis* H37Rv under iron-limiting conditions and displayed significant growth inhibition which rivals isoniazid (Somu *et al*., 2006a). Also, the role of the glycosyl unit of SAL-AMS has been investigated. The ribofuranose ring oxygen was replaced by a CH_2 and this new compound displayed potent both *in vitro* and *in vivo* inhibition activities towards MbtA and whole-cell *M. tuberulosis* assay, respectively. Removal of the both 2'- and 3'-alcohols in the glycosyl domain resulted in complete loss of activity (Somu *et al*., 2006b). Recently, the investigation of the aryl scaffold structural domain has also been explored (Qiao *et al*., 2007). This study demonstrates that substitutions in the *ortho*-hydroxy group and modifications at the C4 of the aryl group are poorly tolerated, a result consistent with the substrates specificities of NRPSs adenylation enzymes. However, modifications only at the C4 with either F (4-fluorosalicyl-AMS) or NH_2 (*p*-aminosalicyl-AMS) led to the synthesis of analogues exhibiting potent both *in vitro* and *in vivo* inhibition activities.

Hence, the compounds investigated in this study must be tested under other conditions to validate their target; low-iron environments in which MbtA is being activated for the early siderophore biosynthesis, over-expressing the *mbtA* in *M. bovis* BCG and *in vitro* enzyme assays will provide more accurate information about activity and specificity. Analyses of their activity against iron-starved bacteria may, however, be difficult to interpret. Bacteria grown this way present an extremely exaggerated response to iron-limitation of a magnitude that has never been observed *in vivo*.

To determine *in vitro* activity and specificity of compounds, *mbtA* gene was cloned in different vectors. Initial attempts to clone the gene into *E. coli* expression vectors resulted in either undetectable yields of gene product (*mbtA*-pTYB1) or insolubility of the recombinant fusion protein (*mbtA*-pMAL-C2X). The early work by Quadri *et al*. (1998) reported that the expression of *mbtA*, containing hexahistidine-tags at its carboxy termini did not show expression. Then, the gene was cloned as a fusion with the maltose binding protein, as well as the His-tags, and this strategy allowed expression and purification of MbtA in *E. coli* BL21 (DE3)pLysS, although no high protein yield was obtained (0.1 mg/L). In the present study, *mbtA* was cloned into *E. coli*-mycobacteria shuttle vectors, pSD26 and pVV16, resulting in expression and purification of the adenylation domain in *M. smegmatis* mc^2155. The pSD26 vector harbours an acetamidase promoter, whereas the pVV16 has the *hsp*60 promoter, but both vectors possess hexa-histidine-tags for ease purification. Since these constructs are over-expressed in mycobacteria, activity of the enzyme is expected. Preliminary *in vitro* assays of *mbtA*-pVV16 showed enzymatic activity, but this must be tested further. Although, the K_M for ATP and SAL have been reported elsewhere (Quadri *et al*., 1998; Somu *et al*., 2006b), the kinetics of the MbtA for both substrates must be again tested since differences in protein-tagging, protein purification strategy, buffer assay, and host source of expression may account for difference in K_M obtained in different set of experiments. Thus, either constructs will be valuable to determine and validate the activity and specificity of the investigated compounds. Furthermore, in the previous section the activation of the carrier proteins of MbtB has been described, which will allow a greater understanding of the enzymology during the early mycobactin biosynthesis.

3.8 Further Work.

The over-expression of *mbtA*, as soluble protein, will allow determination of its kinetics for ATP and SAL substrates. Knowing the K_M for each substrate, *in vitro* inhibition by potential drugs can be precisely assessed. Next, inhibiton of the second stage, formation of salicyl-arylation intermediate, will be also tested since the activated carrier proteins are already available Chapter 2. In parallel, *in vivo* growth inhibition by the inhibitors will be investigated under iron-deficient medium and over-expressing the *mbtA* in *M. bovis* BCG to determine MIC.

4. Probing the *N*-hydroxylation of lysines by MbtG

4.1 The flavin-dependent monooxygenases.

The first committed step in the biosynthesis of various bacterial and fungal siderophores of the hydroxamate type, such as aerobactin (*E. coli* plasmid ColV-K30), alcaligin (*Aerobacter sp*.), ferrichrome (*Ustilago sp*.), involves the *N*-hydroxylation of a primary amino group (Stehr *et al*., 1998). Most of these enzymes are referred to as dehydrogenases, but some are called oxidases, peroxidases, oxygenases or reductases (Horton *et al*., 2002).

The nomenclature for enzymes that catalyse these reactions is often confusing. Oxidase is the general name for enzymes that catalyse oxidations in which molecular oxygen is the electron acceptor. Oxygenases catalyse oxidative reactions in which oxygen atoms are directly incorporated into the subtrate molecule, yielding a new hydroxyl or carbonyl group (Nelson & Cox, 2004). Oxygenases play an important role in both prokaryota and eukaryota. In bacteria, the oxygenases degrade aromatic compounds both by hydroxylating the aromatic ring in preparation for ring-cleavage and by catalysing the ring-fission reaction. In mammals, oxygenases are involved in the synthesis of steroids, neurotransmitters and in detoxication (Harayama *et al*., 1992).

The oxygenases are diverse and they differ in structure, mechanism, and coenzyme requirements. They can be simplified into two groups based on the number of oxygen atoms being incorporated into the substrate. Hence, the oxygenases are divided into dioxygenases (two oxygen atoms) and monooxygenases (one oxygen atom) (Gunsalus *et al*., 1975; Harayama *et al*., 1992; Mason & Cammack, 1992). The monooxygenases are a very abundant enzyme class and this is reflected in their diversity. The best-known monooxygenases are the P450 heme-containing oxygenases (EC 1.14.14 and EC 1.14.15), which are abundant in mammals and present in prokaryota, which can catalyse hydroxylation in unactivated carbon atoms. Other monooxygenases include the non-heme (EC 1.14.16) and a variety of copper-dependent (EC 1.14.17 and EC 1.14.18) variants. Monoxygenases use different co-substrates, such as reduced flavin nucleotides ($FADH_2$ or $FMNH_2$) or reduced nucleotides (NADH or NADPH) (van Berkel *et al*., 2006). Monoxygenases that use $FADH_2$ or $FMNH_2$ act as prosthetic groups are often called flavin-dependent monooxygenases, flavoenzymes or

flavoproteins (EC 1.14.12 and EC 1.14.13) (Horton *et al*., 2002). However, some monooxygenases do not require cofactors, such as the polyketide monooxygenase ActV-OrF6, involved in actinorhobdin biosynthesis in *Streptomyces coelicolor* (Sciara *et al*., 2003), and YigN, a quinol monooxygenase from *E. coli* that oxidizes multiringed aromatic substrates (Adams & Jia, 2005). Despite this diversity, the monooxygenases catalyse the hydroxylation in the following general mechanism:

$$AH + BH_2 + O\text{-}O \longrightarrow A\text{-}OH + B + H_2O$$

where AH is the main substrate and BH_2 the co-substrate. Since, most monoxygenases catalyse reactions in which the main substrate becomes hydroxylated, they are also called hydroxylases, mixed-function oxidases or mixed-function oxygenases. The latter indicates that they oxidise two different substrates simultaneously (Nelson & Cox, 2004). For instance, the *p*-hydroxybenzoate hydroxylase (PHBH), a flavin monooxygenase of *Pseudomonas aeruginosa* PAO1, catalyses the hydroxylation of aromatic substrates, incorporating an atom of oxygen using O_2, NADPH and FAD giving 3,4-dihydroxybenzoate, water, and NADP (Figure 33) (Palfey *et al*., 1999).

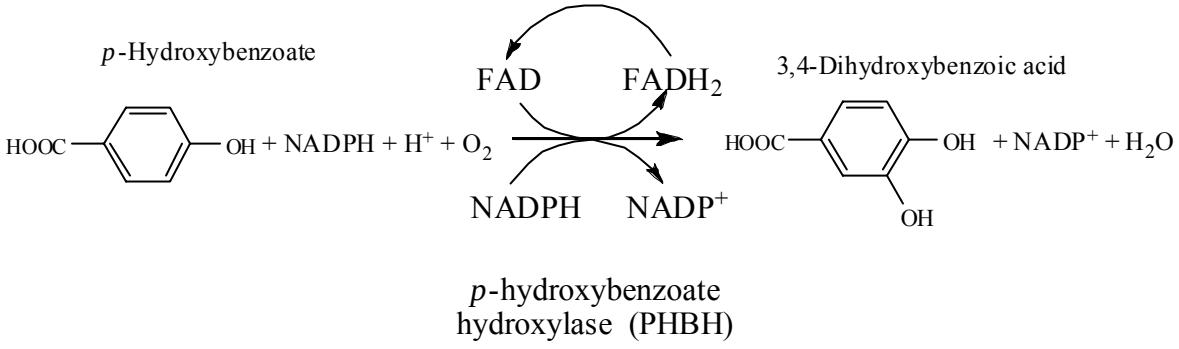


Figure 33. **Reaction catalysed by *p*-hydroxybenzoate hydroxylase.** *p*-Hydroxybenzoate hydroxylase with its co-factor FAD catalyses the incorporation of an atom of oxygen into *p*-Hydroxybenzoic acid to produce 3,4-dihydroxybenzoic acid (Palfey *et al*., 1999).

Intramolecular reactions between O_2 and other molecules induce a number of interesting phenomena. The electronic ground state (lowest-energy state) of dioxygen is a triplet $^3O_2$, in which two unpaired electrons are present. Pairing these electrons to give one with "up" spin and one "down" gives a single state $^1O_2$ (singlet oxygen). In the ground state, the two highest energy electrons reside in identical molecular orbitals with the same spin and therefore they cannot occupy the same region of space (the Pauli exclusion principle). Electrons with opposite spin, as in O_2, can occupy the same region in space, that renders greater repulsion between them to react with other molecules, but this requires energy and the reactions are kinetically slow (Pryor *et al*., 2006). When oxygen oxidises organic molecules, it is itself reduced. By adding electrons to the molecular orbitals of the ground state of dioxygen, a stepwise reduction of oxygen products results. By addition of one electron, superoxide (O_2^-) is formed, whereas addition of two electrons produces peroxides (HO_2, H_2O_2) are produced. These small molecules are highly reactive oxygen intermediates (ROIs) that can inactivate many enzymes, membrane lipids and nucleic acid, with subsequent damage of cells (Valko *et al*., 2007). Hence, the activation of dioxygen not only requires energy, but also a safe chemical reduction process to avoid ROS (van Berkel *et al*., 2006). In heme-enzymes, e.g. P450 monooxygenases, transition metals are required to activate molecular oxygen (Nelson & Cox, 2004). On the other hand, the flavin-dependent monooxygenases employ the reduced flavin cofactor for oxygenation reactions (van Berkel *et al*., 2006). To avoid confusion in the subsequent paragraphs, hereafter the flavin-dependent monooxygenases will be denominated FDMOs. The FDMOs carry out the oxygenation in three common chemical processes: (i) reduction of flavin by NAD(P)H; (ii) reaction of reduced flavin with O_2 to provide a C4a-flavin (hydro)peroxide, which is the oxygenating reagent (it is peroxide for electrophilic substrates and the hydroperoxide for nucleophilic substrates); and (iii) binding, orienting, and activating the substrate for its oxygenation by the C4a-flavin (hydro)peroxide (Figure 34) (Ballou *et al*., 2005). However, the common mechanisms differ according to the number of polypeptides involved in oxygenation. The single-component FDMOs carry out all catalytic reactions in which three conformational changes in which both protein and FAD are important for catalysis (Ballou *et al*., 2005; Malito *et al*., 2004). The two-component FDMOs divide the catalytic tasks by employing two different polypeptides. Firstly, a reductase which generates

reduced flavin and secondly a hydroxylase using reduced flavin to catalyse substrate monooxygenation (Ballou *et al*., 2005; van Berkel *et al*., 2006).

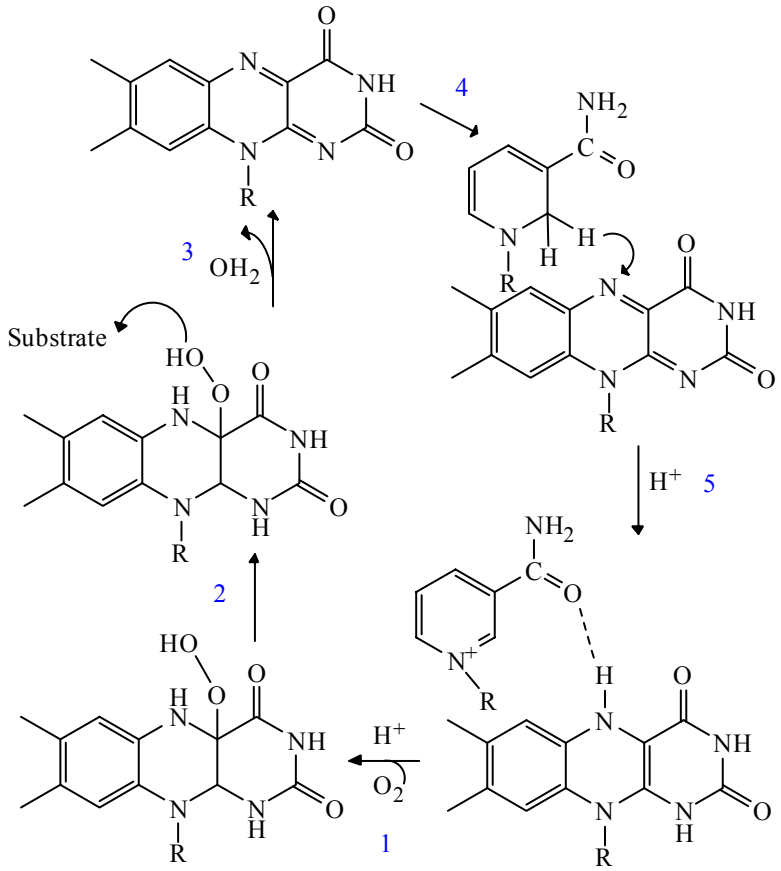


Figure 34. General mechanism for FAD–dependent oxygenation. FDMOs oxygenate substrates by initially forming a flavin hydroperoxide at the enzyme active site (1), which then transfers the terminal oxygen atom of the hydroperoxide (OH) to the substrate (2). The remaining "hydroxyflavin" then reoxidizes with the release of water (3). For 'external' FDMOs (illustrated here), NADPH is then used to regenerate the FAD (4 & 5) for the next round of catalysis; the flavins of internal FDMOs are reduced by the substrate itself.

Although, most enzymes are specific for one electron acceptor, some display dual specificity. Sequence aligment of a set of Baeyer-Villiger monooxygenases (BVMOs) with a

preference for NADPH using 4-hydroxy-acetophenone monooxygenase (HAPMO) as a prototype and mutagenesis analysis of the latter, revealed three amino acid residues that were important for co-enzyme specificity in HAPMO (Arg339, Lys439 and Arg440) (Kamerbeek *et al*., 2004). The mutants Arg339Ala and Lys439Ala showed decrease affinity for NADPH. Furthermore, replacing the lysine (Lys329) in another Type I BVMO, the cyclohexanone monooxygenase, which is analogous to Lys439 in HAPMO, changed the co-enzyme specificity towards NADH (Kamerbeek *et al*., 2004). The electron acceptor may be an integral part of the enzyme as shown in the case of the monooxygenase of *Methylophaga* sp. strain SK1 (Alfieri *et al*., 2008), or replaced during oxygenation of the substrate as in the monooxygenase of *Schizosaccaromces pombe* (Eswaramoorthy *et al*., 2006).

Although, the flavin nucleotide in most FMDOs is not covalently bound to the protein, some enzymes have covalently bound FMN or FAD as prosthetic groups (Mewies *et al*., 1998). The FDMOs are involved in a wide variety of cellular processes including drug detoxification, biodegradation of aromatic compounds in the environment, biosynthesis of antibiotics and siderophores. Oxygenation reactions they catalyse include hydroxylations, epoxidations, Baeyer-Villiger oxidations and sulfoxidations (Figure 35).

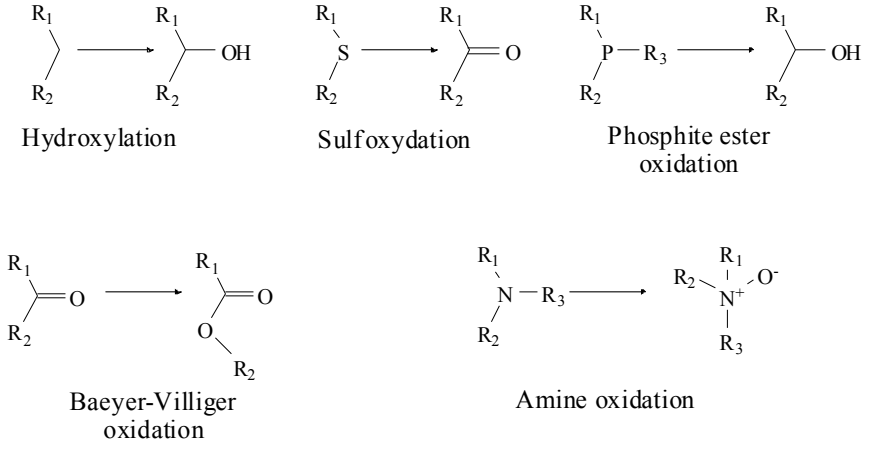


Figure 35. **Reactions catalised by flavoprotein monooxygenases.**

The specific type of oxygenation and selectivity depends on the shape and chemical nature of the active site of each specific monooxygenase. However, based on amino acid sequence and structural homology, the topology of the enzymes play an important role in the type of oxygenation catalysed by these enzymes (van Berkel *et al*., 2006). The FDMOs can be subdivided as external and internal oxygenases. In the external FDMOs, the flavin cofactor reacts with the molecular oxygen atom when it is in the reduced form and NADH or NADPH are consumed. On the other hand, in the internal FDMOs the flavin is reduced by the substrate itself (van Berkel *et al*., 2006). Furthermore, based on amino acid sequence, structure and mechanism, the external monooxygenases are classified in six subclasses (Table 15).

Table 15. **Classification of external flavoprotein monooxygenases.** [a]The most commonly found *in vivo* activities are given (van Berkel *et al*., 2006).

Sub-class	Prototype	Reactions[a]	Cofactor	Coenzyme
A	4-OH-benzoate hydroxylase	Hydroxylation epoxidation	FAD	NAD(P)H
B	Cyclohexanone monooxygenase	Baeyer-Villiger; *N*-oxidation	FAD	NADPH
C	Luciferase	Light emission; S-oxidation		FMN NAD(P)H
D	4-OH-phenylacetate hydroxylase	Hydroxylation		FAD NAD(P)H
E	Styrene monooxygenase	Epoxidation		FAD NAD(P)H
F	Tryptophan 7-halogenase	Halogenation		FAD NAD(P)H

The B flavoprotein monooxygenases are multifunctional enzymes that oxidise carbon atoms and other (hetero)atoms (Fraaije *et al*., 2002). Moreover, this subclass consist of three sequence-related monooxygenase subfamilies: flavin-containing monooxygenases (FMOs), microbial *N*-hydroxylating monooxygenases (NMOs) and Type I BVMOs. These enzymes are single-component FAD-containing enzymes and are specific for NADPH (Fraaije *et al*., 2002). Sequence aligment of the well studied HAPMO, a BVMO from *P. fluorescens* and related FDMOs revealed three conserved motifs in this subclass; two Rossman-fold motifs [GXGXX(G/A)] for the two binding domains of FAD and NADPH, and the specific

fingerprint [FXGXXXHXXXW(P/D)] located before the second Rossman motif. The latter motif is strictly conserved in FMOs and NMOs (Fraaije *et al*., 2002). Mutation analysis of the His and Trp residues leads to insolubility and loss of the FAD co-factor of the enzyme, respectively, indicating that these residues are essential for enzyme activity (Fraaije *et al*., 2002).

Amino acid sequence comparison of NMOs from *E. coli* (aerobactin, AerA, IucD), *P. aeruginosa* (pyoverdine, PvdA, PsbA), *Bordetella bronchiseptica* (alcaligin, AlcA) and *Ustilago maydis* (ferrichrome, Sid1), and 30 sequences of FMOs revealed amino acid differences in the two Rossman motifs, as well as another motif (Ambrosi *et al*., 2000; Stehr *et al*., 1998). In the first Rossman motif (located in the N-terminal of the protein), replacement of the last glycine for proline was detected and it was assumed to cause weak binding of FAD to lysine N^6-hydroxylase (Macheroux *et al*., 1993). In AlcA and PvdA an alanine residue and in Sid1 a serine residue replace the last glycine in the second Rossman motif (Ambrosi *et al*., 2000; Stehr *et al*., 1998). Another motif, [(L/F)ATGYXXXXP)] located at the C-terminal of the protein, was predicted to be for substrate binding, since this region is highly hydrophobic (Ambrosi *et al*., 2000; Stehr *et al*., 1998). However, the crystal structure of a FMO from *S. pombe* shows that the FAD is tightly bound to the enzyme, whereas the NADPH may be replaced by the substrate (Eswaramoorthy *et al*., 2006). In the enzyme-FAD-substrate crystal, the substrate is bound to FAD in the isoalloxazine ring and occupies the NADPH binding site, indicating that there are no more interactions with the protein (Eswaramoorthy *et al*., 2006).

4.2 The *N*-hydroxylation in siderophores.

Many microorganisms utilise hydroxamates and carboxylate siderophores for sequestering iron, and their biosynthesis mainly rely in NRPS-independent systems. The biosynthesis of these siderophores depends on enzymes, such as NMOs, decarboxylases, aminotransferases and aldolases (Challis, 2005). It has been proposed that the formation of hydroxamates, in which functional groups are derived from diamino acids, such as L-ornithine and L-lysine, is the first step in the biosynthesis of the corresponding siderophore (Miethke & Marahiel,

2007). Biochemical data support the above hypothesis, in which L-ornithine N^5-hydroxylases, such as the CchB involved in *Streptomyces coelicolor* coelichelin siderophore biosynthesis (Pohlmann & Marahiel, 2008), PvdA *P. aeruginosa* in pyoverdin biosynthesis (Ge & Seah, 2006) and *E. coli* K-12 Lysine N^6-hydroxylase (IucD) is required in aerobactin (de Lorenzo *et al*., 1986; Plattner *et al*., 1989), catalysed the hydroxylation of their respective substrates as the first biosynthetic step in siderophore synthesis (Figure 36). Other NMOs genes have been identified in diverse microorganisms. *Aspergillus nidulans*, a filamentous ascomycete, produces ferricrocin that requires *sidA* that encodes L-ornithine N^5-monooxygenase as the first step in the siderophore biosynthesis (Eisendle *et al*., 2003). In *Ustilago maydis*, *sid1* encodes for L-ornithine N^5-oxygenase which also catalyses the hydroxylation of L-ornithine as first step of the biosynthesis of ferrichrome and ferrichrome A (Mei *et al*., 1993).

In the gene cluster for mycobactin biosynthesis, a lysine *N*-oxygenase (*mbtG*, Rv2378c) was found to share homology with ornithine and lysine *N*-oxygenases activities that would be required for *N*-hydroxylation of the two lysines at some stage during mycobactin synthesis (Quadri *et al*., 1998). In the mature mycobactin molecule, two hydroxylysine residues are present. One is found in a central position and one is cyclised and terminally situated (Figure 34). Mycobactins contain both hydroxamates and catechols as iron chelating groups (Quadri *et al*., 1998; Ratledge, 2004). The catechol group is introduced during the early steps of mycobactin biosynthesis (Quadri *et al*., 1998; Ratledge & Dover, 2000). The same biological process occurs during the biosynthesis of yersiniabactin (Gehring *et al*., 1998a) and pyochelin (Quadri *et al*., 1999), siderophores of *Yersinia sp*. and *P. aeruginosa*, respectively. The hydroxamates of mycobactins are derivatives of lysine and hydroxylation of these amino acids is required to produce the chelating groups for Fe (III) (De Voss *et al*., 1999; Ratledge, 2004; Ratledge & Dover, 2000). However, the hydroxylation of lysines in the core structure of mycobactin may not occur during the early stage of biosynthesis (Moody *et al*., 2004).

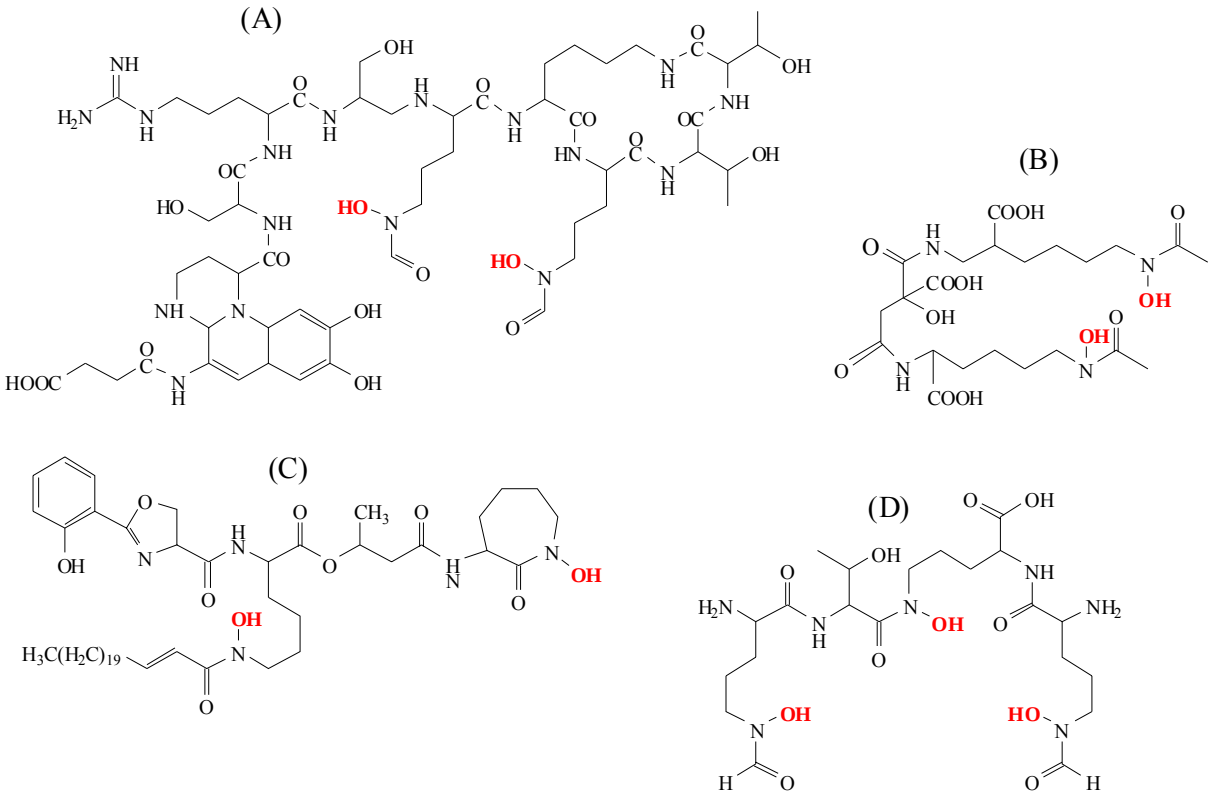


Figure 36. **Structures of (A) pyoverdine, (B) aerobactin, (C) mycobactin, and (D) coelichelin siderophores.** Hydroxamate groups (highlighted in red) are introduced by NMOs. For pyoverdine, aerobactin, and coelichelin the hydroxylation occurs as first step, whereas in mycobactin the hydroxylation is hypothesised to take place in the late stage of its biosynthesis.

Different hypotheses have been postulated to understand how the lysines are incorporated, either as unmodified amino acids or hydroxylated, and finally added to the growing mycobactin. Firstly, MbtG might hydroxylate both lysine residues before addition to the growing mycobactin and then these would be activated by the adenylation domains of MbtE and MbtF proteins, which also suggest that lysines are modified without requirement of

the PCP domains. In most NRPSs modules, once the amino acid is activated and transferred to PCP domain it can, without losing the covalent linkage, be modified by optional domains such as an epimerazation domain. However, the discovery of a compound structurally related to mycobactin T, which lacked the oxygen atoms in both lysines, has suggested another hypothesis (Moody *et al*., 2004). Screening for antigens presented by CD1a-restricted T cells from *M. tuberculosis*, a compound initially named 838 based on its nominal mass elucidated by mass spectrometry analysis was elucidated. Further structural analyses yielded product ions at *m/z* 642 and *m/z* 197, which were each 16 u (the mass of oxygen) smaller than the previously described mycobactic acid and cobactin fragments from mycobactin T (Snow, 1965a). The compound was named didehydroxymycobactin (DDM)-838 (Figure 37). Identification of MS/MS products at *m/z* 727 and 84 determined unmodified lysines in the proposed structure of DDM (Moody *et al*., 2004). According to Krithika *et al*. (2006), MbtG was able to catalyse the hydroxylation on both acetylated lysine and L-lysine, although the preference was for the former by 5-fold. This result also suggests that the hydroxylation of lysines may be the last step for mycobactin biosynthesis, and the discovery of the didehydroxy analog of mycobactin isolated from mycobacteria supports this hypothesis (Moody *et al*., 2004). Therefore, these studies allow the speculation that unmodified lysines are incorporated in the growing chain by the adenylation domains of MbtE and MbtF. The central lysine is then *N*-acylated and the terminal lysine is cyclised, and MbtG subsequently carries out hydroxylation to produce a functional mycobactin.

On the other hand, the hydroxylation reaction in some NRPS occurs during the biosynthesis before yielding the final product. A number of nonribosomal peptide antibiotics contain β-hydroxy amino acids, including vancomicyn and bleomycin (Walsh *et al*., 2001). One antibiotic of this group is novobiocin, produced by *Streptomyces spheroides*, in which the core structure, essential for activity, is formed by 3-amino-4-hydroxy-coumarin. Two open reading frames, NovH and NovI, have been predicted to participate in the synthesis of the core structure. NovH is a two-domain A-PCP protein, whereas NovI is a heme protein of the cytochrome P450 monooxygenase class. The NovH catalyses L-tyrosyl-AMP formation by its adenylation domain, which can be transferred on the pantetheinylated PCP domain, producing

the covalent L-tyrosyl-S-NovH intermediate on the holo PCP domain. Then NovI installs one atom from O_2 at the C_3 of the Tyr-S-NovH to yield (3R)-3-OH-tyr-S-PCP-NovH (Chen & Walsh, 2001; Steffensky *et al*., 2000). Thus, NovI recognises the L-tyrosyl moiety, presented on the pantetheinyl arm of the PCP domain of NovH, NovI acts catalytically on its tyrosyl-S-NovH partner but the L-OH-Tyr product stays covalently attached on the NovH PCP domain. Homologues of the NovH/NovI enzymes are found in other microorganisms that produce antibiotics containing L-hydroxyl amino acid moieties, such as vancomycin and nikkomycin. This suggests that hydroxylation of a tethered aminoacyl-S-PCP, before yielding the final product, may be a general strategy for diversion of a fraction of the amino acid pool to modifications that provide building blocks for antibiotic biosynthesis (Chen & Walsh, 2001).

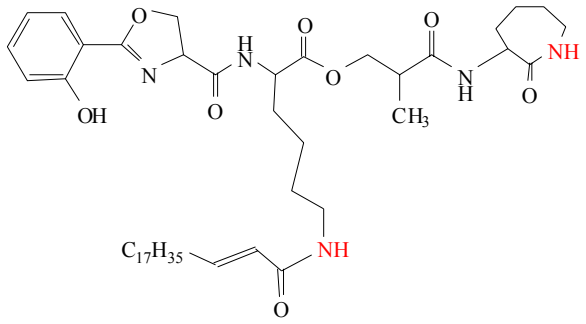


Figure 37. Structure of the CD1a-presented antigen, didehydroxymycobactin (DDM)-838. The DDM structure was deduced from the MS/MS spectrum of $[M+H]^+$ *m/z* 838. DDM might function as an intermediate in mycobactin synthesis and this suggest that the lysines are incorporated into the peptide and subsequently hydroxylated by MbtG to produce functional mycobactin [Adapted from Moody *et al*., (2004)].

Despite this partial characterisation of MbtG, there are some biochemical questions in mycobactin biosynthesis that remain unanswered. Firstly, is the hydroxylation of lysines the last step for mycobactin biosynthesis? If this is the case, the adenylation domains, both MbtE and MbtF, will prefer lysines to acylated or hydroxylated lysines. It has been assumed that the

major fidelity or editing functions for amino acid selection and incorporation into elongation peptide chain reside in the A domain of NRPS (Pavela-Vrancic *et al*., 2004). Despite this specificity, some NRPS systems exhibit relaxed amino acid substrate specificity, allowing the production of a variety of isoforms of the respective product. The VibF module, a protein for vibriobactin biosynthesis, which contains two heterocyclisation domains, one adenylation domain, two condensation domains and peptidyl carrier protein, was able to utilise various L-threonine analogues, as well as serine and cysteine as alternate substrates in aminoacyl-AMP formation (Walsh *et al*., 2001). For several NRPS systems, like tyrocidine, surfactin, cyclosporine and enniatin, nutrient availability conditions can affect the constitution of the final product, either *in vivo* by adding certain amino acids to the medium or *in vitro* in cell-free systems with arbitrarily chosen substrate concentrations (Mootz and Marahiel, 1997). The relaxed specificities of these domains are likely to respond to biological requirements as they can switch from one substrate to another to yield a different product.

On the other hand, the discovery of the DDM compound might be considered as an initial step for mycobactin degradation or as defense mechanism against innate immunity of the host. In general, once Fe^{3+} is mobilised and captured by siderophores, it is already accessible for cellular uptake (Ratledge & Dover, 2000). The ferric-siderophore complex must be dissociated and iron reduced to its soluble form (Fe^{2+}) to be available for subsequent cellular processes (Ratledge & Dover, 2000). The *E. coli* ferric enterobactin esterase (Fes) was the first ferric-siderophore esterase to show hydrolytic activity on the Fe-enterobactin complex followed by ferric iron reduction (Brickman & McIntosh, 1992; O'Brien *et al*., 1971). Recently, two other esterases of *Salmonella enterica* have been biochemically characterised, IroD and IroE, with similar catalytic function as Fes (Lin *et al*., 2005); their genes are part of a functional operon mediated by Fur (Baümler *et al*., 1998). IroD could hydrolyse both *apo* and ferric-siderophore, whereas IroE prefers *apo*-siderophores as substrates, although another study showed different results about substrate specificity of these esterases (Zhu *et al*., 2005). This study showed different substrates specificities for these esterases: IroD preferred free iron enterobactin and salmochelin substrates over the ferri-siderophores, while IroE hydrolysed both free iron-siderophores and iron-charged diglucosylated enterobactin (Zhu *et*

al., 2005). Although, indirect reduction of ferric-siderophore by soluble flavin reductases has been observed in prokaryotes, their ferric-siderophore reductase activity is not considered specific as the enzymes are neither substrate specific or iron regulated (Halle & Meyer, 1992). Hence, the experimental data suggests that degradation of siderophores may be a required step to remove iron from the complex, facilitating reductive release of the metal, a similar degradative scenario might be envisaged for the ferric-mycobactin complex, and thus the DDM might be a degradation compound.

Hence, different hypothetical scenarios might occur during *in vivo* mycobactin biosynthesis. Here, the aim of this chapter was to investigate at which stage hydroxylation might occur in the lysine residues. To assess the hypothesis that DDM is an intermediate product prior mature mycobactin, the *mbtG* and adenylations gene domains of MbtE and MbtF were cloned and expressed for their further biochemical characterisation.

4.3 Material and methods.

4.3.1 Molecular cloning of *mbtG* and adenylation domains of MbtE and MbtF.

Amplification of the *mbtG* gene was performed with Vent DNA polymerase (New England Biolabs) using *M. tuberculosis* H37Rv chromosomal DNA and the following primers: pET23b*mbtGu* 5'-GATCGATCCATATGAATCCGACGCTCGCGGT-3' and pET23b*mbtGl* 5'- GATCGATCGCGGCCGCTCGAAAGGATTGGTGCTCAC-3' (*Nde*I and *Not*I recognition sites underlined). The PCR product was then directly ligated at 16°C for 16 hours with T4 DNA ligase (New England Biolabs) into pET23b (Novagen), which had been cut by *Nde*I and *NotI* (New England Biolabs), thus giving rise to *mbtG*-pET23b. The ligation mixture was used to transform *E. coli* TOP10 and the nucleotide sequence of plasmid DNA from recombinant transformants confirmed by sequencing. The *mbtG*-pET23b plasmid was then subsequently used to transform *E. coli* C41 (DE3) enabling the production of the recombinant MbtG protein.

To clone the adenylation domains of *mbtE* and *mbtF*, denominated hereafter as *ambtE* and *ambtF*, respectively, amino acid sequence aligment of the adenylation domain of bacillibactin, DhbE, whose crystal structure has been determined (May *et al*., 2002), was performed to define important structural regions for appropiate folding of the truncated proteins. Hence, primers were designed to clone the adenylation domains in a range of expression vectors (Table 16). Amplification of the adenylation domains was performed with Phusion High Fidelity Polymerase (New England Biolabs) using *M. tuberculosis* H37Rv chromosomal DNA. Amplicons and vectors were digested with their appropriate restriction endonucleases (New England Biolabs), followed by ligation with T4 DNAligase (New England Biolabs) at 16°C for 16 hours. The ligation mixture *ambtE*-pET28b was used to transform *E. coli* TOP 10. The ligation mixture of *ambtF* in each of the vectors was used to transform *E. coli* NEB 5-alpha F´*Iq* (New England Biolabs).

Table 16. **Primers designed to clone the adenylation domains both *mbtE* and *mbtF* genes into pET and pVV16 vectors**.

Gene	Vector	Primers	Restriction site (underlined)
ambtE	pET28b	Up-5'-GATCGATCCATATGGGGATAGGCAGCGAAGACCGG-3'	NdeI
	pET28b	Down-5'-GATCGATCGCGGCCGCTTACCGCACACCGACATCGACGCCGAACGC-'3	NotI
	pET23a	Up-5'-GATCGATCCATATGGACGTGAGTCGCGAGACCCCG-3' (a)	NdeI
ambtF	pET23a	Down-5'-GATCGATCGCGGCCGCCGCAATCCCACGACGGCGCGCGGCCTG -'3	NotI
	pET28b	Down-5'-GATCGATCGCGGCCGCTTACGCAATCCCACGACGGCGCGCGGCCTG -'3	NotI
	pVV16	Up-5'- GATCGATCCATATGCGCAATCCCACGACGGCGCGCGGCCTG-3'	NdeI
		Down-5'- GATCGATCAAGCTTCGCAATCCCACGACGGCGCGCGGCCTG-3'	HindIII

(a) In both genes, the up primers are the same for both vectors.

Selection of *mbtG*-pET23b, *ambtF*-pET23b, *ambtE*-pET23b, *ambtF*-pTYB2 was performed on LB agar plates containing Ampicillin (100 μg/mL), whereas for *ambtF*-pET28b, Kanamycin (25 μg/mL) was added to the solid media. For *ambtF*-pVV16 Hygromycin (200 μg/mL) was added to LB agar. Screening was performed by double digestion followed by electrophoresis on agarose gels and stained with EtBr. Integrity of genetic constructs was verified by nucleotide sequencing.

4.3.2 Gene expression and protein purification of MbtG and adenylation domains.

To seek gene expression, *E. coli* C41(DE3) was transformed with each of the following constructs: *mbtG*-pET23b, *ambtF*-pET23a, *ambtE*-pET28b, and *ambtF*-pET28b, whereas *E. coli* ER2566 was used for *ambtF*-pTYB2. Gene expression analysis was determined by testing different temperatures, incubation time and IPTG concentrations as described in (Chapter 2, section 2.2.3). For *ambtF*-pVV16 *M. smegmatis* mc^2155 was also used as heterologous host and gene expression analysis was determined as described in Chapter 3, section 3.5.3. Gene products cloned into pET and pVV16 vectors were purified by affinity chromatography using a His-Trap column as described in Chapter 2, section 2.2.5, whereas the adenylation domain

of MbtF cloned into pTYB1 was purified by affinity chromatography using a chitin column as described in Chapter 2, section 2.2.7.1. The purity of proteins was assessed by 12% SDS-PAGE and Coomasie blue staining. Purified protein fractions were pooled and dialysed against against appropriate buffer, and concentrated using Centricon YM-30 filter unit (Millipore), and stored at -80 ºC until use. Protein concentration were estimated by the BCA protein assay (Pierce).

4.3.3 *N*-Hydroxylation of MbtG on DDM.

Krithika *et al*. (2006) reported that MbtG could hydroxylate acylated lysines as the preferred substrate. Thus, MbtG will be biochemically characterised using protocols reported elsewhere (Plattner *et al*., 1989) to seek activity of the protein. Hydroxylation will be attempted using DDM compound provided by our collaborators Cressida Madigan and Branch Moody (Brigham and Women's Hospital, Division of Medical Sciences, Harvard University).

4.3.4 Biochemical activity of the adenylation domains.

To investigate substrate preference by the adenylation domains, ATP-PPi exchange assay will be performed as reported by Linne and Marahiel (2004).

4.4 Results.

4.4.1 Expression and purification of MbtG.

Gene expression analysis of *mbtG*-pET23b was carried out to determine the optimal conditions for *mbtG* over-expression, which involved incubation with 1mM IPTG at 16°C for 16 hours. The purification of protein was carried out by nickel affinity chromatography. A protein was purified to > 95% purity on the Ni^{2+}-charged resin consistent with the predicted molecular mass calculated for MbtG of 46 kDa (Figure 38). During elution of MbtG, there were two problems, firstly the protein either precipitated quickly, or secondly, lost its cofactor FAD.

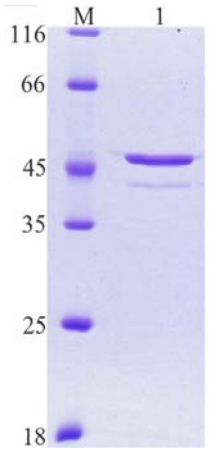


Figure 38. Coomasie Blue stained 12% SDS-PAGE of MbtG purified. M= protein molecular marker. Lane (1) MbtG purified.

4.4.2 Cloning and expression of adenylation domains of MbtE and MbtF.

As was outlined the initial aim of the project was to elucidate the biochemical function of MbtG. Recent studies have shown the predicted function of this protein (Krithika *et al*., 2006), and the discovery of DDM suggests that hydroxylation is the last step in mycobactin synthesis (Moody *et al*., 2004). This information suggests that unmodified lysines are activiated by the adenylation domains of MbtE and MbtF, and then subsequently incorporated into the growing

core structure of mycobactin. To investigate this hypothesis, the adenylation domains of MbtE and MbtF were cloned and their over-expression examined.

Initial attempts were to clone the domains into pET vectors as fused hexahistidine tag to facilitate protein purification. The region coding the adenylation domain of MbtE, *ambtE* was cloned into pET28b, whereas the equivalent *ambtF* was cloned into pET23a. The AMbtE protein was over-expressed as an insoluble protein at 37°C in all IPTG concentrations examined. When gene expression was induced at this temperature, a heavily staining band having migration compatibility with the expected molecular mass of 55.5 kDa, suggested the recombinant protein was present in the cell lysate, but no detectable protein remained in the supernatant after centrifugation for 30 minutes at 15,000 rpm as determined by 12% SDS-PAGE (Figure 39). Expression of the protein was not observed in other growth, time and temperature conditions as well as different concentrations of IPTG (data no shown). Further attempts to clone *ambtE* were made using different expression vectors but without success.

In addition, similar problems were also found for *ambtF* expression. Different conditions were attempted to allow expression of protein, but again without success. Mycobacterial genes can sometimes be difficult to clone and express due to the high G+C content, and different strategies have been used to overcome this problem. The codon alteration strategy, in which the first codons of the gene are modified in order to prevent the formation of secondary structures that might inhibit translation, was successfully used to obtain considerable mycobacteria DapF protein expression (Usha *et al*., 2006). It is likely that the high G+C content in the native sequence results in the formation of secondary structures in the encoding mRNA. The formation of such a complex can block the binding site of the ribosome and eventually lead to the failure of protein expression, i.e. blocking translation. Alteration of the DNA nucleotide sequence, and consequently the mRNA, might avoid the formation of this complex.

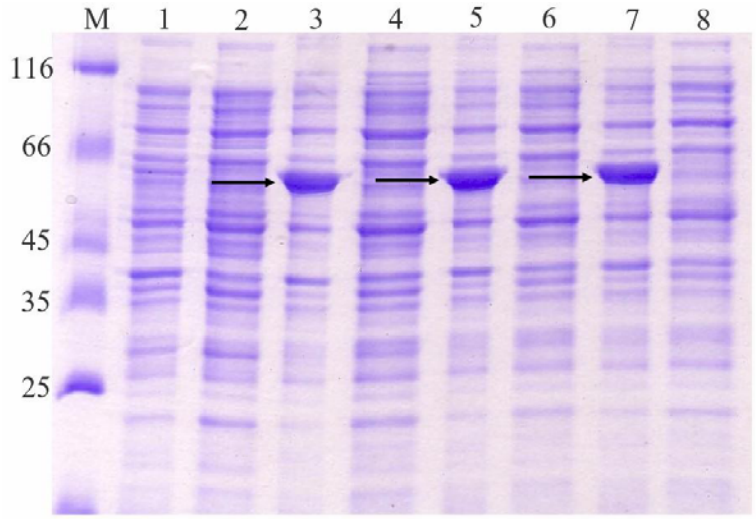


Figure 39. **Expression analysis of AMbtE.** *E. coli* C41(DE3)-*ambtE*-pET28b was grown at 37°C, when $O.D_{600}$=0.5-0.6, different concentration of IPTG were added to seek expression of the adenylation domain. M=protein molecular marker. Lane 1 and 2, crude lysate and supernatant, respectively, of negative control [no inducer added to *E. coli* C41(DE3) transformed with only pET28b]; Lanes 3 to 8 samples loaded with *E.coli* C41(DE3)-*ambtE*-pET28b. Lane 3 and 4, crude lysate and supernatant, respectively, induced with 1 mM IPTG. Lane 5 and 6, crude lysate and supernatant, respectively, induced with 0.5 mM IPTG. Lane 7 and 8, crude lyaste and supernatant, respectively, induced with 0.1 mM IPTG. In lanes 3, 5, 7 a band migrated with the expected size of the protein (55.5 kDa) indicating thus over-expression of the adenylation domain, although in its insoluble form.

There are 64 codons that only encode only 20 amino acids, so alteration of these codons, without altering the amino acids sequence, would help the expression of the protein. Thus, by adjusting only the primer up of *ambtF* (altering the native nucleotide sequence) for cloning into both pET23a and pTYB2 vectors, one might expect to increase yields of recombinant protein at lower temperatures and/or IPTG concentrations which could ultimately lead to a soluble protein preparation. Primers were re-designed to clone *ambtF* into different vectors. Thus, *E. coli* expression vectors were tested using the same codon alteration strategy (Table 17).

Table 17. **Primers were re-designed allowing the cloning of *ambtF* into two different vectors.** (a) The sequence of the mutation region of the *ambtF* forward (For) oligonucleotide primer used to amplify *ambtF* is displayed below the sequence of the corresponding native sequence. The first 10 codons of the *ambtF* mRNA were modified to prevent secondary structures that might affect translation, whereas the reverse (Rev) oligonucleotide was designed to be homologous to the *M. tuberculosis* H37Rv to promote efficient priming. (b) Restriction sites are underlined.

Native amino acid sequence	Asp	Val	Ser	Arg	Glu	Thr	Pro	Val	Ala	Val
Native nucleotide sequence	GAC	GTG	AGT	CGC	GAG	ACC	CCG	GTG	GCC	GTC
(a) Mutated sequence	GAT	GTT	TCT	AGA	GAA	ACT	CCT	GTT	GCT	GTT

Vectors	Pimers	(b)
pTYB2	For 5'-GATCGATCCATATGGATGTTTCTAGAGAAACTCCTGTTGCTGTCGCGCTGTCCCGTGGT-'3	NdeI
	Rev 5'-GATCGATCCCCGGGCGCAATCCCACGGCGCGCGGCCTG-'3	SmaI
pET23a	For-5'-GATCGATCCATATGGATGTTTCTAGAGAAACTCCTGTTGCTGTTGCGCTGTCCCGTGGTCCG-'3	NdeI
	Rev-5'-GATCGATCGCGGCCGCCGCAATCCCACGACGGCGCGCGGCCTG -'3	NotI

Hence, *ambtF* was successfully cloned into pTYB2 vector but not in pET23a using *E. coli* TOP 10 as cloning host. However, when the integrity of the construct was verified by nucleotide sequencing, point mutations were observed in different samples. As a result, another cloning host was used, *E. coli* NEB 5-alpha F´*Iq*, which allowed successful cloning of the gene without point mutations. The protein expression analysis was carried out by transforming *E. coli* C41(DE3) with *ambtF*-pTYB2. Cultures were grown initially at 37˚C, then the temperature was shifted to 16˚C and 0.5 mM IPTG added to induce expression. After purification using a chitin column, a band migrated at the expected size of the truncated protein on 12% SDS-PAGE gels (Figure 40A); the adenylation domain of MbtF is expected to have a mass of 56 kDa. To confirm the identity of the truncated protein MALDI-MS was carried out. Instead of the mycobacterial protein, an *E. coli* protein (GroEL, 60 KDa) was found to be the expressed and purified. This result is in accordance with the vector

manufacturer's suggestion that either this chaperon or the intein tag (55 KDa) may appear in the elution following the on-column cleavage reaction. *ambtF* was also cloned into pVV16 followed by transformation into *M. smegmatis* mc^2155. Transformed cells were grown at 37°C until an OD_{600}=0.5-0.6 and further incubated for 4 hours. Cells were disrupted by sonication followed by centrifugation and the supernanant subjected to affinity chromatography using a His-Tag column. After purification, a band migrated on 12% SDS-PAGE with the expected size of AMbtF (Figure 40B), however, MALDI-MS analysis of the constituted peptides of the purified protein showed the identity of a chaperonin (GroEL1). GroEL1 has an approximate similar MW (~60 kDa) to that of AMbtF (55 kDa), and possess a histidine-rich C-terminal region, which would also bind to the nickel column during purification.

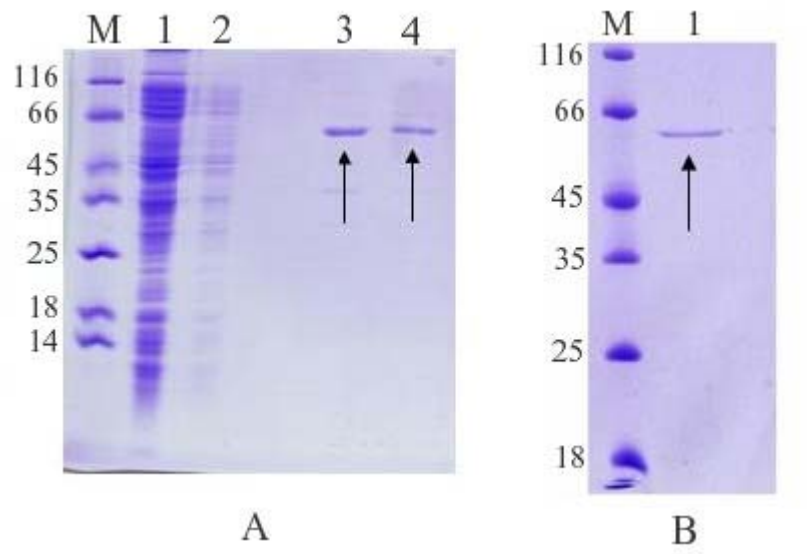


Figure 40. Expression analysis of AMbtF. A) The *ambtF* was cloned into pTYB2 and the plasmid transformed in *E. coli* C41(DE3). Protein purification was performed using a chitin column. B) The *ambtF* was also cloned into pVV16 and the plasmid transformed into *M. smegmatis* mc^2155, followed by purification using a His-tag column. In both cases, a band migrated (pointed with arrows) on 12% SDS-PAGE with the expected size of AMbtF, however MALDI-MS analysis shown the identity of different proteins. M= molecular protein marker.

4.5 Disscusion.

Siderophores depend on three chemical functional groups for iron chelation: catechols, hydroxamates, or α-hydroxy-carboxylates (Miethke & Marahiel, 2007). Biosynthesis of hydroxamates from diamino acids involves the hydroxylation of the terminal amino group by an *N*-hydroxylating monooxygenases (NMOs) to form a hydroxylamine (van Berkel *et al*., 2006). Few NMOs have been biochemically studied due to difficulties in cloning and expression of these enzymes (Putignani *et al*., 2004; Thariath *et al*., 1993) as well as the low affinity for FAD, hindering mechanistic studies (Stehr *et al*., 1999). Early studies with L-lysine-N^{ε}-oxygenase (IucD) suggested that this class of monooxygenase is normally attached to the cytoplasmic membrane due to the presence of a hydrophobic amino acid sequence (Herrero *et al*., 1988), hampering its isolation or expression (Plattner *et al*., 1989). Although, discrepancies do exist and the gene could be cloned and its product obtained as a cytoplasmic protein (Thariath *et al*., 1993). In addition, similar problems have also been reported for PvdA, an *L*-ornithine N^{δ}-oxygenase from *Pseudomonas* species (Ge & Seah, 2006; Meneely & Lamb, 2007; Putignani *et al*., 2004) and the *L*-ornithine N^5-hydroxylase from *S. coelicolor* (CchB) (Pohlmann & Marahiel, 2008). In this study, the *mbtG* gene was also cloned and expressed as a soluble protein using *E. coli* C41(DE3) with good protein yield.

During MbtG purification the characteristic yellow colour due to the FAD coenzyme was observed, which suggests that the protein retains its coenzyme. It has been reported that the coenzyme in NMOs weakly bind to the enzyme because of the exchange of the last glycine to proline in the FAD-binding motif, a residue which might fulfil an important structural role (Stehr *et al*., 1998; 1999). The lysine N^6-hydroxylase IcuD of *E. coli* is always isolated in its apo-form indicating that the enzyme binds to its coenzyme rather weakly (Macheroux *et al*., 1993). This enzyme was incubated with 20-fold excess of FAD followed by purification using a Sephadex column. The absorption spectrum of the treated protein was similar to that of free FAD enzyme, suggesting that the coenzyme is lost during purification. This addition of FAD after purification, led to enhancement of protein activity (Macheroux *et al*., 1993). Similar results are also obtained during purification of PvdA, which has a low dissociation constant for flavin (Ge & Seah, 2006). Multiple alignment of the sequences

among NMOs of *M. tuberculosis* (MbtG-H37Rv), *M. smegmatis* (MbtG-MS), *Pseudomonas* (PvdA), *S. coelicolor* (CchB) and *E. coli* (IucD), revealed that the FAD-binding motif, the NADP-binding motif, and the FATGY motif are present in all these types of monooxygenases (Figure 41). However, the BVMO motif proposed by Fraaije *et al*. (2002) is not highly conserved amongst the NMOs. In the FAD-binding motif, the first two glycines are conserved, whereas the last glycine varies in the sequences aligned. In *M. tuberculosis* H37Rv, the FAD-binding motif has an alanine instead of glycine (GAGAKA), but this amino acid exchange might not affect the binding of the coenzyme. Interestingly, the MbtG of *M. smegmatis* mc^2155 conserves the third glycine in this motif, indicating that the co-enzyme should be tightly bound to MbtG.

The hydroxylation of lysines or ornithine residues in hydroxamates siderophores are a key step since they are essential for the generation of the chelating groups for Fe (III). *L*-ornithine N^5- oxygenase from *Burkholderia cepacia* is an important enzyme for hydroxylation of ornibactin. A mutation in *pvdA*, which encodes for the enzyme, did not carry out the hydroxylation of *L*-ornithine in ornibactin synthesis. However, ornibactin synthesis in the mutant was partially restored when the precursor *L*-N^5-OH-ornithine was added to the culture medium (Sokol *et al*., 1999). Three mutants in *pvd*, were designed to understand the function of the enzyme. The first mutant, *pvd*-1, was unable to hydroxylate *L*-ornithine in a cell-free system. The two other mutants appear to block further steps in the biosynthetic pathway leading to pyoverdin (Visca *et al*., 1992). The product of the *iucD* gene, a 53 kDa protein, is required for the hydroxylation of N^{ε}-hydroxylysine in the biosynthesis of the *E. coli* K-12 aerobactin (de Lorenzo *et al*., 1986).

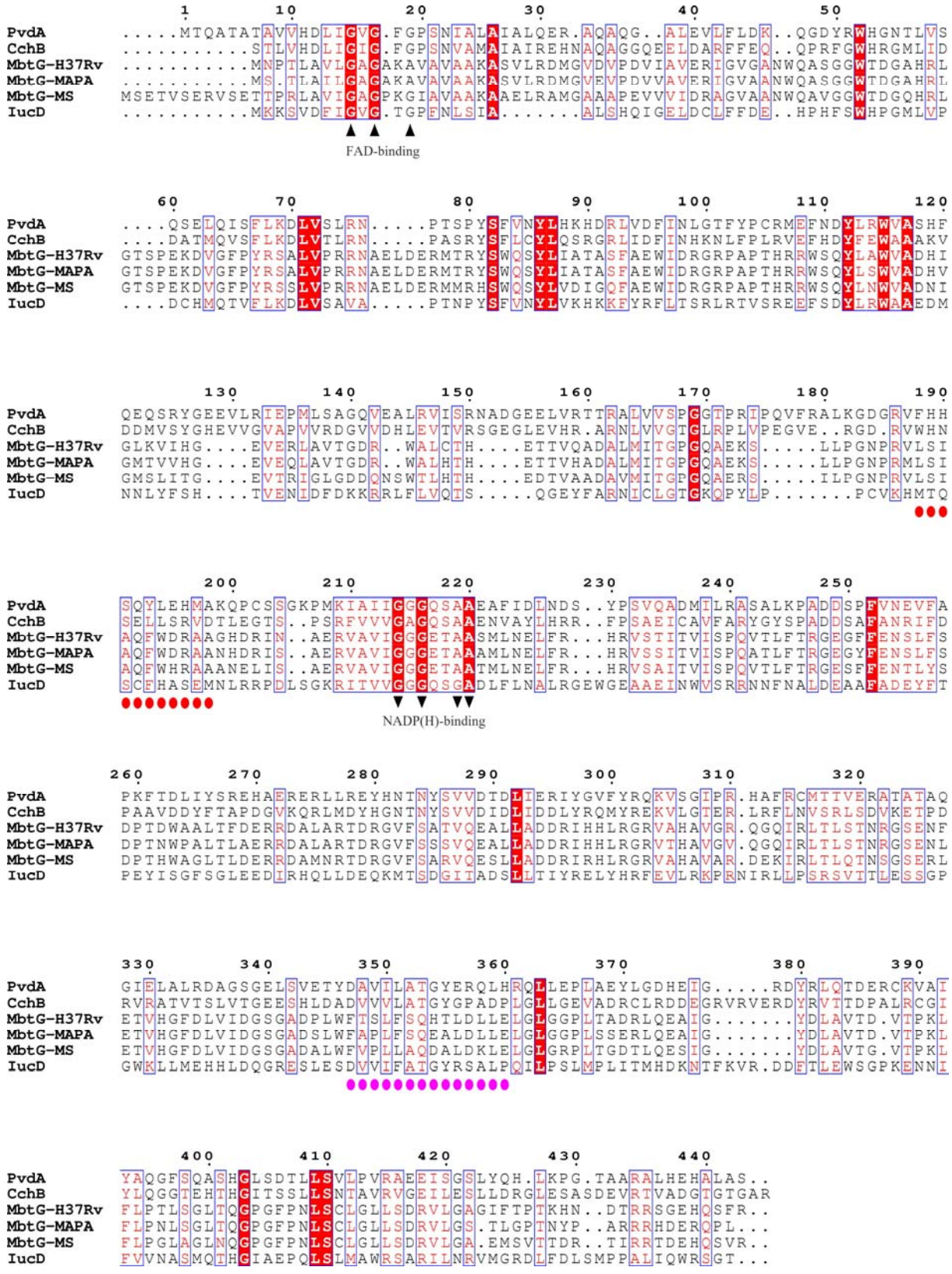

FAD-binding
NADP(H)-binding

Figure 41. **Multiple sequence alignment of NMOs.** The sequences were aligned using ClustalW2 (default settings) and Esprit. The FAD-binding motif is indicated by (▲), whereas the NADP(H)-binding motif is indicated by (▼). Red closed-circles indicate the predicted specific motif of BVMOs as described by Fraaije *et al*. (2002). Pink closed-circles shows the predicted hydrophobic motif described by Stehr *et al*. (1998). PvdA (*L*-ornithine N^5-oxygenase) from *P. aeruginosa*; CchB (*L*-ornithine N^5-hydroxylase) from *S. coelicolor*; MbtG-H37Rv, MAPA, and MS, from *M. tuberculosis* H37Rv, *M. avium* subsp. *paratuberculosis* and *M. smegmatis*, respectively; IucD (lysine N^6-hydroxylase) from *E. coli*.

Different hypotheses have been postulated to understand how the lysines are incorporated into the core structure of mycobactins. It was thought that hydroxylation occurs prior to the amino acids being incorporated into the growing mycobactin by MbtE and MbtF. Another scenario was hypothesised, in which the amino acids are first activated by the adenylation domains followed by hydroxylation, then further modifications on the lysines, acylation and cyclisation in the central and terminal residues, respectively, were likely to occur. However, the discovery of DDM-838 (Moody *et al*., 2004) with lysines unmodified in the core structure of mycobactin T has suggested that hydroxylation of these amino acids occurs as the last step in mycobactin biosynthesis. Biochemical data supports this hypothesis, in which the the MbtG could hydroxylate an acetylated lysine preferentially over lysine (Krithika *et al*., 2006) suggesting thus that DDM is an intermediate compound. On the other hand, the Lysine N^{ω}-hydroxylase (IucD) of *E. coli*, prefers lysine as a substrate (Marrone *et al*., 1996). Lysine analogues, in which the ω-amino position are changed to methyl or acylated groups, were not hydroxylated by the enzyme, whereas modification at the α-position results in hydroxylation (Marrone *et al*., 1996). In addition, PvdA is also specific for its substrate, L-ornithine, although, the enzyme could carry out NADPH oxidation on lysines without formation of the hydroxylated product (Ge & Seah, 2006; Meneely & Lamb, 2007). Perhaps the topology of the enzyme might be the key of the mechanistic reaction observed in MbtG, which is able to hydroxylate both lysines and acetylated lysines.

If DDM is a true intermediate, it is logical to assume that the adenylation domains of MbtE and MbtF should activate first unmodified lysines, and then the residues are modified (acylation and cyclisation) in their corresponding NRPS, followed finally by hydroxylation by MbtG to produce a functional mycobactin. To investigate this hypothesis, cloning and expression of the adenylation domains were attempted in this study. Thus, *ambtE* was successfully cloned and expressed in pET28b using *E. coli* C41(DE3) as heterologous host, although, the protein was insoluble. As outlined before, cloning domains of NRPSs and PKSs is always difficult since the exact borders of conserved and less conserved amino acids in interdomains regions are not easy to define. Misfolding and solubility problems might arise from incorrectly designed domains when trying to express recombinant peptide synthetases in heterologous hosts. In both cases, the result might be the interruption in the intramodular communication, incorrect folding and therefore affecting enzymatic activity (Weber *et al*., 2000). Furthermore, over-expression of foreign proteins from cloned genes in heterologous hosts, such as *E. coli*, often leads to the formation of insoluble intracellular aggregates of the expressed protein, the so-called inclusion bodies. Evidence suggests that inclusion bodies arise by the inappropriate aggregation of partially folded or misfolded polypeptides, rather than insolubility or instability of the native protein (Baneyx & Mujacic, 2004).

The *ambtF* was also cloned into pET23a, however, the gene was not expressed despite different conditions attempted. As an alternative method, the codon alteration strategy was employed to overcome this problem (Usha *et al*., 2006). The gene was successfully cloned into pTYB2 but not in pET23a. Expression analysis of *ambtF*-pTYB1 using *E. coli* ER2566 as heterologous host, no evidence for *ambtF* expression was found. *M. tuberculosis* possesses a high G+C content around 65 mol% (Cole *et al*., 1998). This G+C richness is reflected in a strong bias towards C- and G-ending codons for every amino acid with an overall G+C content at the third position of codons of 83%. However, there is a significant variation in codon usage patterns among genes, which appears to be associated with gene expression level (Andersson & Sharp, 1996). Although several codons can encode the same amino acid, *E. coli* contains tRNA for certain high-usage codons than other low-usage codons (Ikemura, 1985). As codons usage is thought to have coevolved with tRNA population, the use of heterologous

hosts phylogenetically closer to *M. tuberculosis*, such as *Streptomyces*, *Corynebacteria*, and the rapidly growing and non-pathogenic *M. smegmatis,* may solve gene expression. Since *M. tuberculosis* tRNA populations are closer in *M. smegmatis*, *ambtF* was further cloned using its native forward primer into pVV16 and expression analysis was carried out in *M. smegmatis* mc^2155, however, gene expression was not observed. Different growth conditions might give positive result, but this has not been evaluated yet.

There is another way to test the above hypothesis by deleting the *mbtG* gene (gene knockout) from the chromosome of any mycobacteria species that produces mycobactin, such as *M. tuberculosis* or *M. bovis*. Thus, deleting the gene and growing the mutant in iron-deficient conditions, the mycobacteria phenotype should produce the intermediate mycobactin (DDM), in which the lysines are not hydroxylated. This approach has been attempted; however, re-generation of the mutant has been hindered (Dr. Apoorva Bhatt, personal communication). The *mbtG* gene was deleted from *M. bovis* BCG chromosome by especialised transduction (Bardarov *et al*., 2002). To further study this phenotype, several attempts to culture the mutant in different types of medium was not afforded.

4.6 Further work.

Since MbtG is not stable for a long period, conditions (pH, buffer, temperaure) will be investigated to obtain a stable enzyme. In order to analyse the hydroxylation of DDM by MbtG, first enzymatic activity of MbtG will be investigated by oxidation and hydroxylation assays with lysine and modified lysines as reported for PvdA and IucD (Macheroux *et al*., 1993; Meneely & Lamb, 2007). Then, hydroxylation of the unmodified lysines present in DDM compound will be attempted. Furthermore, expression trials will be investigated to obtain soluble and active AMbtE. Since adenylation domains are responsible for amino acid recognition, ATP binding and amino acyl adenylate formation, AMbtE substrate preference (unmodified or modified lysines) can be assessed using the ATP:PPi exchange assay (Linne & Marahiel, 2004).

5. General Materials and Methods.

5.1 Molecular Biology Techniques.

5.1.1 Polymerase chain reaction (PCR).

Amplification of genes by PCR was carried out using *M. tuberculosis* H37Rv chromosomal DNA. Primers were designed *in house* and purchased from Eurofins MGW Operon. A typical PCR reaction contained 100 µL of final volume, in which the ingredients were mixed well and DNA polymerase was added last to initiate the reaction. Different DNA polymerases were used for cloning the genes, which are mentioned in each chapter. The basic PCR reaction described below was used for cloning some genes and modifications in some conditions were done according to the needs. Multigradient step PCR reactions was used for cloning difficult genes, such as *ArCP*, *mbtA*, and *ambtF*.

A) Recipe

2 µL	5’ Cloning primer (100 pmole)
2 µL	3’ Cloning primer (100 pmole)
1 µL	Genomic DNA (H37Rv) (1 µg/µL)
2 µL	dNTP’s
10 µL	Thermostable buffer (10X)
8 µL	DMSO (optional)
2 µL	$MgSO_4$ (optional) (100 mM)
1 µL	DNA polymerase[1]
72 µL	ddH_20 (for molecular biology)

B) PCR conditions

95ºC	3 minutes	
95ºC	1 second	
60-70ºC	30 seconds	35 cycles
68ºC	0.5 minutes	
68ºC	10 minutes	
4ºC	∞	

5.1.2 Isolation of plasmid DNA.

Plasmid DNA was isolated using the Quiaprep spin miniprep kit (Qiagen) according to the manufacturer's instructions.

5.1.3 Restriction enzyme digestion of DNA.

The restriction endonuclease digestions were perfomed according to manufacturer's recommendations. Buffer and temperature of digestion were chosen based on optimal function of the used enzyme. The final volume for a typical digestion was 20 μL.

Restriction enzyme digest	Total reaction volume (20 μL)
DNA	5 μL
Buffer 10X	2 μL
Restriction enzyme 1	1 μL
Restriction enzyme 2	1 μL
[a]dH_2O	11 μL

[a]Molecular grade dH_2O was used for reactions.

5.1.4 Electrophoresis of DNA.

PCR products and restriction digests were analysed by horizontal slab agarose gel eletrophoresis (Hoefer). Percentage of agarose was prepared according to the size to the analysed DNA. Agarose gels were prepared by dissolving electrophoretic grade agarose (Bioline) in 1 X TAE (Tris-acetate-EDTA buffer). 1 X TAE was made up to 40 mM Tris-acetate and 1 mM EDTA. DNA samples for electrophoresis were prepared by mixing with $1/5^{th}$ volume of the 5X DNA loading dye [0.25 % bromophenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in dH_2O]. The samples were run at 110 volts until the dye migrated the required distance. The gel was stained with 0.5 μg /mL solution of ethidium bromide for 20 minutes, and visualised in a UV transiluminator. The gel was photographed using the Bio-rad gel software.

5.1.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Expression analysis and purification of proteins were analysed by subjecting on 10-15 % SDS-PAGE gel by the discontinous gel method (Laemmli, 1970). A Hoefer "mighty small" SE 200 vertical slab gel system was used. Resolving and stacking gels and the acrylamide/bis/water mix were prepared as follows:

Resolving gel:

Composition	For two gels
[a]4 X resolving gel buffer	3.75 mL
[b]Acrylamide/bis/water mix	11.25 mL
[c]TEMED	30 mL
10% ammonium persulfate	75 mL

[a]The 4 X resolving buffer consists of 1.5 M Tris-HCl, pH 8.8 and 0.4% SDS.

[b]Percentage of acrylamide/bis.water was varied according to needs.

[c]TEMED (Tetramethylethylenediamine).

Stacking gel:

Composition	For two gels
[d]4 X stacking gel buffer	1.25 mL
Acrylamide	0.65 mL
TEMED	15 mL
10% ammonium persulfate	75 mL
H_2O	3.05 mL

[d]The stacking gel buffer consists of 0.5M Tris-HCl, pH 6.8 and 0.4% SDS.

Protein samples were prepared by mixing with 1/5th the volume of SDS loading buffer (360 mM Tris-HCl, pH 8.8, 9% (w/v) SDS, 0.9% (w/v) bromophenol blue, 15% (w/v) β-mercaptoethanol and 30% glycerol) and boiled at 95°C for 5 minutes. The samples were loaded onto the gel and run at 25 mA for approximately an hour. The running buffer was made up of 25 mM Tris-HCl, 190 mM glycine and 4 mM SDS. Protein gels were stained with Coomasie brilliant blue R-250 stain (5% glacial acetic acid, 0.025% Coomasie brilliant blue (w/v) and 50% methanol) and left for 30 minutes. The excess stain was removed with a destaining solution (10% glacial acetic acid and 30% methanol). The gels were photographed using the Bio-rad software. Gels were dried between two sheets of cellophane.

5.1.6 Estimation of protein concentration.

The concentration of total protein concentration present in cell wall material and membranes were measured using the Bicinchoninic protein assay (BCA) kit (Pierce). The assay was performed according to the manufacturer's protocol. The assay was calibrated over the range 3.5-5.0 ug/mL by using a range of standards derived from a 2 mg/mL solution of bovine serum albumin (BSA) (Pierce). Samples, standards and blanks were assayed in duplicate. Samples were also diluted 1 in 5 to ensure a reading was obtained within the limits of the standard curve. The assay was incubated at 37°C for 30 minutes and the colorimetric change was measured at 562 nm.

5.2 Culture Media.

5.2.1 Luria-Bertani (LB) medium.

Composition	Per liter
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

LB broth and LB agar were obtained from Sigma-Aldrich Company. Medium solutions were prepared following the manufacturer's instructions. Media were sterelised by autoclaving at 121°C for 15 minutes.

5.2.2 Terrific Broth (TB) medium.

Solution A

Composition	Per 900 mL
Bacto-tryptone	12 g
Bacto-yeast extract	24 g
Glycerol	4 mL

Solution B

Composition	Per 100 mL
KH_2PO4	2.3 g
K_2HPO4	12.5 g

Solutions were prepared separately followed by autoclaving at 121°C for 15 minutes. To prepare the final medium, 100 mL of solution B was added to solution A.

5.2.3 Proskauer and Beck (PB) medium.

Glassware for preparing media was deferrated: glassware were filled with alcoholic KOH (2%) and left overnight. Then, glassware was washed with deionised water, followed by filling with 8 M HNO_3 and leaving overnight. Glassware was autoclaved at 127ºC for 15 minutes prior to using. Afterwards, PB medium was prepared in the deferrated glassware. Since *M. bovis* BCG cannot use glucose as carbon source, sodium pyruvate (1%) was used instead. The *glpK* gene, which encodes a glycerol kinase, required for phosphorylation of glycerol, is a pseudogene in this strain.

Composition	Pero liter
Monopotassium phosphate	5 g
Asparagine	5 g
Magnesium sulphate $7H_2O$	0.6 g
Magnesium citrate	2.5 g
Sodium pyruvate	10 g

Ingredients were dissolved in that order, being certain that each compound was solubilised before adding the next one. The pH was adjusted to 7.8 by the addition of 40% NaOH followed by autoclaving at 121°C for 15 minutes after the pH should be 7.4. Next, the medium was filtered throught Whatman Filter to remove precipitation; aliquots were prepared according to the desired volume and autoclaved again at 121°C for 15 minutes.

5.3 Strains and vectors.

5.3.1 Strains.

E. coli TOP 10 (Invitrogen), *E. coli* XL1 Blue (Strategene) and *E. coli* NEB 5-alpha F´*Iq* (New England Biolabs) were used for plasmid maintenance, whereas *E. coli* C41(DE3) (OverExpress), *E. coli* K12 and *E. coli* ER566 (New England Biolabs) were used for protein expression. *E. coli* strains were grown at 37°C in an orbital shaker at a speed of 180 rpm. *M. bovis* BCG Pasteur was used for whole-cell assay, whereas *M. smegmatis* mc^2155 was employed for heterologous expression.

5.3.2 Vectors.

The vectors used in this study are the following:

Vector	Concentration of antibiotic used for selection (μg/mL)
pET23a	Ampicillin (100)
pET23b	Ampicillin (100)
pET28b	Kanamycin (25)
pCDFDuet1	Streptomycin (100) and Spectinomycin (100)
pSU	Chloramphenicol (30)
pTYB series	Ampicillin (100)
pMAL-C2X	Ampicillin (100)
pMV261	Kanamycin (50)
pVV16	[a]Hygromycin (200) and Kanamycin (50)
pSD26	[a]Hygromycin (200)

[a]For *E. coli* strains Hygromycin (200 μg/mL) was used, whereas for *M. smegmatis* mc^2155 (50 μg/mL) was utilised.

5.3.3 Preparation of competent cells.

5.3.4.1 Chemical competent cells.

The rubidium chloride protocol was used for preparing chemical competent cells. Original *E. coli* glycerol stocks were inoculated in 5 mL of fresh LB medium. Cells were grown overnight at 37ºC in an orbital shaker at 180 rpm. The following day, the entire overnight culture was transferred to 50 mL of LB medium containing 20 mM $MgSO_4$. Cells were grown at 37ºC in an orbital shaker at 180 rpm until OD_{600}=0.5-0.6. The cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4ºC. The pelleted cells were gently resuspended in 0.4 volume (based on the original culture volume) of ice-cold TFB1 buffer. The resuspended cells were incubated on ice for 30 minutes at 4ºC. The cells were pelleted again by centrifugation at 5, 000 rpm for 10 minutes at 4ºC. Cells were gently resuspended in 0.04 of the original volume of ice-cold TFB2 buffer, and incubated on ice for 30 minutes at 4ºC. Next, cells were

aliquoted and flash-frozen as 50 µL stocks, using liquid nitrogen, and stored at -80ºC until required.

TFB1 buffer.

Composition	Concentration
Potassium acetate	30 mM
$CaCl_2$	10 mM
$MnCl_2$	50 mM
RbCl	100 mM
Glycerol	15%

The pH is adjusted to 5.8 with 1 M acetic acid. Filter-sterilise (0.2 µm) and store at 4°C.

TFB2 buffer

Composition	Concentration
MOPS or PIPES, pH 6.5	10 mM
$CaCl_2$	75 mM
RbCl	10 mM
Glycerol	15%

The pH is adjusted to 6.5 with 1 M KOH. Filter-sterilise (0.2 µm) and store at 4°C.

5.3.4.2 Heat shock transformation.

A 50 µL stock was thawed on ice for at least 10 minutes, then transferred to a cold-Falcon Tube (14 mL). To the chemical competent cells, 1 µL of plasmid DNA was added and mixed gently, and the mix was incubated on ice for 10 minutes. Next, the tube was heated at 42ºC for 2 minutes, and incubated on ice for 10 minutes. Fresh LB medium (150 µL) was added to the tube, followed by incubation at 37ºC for one hour in an orbital shaker at 180 rpm. The transformed cells were plated in LB agar containing the appropriate antibiotic.

5.3.4.3 Preparation of electro-competent *M. smegmatis* mc^2 155 cells.

A starter culture of *M. smegmatis* mc^{2}155 was grown overnight in LB (100 mL) broth supplied with 0.05% Tween 80 at 37ºC in an orbital shaker at 180 rpm. The following day, a 0.6% inoculum of the overnight culture was transferred to 50 mL of fresh LB medium containing 0.05% Tween 80. The culture was incubated in an orbital shaker at 37ºC until OD_{600}=0.5-0.6. Cells were harvested by centrifugation at 5, 000 rpm at 4ºC, which were then resuspended in 30 mL of ice-cold 10% glycerol. The resuspended cells were incubated on ice at 4ºC for 20 minutes and centrifuged again as above. The process was repeated four times by re-suspending each time with reducing volumes of 10% glycerol (20, 10, 5 and 2.5 mL respectively). The pelleted cells were finally re-suspended in 2 mL of 10% glycerol, aliquoted as 50 µL stocks, flash-frozen using liquid nitrogen and stored at -80ºC.

5.3.4.4 Transformation of electro-competent *M. smegmatis* mc^{2}155 with plasmid DNA.

A 50 µL aliquot of electro-competent *M. smegmatis* mc^{2}155 cells was thawed on ice at 4ºC. Next, plasmid DNA (1 µL) was added to the electro-competent cells and mixed gently; the mix was incubated on ice at 4ºC for 30 minutes. The mix was transferred to an ice-cold electro-cuvette (1 mm cuvette gap) and electroporated using an Eppendorf Electroporator 2510 (settings: 1800 mV, 1000 Ohms, 25 µF). After electroporation, 250 µL of fresh LB medium was added, mixed gently, and incubated at 37ºC for 4 hours without shaking. Next, transformed cells were plated in LB agar containing the appropriate antibiotic and incubated at 37 ºC until single colonies were grown (~ 2-4 days).

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